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Original Article

In-house Optimization Radiolabeling of Recombinant scFv with ^{99m}Tc-Tricarbonyl and Stability Studies

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HIGHLIGHTS

- Radiolabeling of scFv was done directly by ^{99m}Tc-tricarbonyl.
- ^{99m}Tc-tricarbonyl was prepared in house from boranocarbonate.
- 99mTc-Radiolabeled scFv can be used for radioimmunoscintigraphy.

ABSTRACT

His-tagged scFv fragments of monoclonal antibodies have better pharmacokinetic properties than whole antibodies. Radiolabeled scFvs are considered for targeted imaging and treatment. Technetium tricarbonyl provides radiolabeling of scFvs without losing its biological activity in a fast and easy procedure. Technetium tricabonyl was prepared as follows: A freshly eluted solution of $Na^{99m}TcO_4$ was added to a mixture containing sodium carbonate, sodium potassium tartarate, boranocarbonate, sodium borohydride. The mixture was heated for 30 min at 100°C. Radiochemical purity was determined using radio thin lyer chromatography. Then, technetium tricarbonyl was added to a solution of scFv in PBS buffer and incubated for 2 h at 50°C, purified by PD-10 column and radiochemical purity was determined. Results showed that radiochemical purity of technetium tricarbony was over 98%. The best conditions for radiolabeling of scFv was: scFv concentration >2 mg/mL, PBS buffer, 2 h incubation at 50°C, pH 8-9, and high activity concentration of tricarbonyl. The best radiochemical purity of scFv was 70% before purificarion. Radiolabeled scFv was stable in PBS for 24 h incubation and there was no release of technetium in competition with histidine. In this study, we optimized radiolabeling of a scFv with technetium tricarbonyl using house made boranocarbonates. The results are promising and will be used for future studies.

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Introduction

Monoclonal antibodies (mAbs) are prepared against specific targets in the body. They are mostly

immunoglobulin G (IgG) which have been applied as therapeutic agents in clinic. Radiolabeled mAbs are used in nuclear medicine as therapeutic or imaging agents in Radioimmunotherapy (RIT) or Radioimmunoscintigraphy (RIS) procedures, respectively (Kręcisz *et al.*, 2021). The intact antibody with a molecular weight of approximately 150 kDa, has slow clearance from the circulation (24 h), while antibody fragments with lower molecular weight have faster clearance and are more suitable to be used for imaging studies, because of rapid and enhanced penetration into targets, which resulted in higher concentration in the target site compared to non-target (Willuda *et al.*, 1999; Ryman and Meibohm, 2017; Ovacik and Lin, 2018). Antibody fragments can be radiolabeled with short half-life radioisotopes for Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) imaging studies. Single chain variable fraction (scFv) of an antibody contains variable domains of heavy and light chains attached through a flexible peptide link. They are prepared through recombinant technology in bacteria and they have genetically fused to His-Tag at N or C-terminal to be easily purified by immobilized nickel affinity chromatography. scFv fragments are target specific with a desirable affinity for selected targets, which can be used as delivery agents for radioisotopes and toxins targeting to tumors or other target sites in the body. His tags contain 5 to 6 histidine that can be used for direct radiolabeling of scFv with ^{99m}Tc (Willuda *et al.*, 1999; Wang *et al.*, 2013).

Technetium-99m is one of the best radioisotopes for routine clinical SPECT imaging, since its radiation physical characteristics are perfect for radiolabeling and imaging studies [IT, 6 h, 140keV (90%)]. In addition, ^{99m}Tc is easily available through milking ^{99}Mo/^{99m}Tc generators. It is cheap and its chemical properties as a transition metal are well understood (Schibli and Schubiger, 2002). One of the ^{99m}Tc cores is tricarbonyl in which technetium has +1 valence. ^{99m}Tc(I)-tricarbonyl, $[^{99m}Tc(CO)_3(H_2O)_3]^+$, with sphere shape and small size could be coordinated to a range of biomolecules with appropriate functional groups and forms stable octahedral complexes. Heterocyclic rings containing N-atoms such as imidazoles, pyridines, pyrazoles, efficiently form stable coordinate covalent bonds with technetium tricarbonyl. Imidazole ring of histidine molecule as a ligand can form a rapidly stable octahedral complex with technetium tricarbonyl. The complex is very stable since technetium is protected from further ligand attack or re-oxidation (Alberto, 2005). scFvs are radiolabeled with technetium through chelators, while his-tagged scFvs can be directly labeled with technetium needless of chelators because of histidine molecules of his-tag. Studies have shown that radiolabeling at the his-tag of scFv preserves immunoreactivity after radiolabeling (Waibel *et al.*, 1999; Badar *et al.*, 2014; Williams *et al.*, 2021).

$[^{99m}Tc(CO)_3(H_2O)_3]^+$ is prepared from carbon monoxide (CO) which reduces technetium (VII) in $Na^{99m}TcO_4$ to technetium (I). $[^{99m}Tc]^{+1}$ reacts with CO and water molecules and technetium tricarbonyl forms $[^{99m}Tc(CO)_3(H_2O)_3]^+$. In the presence of suitable ligands, water molecules are replaced, and octahedral complexes are formed. Preparation of ^{99m}Tc-tricarbonyl from CO is difficult, and not suitable for routine clinical studies (Alberto *et al.*, 2001; Kodina, *et al.*, 2005). The Isolink kit (Mallinckrodt Pharmaceuticals) provides preparation of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ without using CO gas (Liu, *et al.*, 2004). Boranocarbonate $[H_3BCO_2]^{-2}$, one of the ingredients of the kit, hydrolyzes and produces CO.

In this study, we aimed to radiolabel a his-tagged scFv (anti-HER2 scFv) by technetium tricarbonyl. The scFv was provided by biotechnology department at school of Pharmacy, Shahid Beheshti University of Medical Sciences. Since we had no access to the Isolink kit, in the present study, we prepared ^{99m}Tc(I)-tricarbonyl from boranocarbonate produced in house. The radiochemical purity (RCP) of tricarbonyl were determined. His-tagged scFv was radiolabeled with technetium tricarbony in different conditions. RCP and stability of radiolabeled scFv in phosphate buffer saline and in competition with histidine were determined.

Materials and Methods

Preparation of ^{99m}Tc-tricarbonyl complex

Technetium tricarbonyl $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was prepared using boranocarbonate (provided by Dr. abdolreza Yazdani) based on published papers and according to the previously reported methods (Malone *et al.*, 1967; Alberto *et al.*, 2001). Briefly, a freshly eluted solution of $Na^{99m}TcO_4$ (20-140) mCi in one mL of saline was added to a purged vial with N_2 gas containing 15 mg of Na_2CO_3, 22 mg of Na-K tartrate, 10 mg of $Na_2H_3BCO_2$, and 20 mg of $NaBH_4$. The vial was heated for 30 min at 100°C. After this time, the vial got cooled to room temperature and the pH of the solution was neutralized to approximately 6-7 with HCl. The RCP of the produced complex was evaluated by Radio-TLC (radio-thin layer chromatography) with a mobile phase of 1% HCl in methanol.

Radiolabeling of His-tagged scFv with ^{99m}Tc-tricarbonyl complex

Briefly, 90-500-µL solution of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was added to 5-10 µL of scFv (2-5 mg/mL in PBS/ sodium citrate/water) which were provided by Dr Elham Mohit. The radiolabelling reaction was carried out at 37°C and 50°C for 2 h and RCP were determined at 30, 60, and 120 min using Radio-TLC and 1% HCl in methanol and citrate buffer 0.1 M, pH 6 as the mobile phases and a gamma counter. These mobile phase systems provided a good separation between the radiolabeled scFv (R_f = 0), unbound $[^{99m}Tc(H_2O)_3(CO)_3]^+$ (R_f = 0.2- 0.6) and free $^{99m}TcO_4$ (R_f = 1). Moreover, for later evaluations, the radiolabeled scFv was purified by a PD-10 column (Sephadex G-25, GE Healthcare). The radiolabeled scFv was loaded into the column, eluted with PBS, fractions collected according to the instructions of manufacturer and counted using a gamma counter. In order to the radiolabeling optimization, the influence of the type of buffer, final volume, pH, scFv concentration, and temperature of the radiolabelling reaction were evaluated.

Stability studies of radiolabeled scFv

The purified radiolabeled scFv in PBS was incubated for 24 h at 37°C and the stability was evaluated at different times up to 24 h by Radio-TLC analysis. In another study, a mixture of 100 µL radiolabeled scFv and 100 µL 0.1 M Histidine solution was incubated for 24 h at 37°C and RCP was evaluated at different time points (1, 4, 6, and 24 h).

All the experiments were done at least for three times in 3 different days. The results are presented as mean values with standard deviation (SD) from at least three independent studies.

Results

Technetium tricarbonyl $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was prepared in this study from boranocarbonate and used for radiolabeling of scFv containing his-tag. His-tagged scFv was radiolabeled in a two-step procedure. First, $[^{99m}Tc(H_2O)_3(CO)_3]^+$ complex was obtained under a mild alkaline and high-temperature condition with a high RCP (more than 98%). In the second step, radiolabeling of His-tagged scFv was performed by the addition of fresh tricarbonyl complex with high RCP at pH 6-7 to the solution of the scFv followed up for 2 h. The RCP of reaction mixture was about 40-70% which reached to 99-100% after purification using PD-10 column. Fig. 1 shows the profile purification of reaction mixture of radiolabeled His-tagged scFv. Fractions of 1, 2, and 3 pulled out together and analyzed using Radio-TLC (RCP=100%). The negligible radioactivity in fraction 7 is probably for free pertechnetate and tricarbonyl. RCP was determined using Radio-TLC and two different mobile phases. Radiolabeled scFv stayed at origin (R_f=0), while technetium tricarbonyl moved up with the mobile phase (R_f=0.2-0.6).

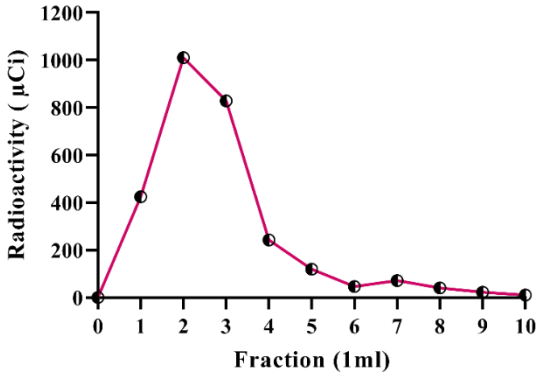

Figure 1. Purification profile of radiolabeled scFv with PD-10 column. The column was eluted with PBS buffer and 1 mL fractions were collected (n = 3 ± SEM).

Radiolabeling of scFv was done at different experimental conditions. In the first experiment, the effect of buffer was analyzed. His-tagged scFv was radiolabeled in PBS buffer/citrate buffer/ and water for 2 h at 37°C and 50°C. Based on Fig. 2A, the reaction performed in PBS buffer had higher RCP compared to citrate buffer and water at 37°C and 50°C. The activity concentration of tricarbonyl per microgram scFv was studied as well. Results showed that the higher radioactivity of tricarbonyl per µg of scFv had better results (Fig. 2B). The volume of reaction mixture had the reverse effect and best result obtaind with 200 µL (Fig. 2C). The effect of pH and temperature was also studied (Fig. 2D and 2E). Based on the results, the optimum conditions for radiolabeling scFv were: PBS buffer, 50°C, 2 h, pH 8-9. Our study showed that the higher the concentration of the scFv solution (>2 mg/mL) and the activity concentration of tricarbonyl (>500 µCi/µL), the higher the RCP would be.

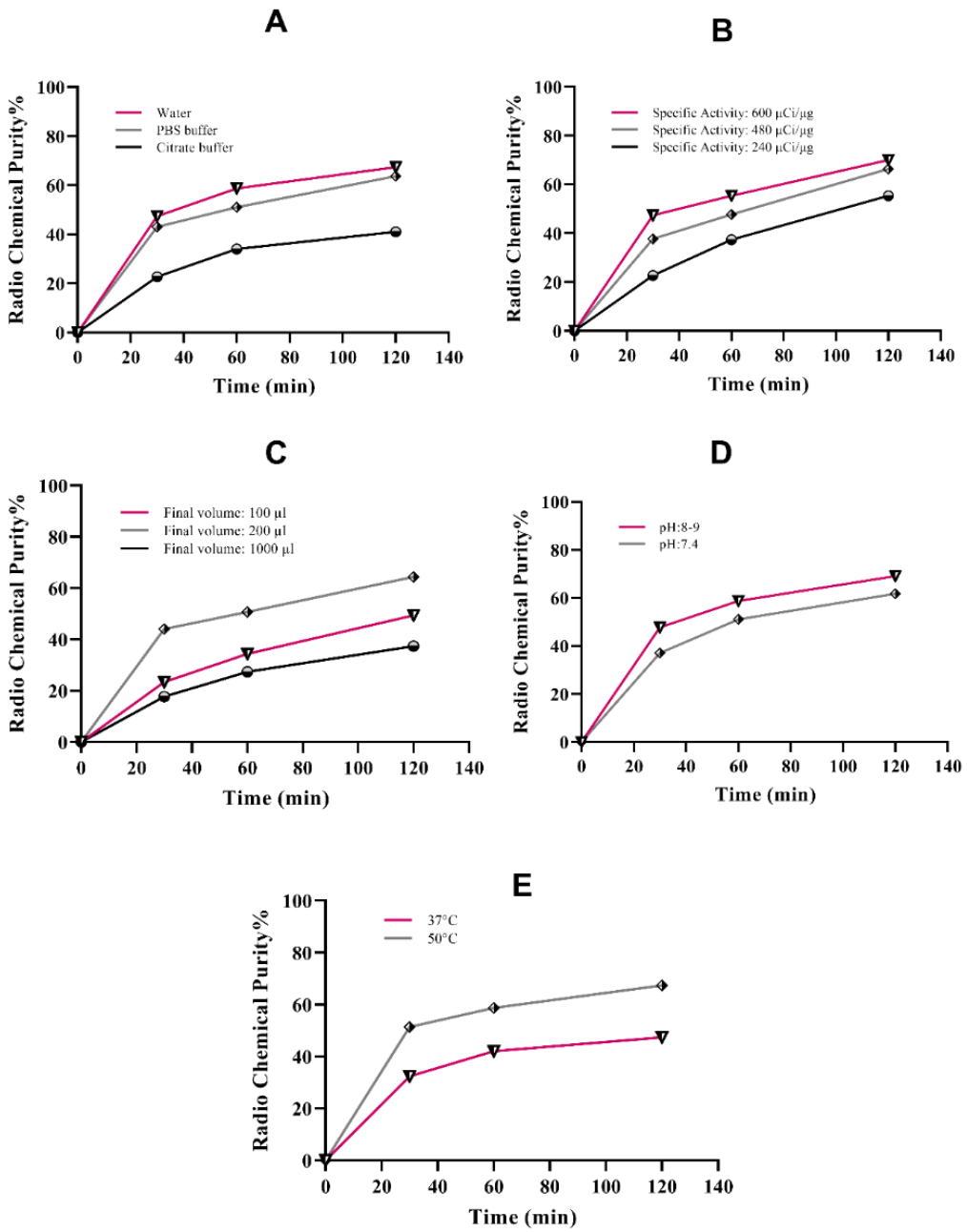

Figure 2. Influence of type of buffer (A), specific activity (B), final volume (C), pH (D), and temperature (E) on radiolabelling of His-tagged scFv with ^{99m}Tc-tricarbonyl. RCP of the radiolabeled scFv (n = 3 ± SEM) was evaluated at 0, 30, 60, and 120 min.

The stability of the purified radiolabeled-scFv was evaluated in PBS buffer and histidine solution at 37°C. RCP was determined at 1, 4, 6 and 24 h. The radiolabeled His-tagged scFv revealed high stability (> 98%) after 24 h of incubation in the PBS buffer (Fig. 3A). In addition there was no release of Tc to histidine (Fig. 3B).

The biological activity of scFv should be preserved during radiolabeling methods. The nitrogen atoms of histidine molecules are useful binding sites for technetium. Nitrogen atoms donor lone pair electrons to the ^{99m}Tc to form coordinate covalent bonds.

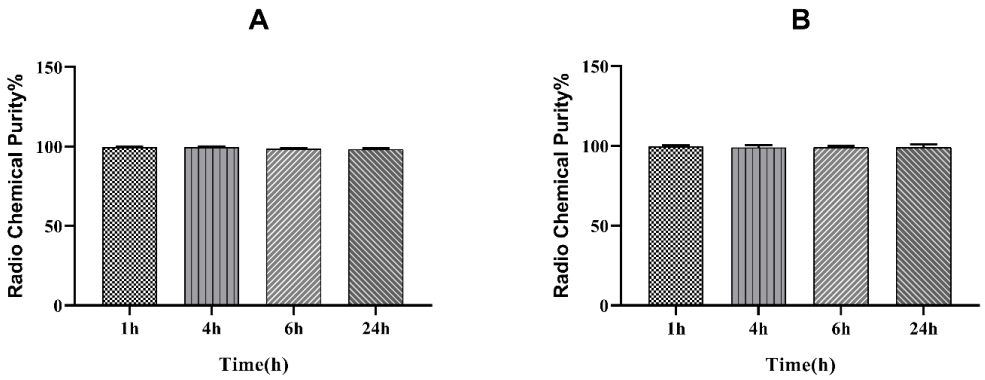

Figure 3. Stability studies of the purified radiolabeled-scFv in (A) PBS buffer and (B) Histidine solution (mean ± SEM, n = 3).

Discussion

Radiolabeling of biological molecules in such a way that the biological activity preserved has led to the introduction of new labeling methods. Radiolabeled monoclonal antibodies or fragments as biological molecules are applied for imaging studies or as therapeutic agents. The radiolabeling procedures should have minimum effect on target binding and biological responses. scFvs are small fragments of monoclonal antibodies prepared through recombinant methods in bacteria and genetically have his-tag at N or C-terminal for purification by immobilized nickel affinity chromatography. His-tag has imidazole rings and scFvs can be easily radiolabeled through his-tag with technetium without chelator.

Technetium tricarbonyl is one of the technetium cores, prepared by reduction of sodium pertechnetate with CO gas to form $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (Alberto *et al.*, 1998). This labeling method requires CO gas and not suitable for nuclear medicine centers. Alberto *et al.* (2001) synthesized Boranocarbonate as in situ CO source for preparation of technetium tricarbonyl. Boranocarbonate as a source of carbon monoxide is solid, stable in air, and can provide a kit formulation for using in nuclear medicine centers. IsoLink, Mallinckrodt Pharmaceuticals provides a kit formulation for preparation of technetium tricarbonyl without using CO gas. $[^{99m}Tc(CO)_3(H_2O)_3]^+$ complex ion has three water molecules, which are prone to substitution with ligands such as imidazole ring of histidine. In the process of radiolabeling his-tagged scFv, the water molecules of tricarbonyl are replaced with histidines present in his-tag, while CO groups of tricarbonyl are tightly bound to technetium and protect complex from further ligand attack or re-oxidation and a stable complex is formed. Since technetium tricarbonyl has small size, the interaction of scFv with proteins faces less steric hindrance and biological activity is preserved (Alberto, 2005).

The aim of this study was to optimize radiolabeling of his-tagged scFv with technetium tricarbonyl. Since we had no access to the IsoLink kits, boranocarbonate prepared in house (by Dr. Yazdani) was used for preparation of technetium tricarbonyl. Technetium tricarbonyl was prepared with RCP >98%. The reaction was easy and fast. Radiolabeling of scFv was done at different conditions. The most important factor in radiolabeling was the concentration of scFv. The RCP was between (40-70)% based on scFv concentration. Low RCP was obtained at concentrations of ≤1 mg/mL, which reached to 70% at concentrations ≥2 mg/mL. Radiolabeled scFv needed subsequent purification by PD-10. Purified radiolabeled scFv showed 99-100% RCP. Results revealed that higher activity concentration of technetium tricarbonyl improved RCP. The radiolabeling reaction had better RCP in PBS buffer, which was in accordance with Williams *et al.* (2021) results that phosphate ions might bridge between cationic residues and $[^{99m}Tc(CO)_3(H_2O)_3]^+$ complex cation. The best incubation time was 2 hr at 50°C, increasing time had not better results. Radiolabeled scFv showed high stability in buffer and no release of technetium in competition with histidine after 24 h incubation.

Conclusion

His-tagged scFv fragments of monoclonal antibodies have better pharmacokinetic properties than whole antibodies. Radiolabeled scFvs are considered for targeted imaging and treatment. Technetium tricarbonyl provides radiolabeling of scFvs without losing its biological activity in a fast and easy procedure. In this study, we

optimized radiolabeling of a his-tagged scFv with technetium tricarbonyl using boranocarbonates. The results are promising and will be used for future studies.

Ethical Statement

This work did not contain any animal and human studies. This work was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (approval code: IR.SBMU.PHARMACY.REC.1401.068).

Competing Interests

The authors declare no conflict of interest.

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Authors' Contribution

S. Shahhosseini, E. Mohit and A. Yazdani developed the idea. S. Joukar, N. Bozorgchami, and D. Hatamabadi did the practical work and analyzed the data. E. Javani and M. Kordi participated in writing the manuscript. All authors contributed in discussion, results, and writing the manuscript.

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