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Original Article

Synthesis, Radiolabeling and Stability Studies of Peptide HYNIC-LIKKP-Pyr-F with ^{99m}Tc as an Apoptosis Imaging Agent

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HIGHLIGHTS

- A noninvasive method for apoptosis imaging is the usage of radiolabeled affinity ligands.
- A new derivative of LIKKPF with an affinity for phosphatidylserine was synthesized.
- The LIKKPF peptide was radiolabeled with ^{99m}Tc.

ABSTRACT

A non-invasive method for detecting phosphatidylserine (PS) exposure on the outer surface of plasma membranes, such as nuclear imaging, could aid in the diagnosis and treatment of diseases associated with apoptosis. Annexin V has been the most researched imaging agent for apoptosis to date. Due to Annexin V's limitations, additional agents, such as small peptides and molecules, have been introduced, including LIKKPF developed by Burtea *et al.* In this study, HYNIC-LIKKP-Pyr-F, a derivative of LIKKPF was prepared using the 9-fluoroenylmethoxycarbonyl (Fmoc) method, radiolabeled with Technetium-99m (^{99m}Tc) with the use of stannous chloride ($SnCl_2$) as a reducing agent and ethylenediamine diacetate (EDDA) and tricine as co-ligands. Radiochemical purity, labeling efficiency, and stability of radiopeptide in normal saline and human plasma were determined using thin layer chromatography (TLC). The partition coefficient of the radiolabeled peptide was measured in a combination of PBS (pH 7.4) and n-octanol. Specific activity was also measured. LC-MS was used to examine the synthesized peptide. The peptide was stable in human serum for at least 4 hours. Peptide was radiolabeled with ^{99m}Tc with radiochemical purity and labeling efficiency over 95% and 90%, respectively. Radiopeptide was stable in saline and human serum for at least 4 hours. The radiolabeled peptide has a great deal of potential as an apoptosis imaging agent for *in vitro* and *in vivo* experiments.

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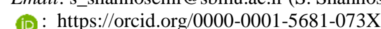
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Introduction

The timely and fast diagnosis of apoptosis is important for the appropriate treatment of diseases, such as

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cancers, atherosclerosis, myocardial infarction and ischemia (Blankenberg *et al.*, 1998, 2000; Hanshaw and Smith, 2005; Stace and Ktistakis, 2006; Blankenberg, 2008; Schutters and Reutelingsperger, 2010). Different targets can be used for imaging the apoptosis, such as phosphatidylserine (PS), phosphatidylethanolamine (PE), depolarized mitochondria membrane, activated caspase, and externalized histone H1. Among apoptosis biomarkers that can be used for imaging studies, PS and PE are better than the others, because of their wide expression, easy accessibility, and fast externalization on the external surface of the plasma membrane of apoptotic cells (Mosayebnia *et al*., 2020). Annexin V is a 35 kDa, endogenous protein with high affinity to PS. Annexin V quickly clears from the body and is unable to detect between dead cells and apoptosis. For these reasons, Annexin V is not suitable for apoptosis imaging (Post *et al.*, 2002; Li *et al.*, 2008). Burtea *et al*. (2009) introduced two peptides that are specifically bound to PS using the phage display method. One of the Peptides, LIKKPF (Leu-Ile-Lys-Lys-Pro-Phe), showed a high affinity for PS through *in vitro* studies.

Since most nuclear medicine procedures are noninvasive, the labeling of targeted ligands with appropriate radioisotopes for apoptosis imaging studies was considered the aim of this study. Radiopeptides are generally developed against specific targets for *in vivo* and *in vitro* studies in nuclear medicine.

In this study, PS was considered the target, since PS is one of the most abundant phospholipids in the inner leaflet of cell membranes and a peptide (LIKKPF) was reported as a lead ligand with a high affinity for PS. Our team has worked on this ligand in several studies (Khoshbakht et al., 2016a; Khoshbakht *et al.*, 2016b; Khoshbakht *et al.*, 2016c).

In another study, new derivatives of LIKKPF were prepared, functionalized with aminooxy (Aoe) at N-terminal and radiolabeled with ^{18}F-FDG (2-deoxy-2-fluoro-D-glucose) (Khoshbakht *et al.*, 2019). The specific activities of these radiolabeled peptides were low. The ^{18}F-FDG -Aoe-LIKKP-Pyr-F showed more affinity to PS compared to other analogs (K_d=0.52 µM), however, it was less than expected and in comparison to ^{99m}Tc-6-Hydrazinonicotinamide (HYNIC)-LIKKPF (K_d=2 nM). The radiopharmaceutical ^{18}F-FDG was also used for the radiolabeling of Aoe-LIKKP-Pyr-F with ^{18}F, which contained glucose, and resulted in low specific activity and higher K_d (low affinity) of radiopeptides. To improve specific activity and affinity, we decided to radiolabel LIKKP-Pyr-F with ^{99m}Tc, which is one of the best radioisotopes for routine clinical single photon emission computed tomography (SPECT) studies. ^{99m}Tc is cheap and easily available through ^{99}Mo/^{99m}Tc generators in addition to its perfect radiation physical characteristics [isomeric transition (IT), 6h, 140 keV (90%)] and its known chemistry (Schibli and Schubiger, 2002).

In this study, the peptide LIKKP-Pyr-F was synthesized, functionalized with HYNIC, and radiolabeled with ^{99m}Tc. The stability of HYNIC-LIKKP-Pyr-F was determined in human plasma. The radiochemical purity, labeling efficiency, log P and stability studies of ^{99m}Tc- HYNIC- LIKKP-Pyr-F were also studied.

Materials and Methods

Amino acids and resin were purchased from Bachem for this study (Bubendorf, Switzerland). Coupling reagents, n-hydroxy benzotriazole (HOBT) and diisopropylcarbodiimide (DIC) were purchased from Sigma-Aldrich (St. Luis, MO, USA). Succinimidyl-N-Boc-HYNIC were purchased from ABX advanced Biochemical compounds GmbH (Radeberg, Germany). All of the chemicals, solvents, and reagents were of analytical quality and could be used right away. Thin layer chromatography (TLC) was performed using Merck Silica gel 60 F254 pre-coated aluminium sheets. $Na^{99m}TcO_4$ was eluted from a ^{99}Mo/^{99m}Tc generator using normal serum (Pars-isotope, Tehran, Iran).

Synthesis of peptide LIKKPF-Pyr-F *functionalized with HYNIC (HYNIC-LIKKPF*-Pyr-F*)*

The peptide LIKKPF-Pyr-F was synthesized based on our previous work (Khoshbakht *et al.*, 2019). Succinimidyl-N-Boc-HYNIC was used to couple HYNIC to the N-terminal leucine (L) amino acid. Briefly, 4-6 equivalent (eq) of Fmoc-(4-pyridyl)-D-Ala-OH was added to 0.5 mg swollen Wang resin along with 2 eq HATU (Hexafluorophosphate azabenzotriazole tetramethyl uranium) and 3 eq DIPEA (diisopropylethylamine) as coupling reagents. Protecting groups were removed by 10% piperidine in dimethylformamide (DMF). Then, the next Fmoc-protected amino acid was coupled using 2 eq HOBT and DIC as coupling reagents. The procedure was repeated until the last amino acid, leucine, was coupled. A solution of 2 eq of Succinimidyl-N-Boc-HYNIC and 2 eq DIPEA (diisopropylethylamine) was used to couple HYNIC to leucine. The identity of the peptide was confirmed by liquid chromatography- mass spectrophotometry (LC-MS) (triple Quad 6410 Agilent Technologies using series 1200 high performance liquid chromatography (HPLC) system, Tokyo, Japan; column: C-18, 250×4.6 mm, 5 µm) using the mobile phase of A: H2O + 0.1% TFA (trifluoroacetic acid), B: acetonitrile at the flow rate of 1 $mL.min^{-1}$, and total run time of 40 min.

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Stability of HYNIC-LIKKPF-Pyr-F *in human serum plasma*

The stability of peptide in human plasma was determined by adding 450 µL fresh human serum to 50 µL peptide and incubated at 37°C for 5, 10, 30, 60, and 120 min and 24 h. Serum proteins were precipitated by adding 500 µL of acetonitrile. The mixture was centrifuged for 10 minutes at 10000 g. Then, LC-MS was used to examine the supernatant.

Radiolabeling of HYNIC-LIKKPF-Pyr-F with ^{99m}Tc

The HYNIC- LIKKP-Pyr-F was radiolabeled with ^{99m}Tc based on the authors' previous works (Khoshbakht, *et al.*, 2016a; Khoshbakht, *et al.*, 2016b). Stannous chloride ($SnCl_2$) was used as a reducing agent. Ethylenediamine diacetate (EDDA) and tricine (N-[tris (hydroxymethyl) methyl] glycine) were used as co-ligands. Sodium pertechnetate ($Na^{99m}TcO_4$) was milked from ^{99}Mo/^{99m}Tc generator. Different concentrations of peptide and co-ligands along with different activities were applied to reach optimal conditions. To a vial containing 100 µL of peptide (1 µg/µL), 0.5 mL of solution C, 30 µL $SnCl_2$ freshly prepared (1 mg/mL of HCl 0.1M), and 185-370 MBq of $Na^{99m}TcO_4$ were added and incubated at 100°C for 30 min. Solution C is a mixture of equal volumes of solution A and B; Solution A: 60 mg tricine in 1.5 mL phosphate buffer pH 5, and Solution B: 30 mg EDDA in 1.5 mL NaOH 0.1 M.

Radiochemical purity (RCP%) was obtained by TLC using silica gel as stationary phase, acetone, acetonitrile:water (1:1), and 0.1 M sodium citrate as mobile phases. $Na^{99m}TcO_4$ and $^{99m}TcO_2$ have R_f≈1 and R_f=0 on mobile phases, respectively, while radiolabeled peptide has R_f=0 in acetone and sodium citrate, R_f=1 in acetonitrile: water. ^{99m}Tc-co ligand appeared at R_f=1 in 0.1 M sodium citrate.

Log P determination

The amount of 3.7 MBq of the radiolabeled peptide was added to a vial containing 0.5 mL n-octanol and 0.5 mL normal saline. The mixture was vortexed for 5 min and centrifuged at 4000 g for 10 min. The activity of 100 µL of aqueous solution (normal saline) and 100 µL of the organic phase (n-octanol) was measured by gamma counter and used for the calculation of log P (ratio of organic phase activity to aqueous phase activity).

Stability of radiolabeled peptide in saline

To a vial containing 450 µL normal saline, 50 µL radiolabeled peptide (37 µBq/µL) was added and incubated at room temperature. At different time points (30, 60, 120 min and 4 h) percent of RCP was determined using TLC.

Stability of radiolabeled peptide in human serum

To a vial containing 450 µL human serum, 50 µL radiolabeled peptide (37 µBq/µL) was added and incubated at 37°C. At different time points (30, 60, 120 min and 4 h), 0.5 mL acetonitrile was added to precipitate plasma proteins and centrifuged for 10 minutes at 10000 g. Percent of RCP in the supernatant was determined using TLC. Counting the activity associated with the precipitate was performed to determine the activity of radiolabels bound to the plasma proteins.

Results and Discussion

During apoptosis or programmed cell death, PS appears on the external surface of the plasma membrane, which can act as a target for apoptosis imaging studies. Detection of apoptosis is important for the evaluation of response to the different treatments (follow-up) of various diseases, such as cancers (chemo/radiotherapy), cardiovascular diseases, etc. (Makin and Hickman, 2000).

PS is one of the best targets for apoptosis detection since it is exposed to the extracellular surface of the plasma membrane a few hours after apoptotic stimulation (Zhao, *et al.*, 2008). Compounds that have affinity and the ability to bind to PS can be radiolabeled and used in noninvasive methods for apoptosis imaging studies. Different compounds including small molecules, peptides, antibodies and their fragments have been introduced to target PS (Thapa *et al.*, 2008; Burtea *et al.*, 2009). LIKKPF is one of the first peptides obtained through the phage display technique with affinity in nano-molar range for PS (Zhao, *et al.*, 2008).

The peptide HYNIC-LIKKPF-Pyr-F (Fig. 1) was synthesized based on the solid phase technique (SPPS) and Fmoc/tBu method. The yield of synthesis was 40% and the identity of the peptide was confirmed by LC-MS. The molecular weight of HYNIC-LIKKPF-Pyr-F (with C44H68N12O8) was calculated as 881.53 Da, which was experimentally detected at m/z=882 [M+H]+. Analytical RP-HPLC also confirmed the peptide with a retention time (R_t) of 6.1 min in 20% A (TFA 1% in water) and 50% B (acetonitrile) as the mobile phase (Fig. 2).

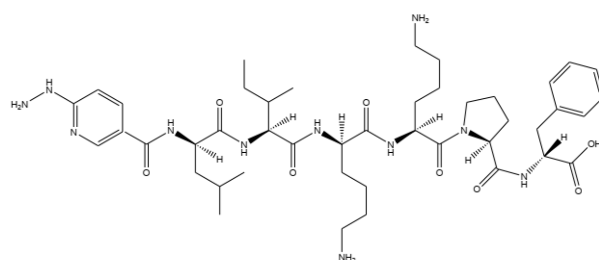

Figure 1. The structure of HYNIC-LIKKPF-Pyr-F.

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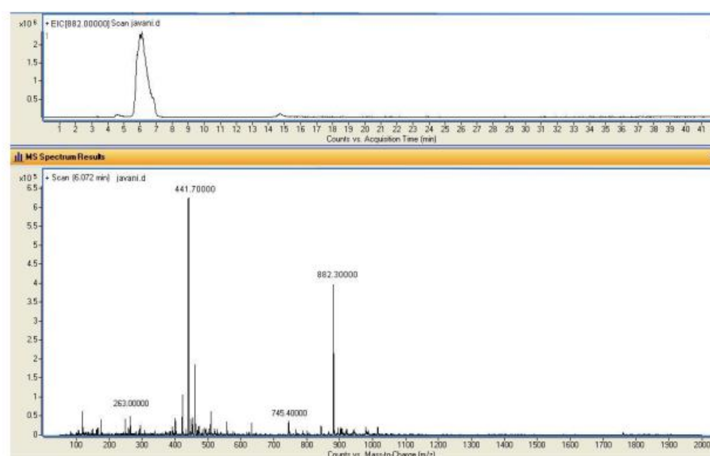

Figure 2. LC-MS chromatogram of HYNIC-LIKKPF-Pyr-F.

The peptide was stable in human plasma at 37°C for at least 4 h. The presence of a peak of 882 at all times confirms the stability of the peptide in human plasma. The log P of radiolabeled peptide (^{99m}Tc-HYNIC-LIKKPF-Pyr-F) was measured in a mixture of normal saline and n-octanol, which was -0.91.

Radiolabeling of the peptide was done in different conditions. The optimum conditions for radiolabeling HYNIC-LIKKPF-Pyr-F with ^{99m}Tc, which its RCP was more than 95%, were (100) µg peptide, 5 mg EDDA, 10 mg tricine, 30 µg $SnCl_2$, 185-370 MBq ^{99m}Tc, pH 5-6, and 30 minutes at 100°C. The specific activity of the product was 14800 GBq/mmol. The radiolabeled peptide was 95 percent intact after 4 hours, with just 10% of the ^{99m}Tc released or transferred to serum proteins (Fig. 3).

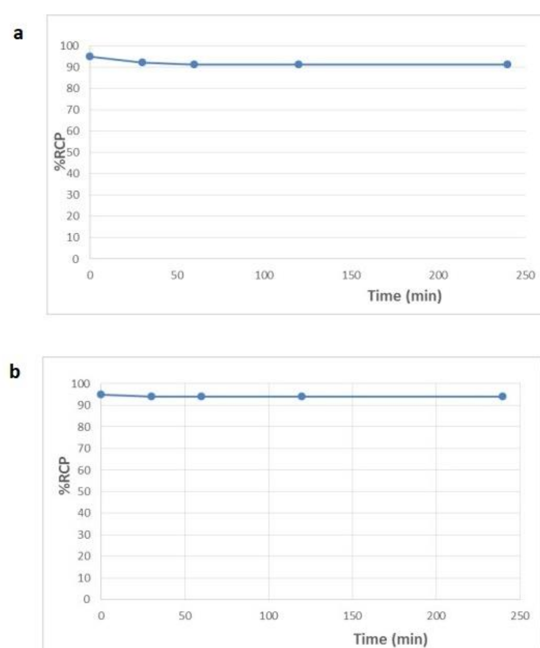

Figure 3. Stability of ^{99m}Tc- HYNIC-LIKKPF-Pyr-F in (a) saline, (b) human plasma.

In this study, a peptide was synthesized and functionalized with HYNIC to be labeled with ^{99m}Tc (HYNIC-LIKKP-Pyr-F). LC-MS was used to confirm the structure. The peptide was stable in human serum plasma for at least 4 h at 37°C. The peptide was radiolabeled with ^{99m}Tc. The radiochemical purity and labeling efficiency were over 95% and 90%, respectively. LogP peptide was -0.91 indicating hydrophilic characters. The radiolabeled peptide was stable in saline and human serum plasma for at least 4 h. Compared to our previous studies on the new derivatives of LIKKPF, the specific activity of the radiolabeled peptide was 14800 GBq/mmol, which was more than ^{18}F-FDG-Aoe-LIKKP-Pyr-F (74 GBq/mmole). In that study, the amino acid phenylalanine (F) was replaced with a synthetic amino acid, 4-pyridyl-D-Ala-OH. The resulting peptide LIKKP-Pyr-F was functionalized with aminooxy (Aoe) and radiolabeled with ^{18}F using ^{18}F-FDG as a prosthetic group. The radiopharmaceutical solution of ^{18}F-FDG contained glucose, which competed with ^{18}F-FDG for radiolabeling of the peptide. The specific activity of that radiolabeled peptide (^{18}F-FDG-Aoe-LIKKP-Pyr-F) was as low as 74 GBq/mmole and resulted in low affinity (K_d=0.52 µM). It might be due to the competition between the radiolabeled peptide and cold peptide for PS. Although the amount of affinity obtained was low, however, it was high enough for specific binding to apoptotic cells *in vitro* and *in vivo* (Khoshbakht *et al.*, 2019). In this study, to improve the specific activity and affinity, we decided to radiolabel peptide with ^{99m}Tc, which is easily available through ^{99}Mo/^{99m}Tc generators, cheap with perfect radiation physical characteristics for imaging studies (Schibli and Schubiger, 2002). We hope that newly synthesized radiopeptide with high specific activity can show high affinity to PS in future *in vitro* and *in vivo* studies.

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Conclusion

Timely and quick diagnosis of apoptosis is important for disease treatment, such as cancer and cardiovascular disease treatment. Radiopeptides, which have an affinity to PS and can specifically bind to it, are the best option for noninvasive nuclear medicine methods. In continuation of our previous research, HYNIC-LIKKP-Pyr-F was synthesized and radiolabeled with ^{99m}Tc. It exhibited high specific activity that makes the radiopeptide a good candidate for *in vivo* and *in vitro* studies. The ^{99m}Tc-HYNIC-LIKKP-Pyr-F can have a great potential to bind to PS with K_d in nano-molar range. More research regarding this radiolabeled peptide is on the way.

Ethical Statements

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.1401.018).

Competing Interests

The authors declare no conflict of interest.

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Authors' Contribution

S. Shahhosseini and S. Khoshbakht developed the idea. E. Javani did the practical work. S. Amidi analyzed some results. S. Khoshbakht and M. Kordi helped in writing the manuscript. All authors contributed to the discussion, results, and writing the manuscript.

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