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Supplementary Information

Ligand-free 99mTc-polyurea dendrimer complexes: Nanoradiotheranostics targeted at ovarian cancer

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The elution of $[^{99m}TcO_4]^-$ was made from a $^{99}Mo/^{99m}Tc$ generator (Drytec®, GE Healthcare). The radioactivity of the technetium-99m solutions was measured in an ionization chamber (Aloka, Curiemeter IGC-3) or in a γ-counter (Berthold-LB 2111, Berthold Technologies, Bad Wildbad, Baden-Württemberg, Germany), depending on the level of the measured radioactivity. The analysis of radiolabelled dendrimers and their radioactive precursors was carried by reversed phase high performance liquid chromatography (RP-HPLC) using a Perkin Elmer system equipped with a reversed phase C18 column, a biocompatible quaternary pump (series 200), a UV-Vis detector (LC290 Perkin Elmer, UV detection at 254 nm) and a γ detector (LB 509, Berthold). The HPLC eluents and gradients are described in Table S1. All the radioactive compounds were also analysed using instant thin-layer chromatography silica gel strips (iTLC-SG) (Pall) with methyl ethyl ketone (MEK) as eluent. The radioactivity distribution on the iTLC-SG strips was detected using a Elsysia-Raytest MiniGITA γ-detector.

1. Synthesis of $PURE_{G4}$-FA_2. The folate targeted dendrimer $PURE_{G4}$-FA_2 was prepared following our reported protocol. Briefly, $PURE_{G4}$ (1.0 equiv.) was conjugated with folic acid (FA) using FA-NHS (2.0 equiv.), in DMSO (0.00025 mmol $PURE_{G4}$/mL), to obtain $PURE_{G4}$-FA_2. The reaction was stirred at RT overnight in the dark. Next, diethyl ether was added to precipitate $PURE_{G4}$-FA_2. The precipitate was dried under vacuum to give a yellow oil. The obtained product was characterized by ^{1}H NMR and was found to be identical to literature.[1]

2. Preparation of *fac*-[^{99m}Tc$(H_2O)_3(CO)_3$]$^+$. [$^{99m}TcO_4$]$^-$ was eluted from a ^{99}Mo/^{99m}Tc generator using a NaCl 0.9% (w/v) aqueous solution. Approximately 3.0 mL of the pertechnetate solution were added to a kit composed of sodium tartrate dihydrate (8.50 mg), sodium tetraborate decahydrate (2.85 mg), sodium carbonate (7.15 mg) and sodium boranocarbonate (4.50 mg). The solution was heated for 30 minutes at 100 ºC. The vial was then allowed to cool to r.t. and the pH value of solution was adjusted to 6 with a 0.1 M HCl solution. The final solution was analysed by iTLC-SG using MEK as eluent and by HPLC using as mobile phase the eluent gradient described in Table S1. to assess the radiochemical purity of the prepared *fac*-[^{99m}Tc$(H_2O)_3(CO)_3$]$^+$ (Figures S1 and S3).

3. Radiolabelling. Approximately 50 µL of a $PURE_{G4}$-FA_2 10^{-3} M aqueous solution were purged in a glass vial. Next, a *fac*-[^{99m}Tc$(H_2O)_3(CO)_3$]$^+$ saline solution (pH 6) (450-950 µL) was added, and the labelling reaction was carried out at 100 ºC for 30 min. The radiochemical purity was controlled by iTLC-SG, using MEK as eluent and by HPLC in the same conditions described in section 2. The same procedure was performed using $PURE_{G4}$.

4. Purification of radiolabelled PURE dendrimers by HPLC. The folate-targeted dendrimer, ^{99m}Tc-$PURE_{G4}$-FA_2, was purified by HPLC by elution with the aqueous 0.1% TFA solution and MeOH gradient, described above. The purification involved the collection of the fractions with RTs in the range 14 to 18 min. This sample was used subsequently in the evaluation of lipophilicity and *in vitro* stability of the radiolabelled dendrimer.

5. Partition coefficients determination. The lipophilicity of HPLC-purified ^{99m}Tc-$PURE_{G4}$-FA_2 was evaluated by determining the partition coefficient in the biphasic system *n*-octanol/0.1 M PBS pH 7.4 ($P_{o/w}$). Briefly, 10 µL of the radiolabelled dendrimer were added to a mixture of *n*-octanol and 0.1 M PBS pH 7.4 (1:1) and then vortexed and

centrifuged (775 *g*, 10 minutes, r.t.) to allow phase separation. The two phases were separated in two different vials and then four aliquots (100 μL) of each phase were collected and counted in a γ-counter. With the measurement of the activity from the different phases, the ratio between the organic and the aqueous phases ($P_{o/w}$) was calculated. The results were expressed as log ($P_{o/w}$).

6. Stability assays. The stability of HPLC-purified ^{99m}Tc-PURE$_{G4}$-FA$_2$, was evaluated under physiologic (saline solution) conditions and in the presence of a cell culture medium. For that, saline or cell culture media (DMEM and RPMI) with and without 0.2% BSA supplementation were added to an Eppendorf containing the radiolabelled dendrimer. The conditions tested were 20 μL radiolabelled dendrimer and 20 μL (non-supplemented cell culture medium) or 40 μL (0.2% BS supplemented cell culture medium). The stability in saline solution was quantified in total volume of 200 μL of radiolabelled dendrimers in PBS. The resulting mixtures were incubation overnight at 37 ºC. The radiochemical purity of the different mixtures was controlled by iTLC-SG (100% MEK).

Table S1. Solvent gradient for HPLC analysis (flow rate: 1 mL/min).

Time (min)	% Solvent A H_2O (0.1% TFA)	% Solvent B MeOH (0.1% TFA)
5	100	0
17	20	80
20	0	100

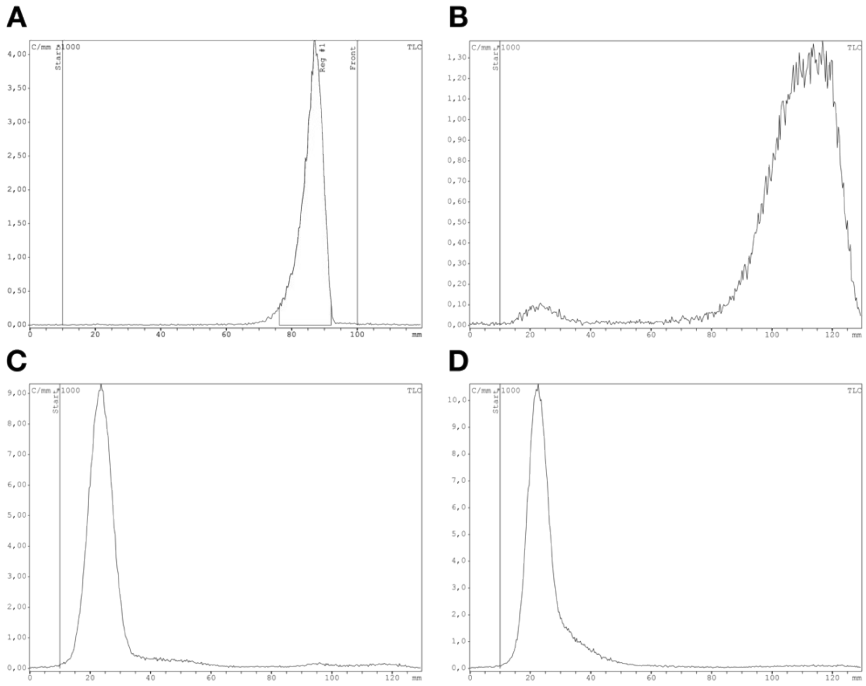

Figure S1. Characterization of radioactive precursors and radiolabelled dendrimers by iTLC-SG (100% MEK): *pertechnetate* $[^{99m}TcO_4]^-$ (R_f= 0.9) (**A**), tri-carbonyl complex *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ (R_f= 1) (**B**), ^{99m}Tc-PURE$_{G4}$ (R_f= 0) (**C**) and ^{99m}Tc-PURE$_{G4}$-FA$_2$ (R_f= 0) (**D**).

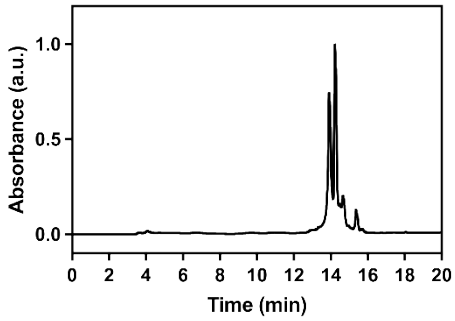

Figure S2. RP-HPLC chromatogram of the PURE$_{G4}$-FA$_2$ dendrimer (RT= 13.90,14.21, major peaks) (UV detection). The sample was eluted using the conditions described in Table S1: water (0.1% TFA) and methanol (0.1% TFA).

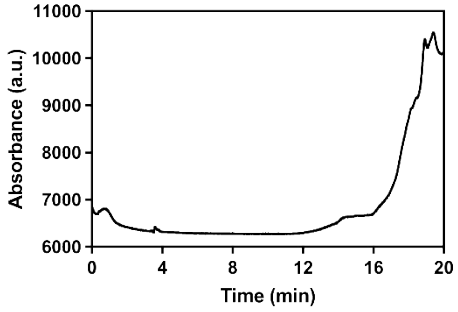

Figure S3. UV-VIS spectra of dendrimer ^{99m}Tc-PURE$_{G4}$-FA$_2$ after purification.

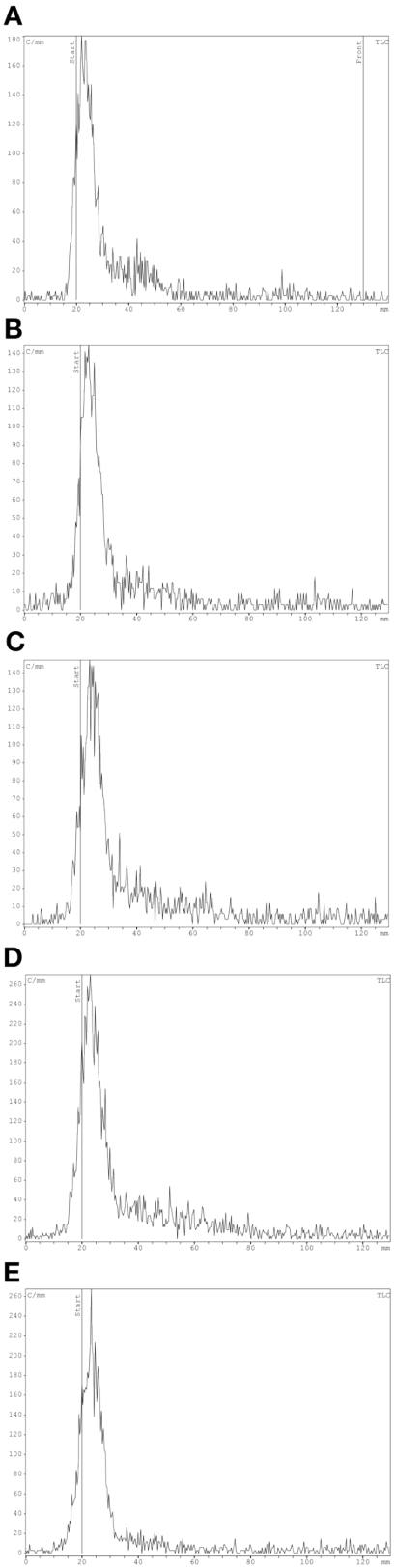

Figure S4. Stability assays of HPLC-purified ^{99m}Tc-PURE$_{G4}$-FA$_2$. The iTLC-SG (100% MEK) chromatograms show the radiolabelled dendrimer after overnight incubation at 37 ºC under physiologic conditions (saline solution) and cell culture media (RPMI and DMEM): saline solution (**A**), RPMI (**B**), RPMI with 0.2% BSA supplementation (**C**), DMEM (**D**) and DMEM with 0.2% BSA supplementation (**E**).

References

[1] P. Mota, R. F. Pires, J. Serpa and V. D. B. Bonifácio, *Molecules* 2019, **24**, 3111.