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Original Article

Synthesis and Labeling of Two Fibrin-Targeted Peptides (HYNIC-GPRPILE, HYNIC-GPKGAAD) Using Technetium-99m and In Vitro Evaluation of Fibrin Binding and Platelet Aggregation

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ABSTRACT

Early detection of thrombus and its location in the body are critical factors for the treatment of thrombosis related diseases. Fibrin is the main component of thrombus, abundant in all thrombi, and is not found in non-pathological conditions. The presence of fibrin in all types of thrombi and its low concentration in blood makes it a sensitive and specific target for imaging studies of thrombus. Fibrin also accumulates in malignant tumors. Thus, fibrin imaging can be used in oncology, atherosclerosis, and thrombosis-related pathologies such as pulmonary emboli and deep vein thrombosis. Different compounds such as antibodies, nanoparticles, and peptides have been studied for fibrin imaging. Among them, peptides are more attractive because of better pharmacokinetics, simple and cheap preparation, and better radiolabeling methods. In this study, two peptides (HYNIC-GPRPILE, HYNIC-GPKGAAD) designed to target fibrin were synthesized. The peptides were identified by LC-MS. The stability and platelet aggregation of peptides were determined. Peptides were radiolabeled with ^{99m}Tc using HYNIC as chelating agent. The release of ^{99m}Tc and fibrin binding of radiopeptides were evaluated. Based on the results, peptides were stable in human plasma for at least 6 h and had no effect on platelet aggregation. Peptides were radiolabeled with pertechnetate at 80°C in 30 min. Radiochemical purity was over 95%. Radiopeptides were stable in human plasma and there was less than 5% release of ^{99m}Tc. The fibrin binding of radiopeptides was 70%>. Since peptides had no platelet binding activity, it can be concluded that binding of radiopeptides to fibrin is specific.

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Introduction

Thromboembolic diseases are the most important causes of mortality and disability in western societies. Early detection of thrombus and its location in the body are critical factors for the treatment of thrombosis related

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diseases. The diagnostic methods which are currently used are: Ultrasonography, CT scan and MRI. In these procedures, only changes in the anatomy of the arteries are examined, and the thrombus must be large enough to be diagnosed (Starmans et al., 2013a; Starmans et al., 2015).

Fibrin is the product of polymerization of fibrinogen and one of the essential components of thrombus. Fibrin is abundant in all thrombi and is not found in non-pathological conditions. The presence of fibrin in all types of thrombi indicates the sensitivity and specificity of fibrin as a target for the detection of thrombus. The low concentration of fibrin in blood makes it a perfect target for imaging studies of thrombus as well (Thakur et al., 2000; Aruva et al., 2006).

Studies have shown that fibrin accumulates in many malignant tumors and plays a vital role in tumor stroma formation, angiogenesis, and metastasis. Thus, fibrin imaging can be used in oncology, atherosclerosis, and thrombosis-related pathologies such as pulmonary emboli and deep vein thrombosis (Starmans et al., 2015).

Different compounds such as antibodies, nanoparticles, and peptides have been studied for fibrin imaging (Rosebrough et al., 1989; Hara et al., 2012; Chung et al., 2014; Kongsuphol et al., 2014). Among them, peptides are more attractive because of better pharmacokinetics, simple and cheap preparation, and better radiolabeling methods (Knight, 2001; Fang et al., 2011; Ciesienski et al., 2013; Starmans et al., 2013; Starmans et al., 2015).

There is currently no radiopharmaceutical for early detection of Thrombus. A radiopharmaceutical that can be bound to thrombus components such as fibrin and accumulate, will be useful for whole body imaging studies of thrombus.

In our previous studies, some peptides were designed and evaluated on docking studies (Rezaeianpour et al., 2017; Rezaeianpour et al., 2018; Mosayebnia et al., 2018). In this study, two peptides (HYNIC-Gly-Pro-Arg-Ile-Leu-Glu: HYNIC-GPRPILE, HYNIC-Gly-Pro-Lys-Gly-Ala-Ala-Asp: HYNIC-GPKGAAD) which showed good affinity on docking studies were selected, synthesized and radiolabeled with Technetium-99m. The stability studies, radiochemical purity, platelet aggregation, and fibrin binding tests were performed.

Materials and Methods

Chemicals

Amino acids and resin were bought from Bachem (Japan). Solvents were purchased from Merck Chemicals and Sigma (USA). NS-HYNIC were bought from Kimia Pajouh Dorsa, Tehran, Iran.

Synthesis of HYNIC-GPRPILE and HYNIC-GPKGAAD peptides

Peptides were synthesized using Fmoc solid-phase method and the authors' previous studies (Rezaeianpour et al., 2017; Rezaeianpour et al.,2018). In brief, the resin 2-chlorotrytil chloride (1 g) was activated by shaking with 1 mL dimethylformamid (DMF) and 9 mL dichloromethane (DCM) for 1 h. The first amino acid was coupled to resin by adding a mixture of 8 equivalent Diisopropyl ethyl amine (DIEA) and one equivalent of Glutamic acid in 10 mL DMF and incubation for 1 h at room temperature. The unreacted amino acids were separated by washing with DMF and the attachment of amino acid to resin was tested using Kaiser test. After removing the Fmoc of first amino acid, a mixture of 3 equivalents of second amino acid (dissolved in 1 mL DCM and 5 mL DMF) and 3 equivalents of DIEA and TBTU [2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyl-aminium tetrafluoroborate] were added to resin and incubated for 2 h. The coupling and washing steps were repeated until all amino acids in sequence were coupled as explained above. Succinimidyl-N-boc-6-hydrazino-niconinamide (HYNIC) was attached as the last amino acid to the sequence. The sequence was cleaved from resin using DCM and trifluoroacetic acid (TFA 1%). The peptides were purified by preparative HPLC on a C-18 reversed phase column (Table 1). The structure of peptides was determined with high resolution LC-MS triple Quad 6410 (Agilent) with a series 1200 HPLC column (Japan, C-18, 250×4.6 mm, 5 µm), mobile phase: A: H2O+0.1% TFA, B: acetonitrile, flow rate: 1 mL/min, volume of injection 20 µL, total run time 40 min.

Table 1. Program of preparative HPLC, A: acetonitrile/water (70/30) and B: NaH_2PO_4/water (10 mM), UV 220 nm

Time (min)	Flow rate (mL/min)	%A	%B
0	15	0	100
2	15	0	100
5	15	20	80
15	15	20	80
17	15	30	70
25	15	30	70
27	15	100	0
35	15	100	0
40	15	0	100

Stability of peptides in human plasma

A solution of Peptides (200 µL, 400 µg) were incubated with 1800 µL human plasma at 37 °C. At different time

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points (2, 4, and 6 h), 500 µL of solution was filtered using micro tube (cut off 10 kDa) at 14000 rpm for 10 min. The peptides were analyzed using LC-Mass.

Platelet aggregation test

In this experiment, the platelet aggregation in response to different inducers in presence of peptides is evaluated. The effect of peptides on platelet aggregation was determined using light transmission aggregometry. In brief, platelet-rich plasma (PRP) and platelet-poor plasma were prepared from citrated blood samples. PRP (200 µL) and peptide (1 µL of 0.05 and 0.1 mM) were incubated in test cuvettes at 37 °C in APACT-4004 aggregometer (LABiTec, Ahrensburg, Germany) for 5 min. Control cuvettes were incubated with 1 µL of DMSO instead of peptide. At the end of incubation, Arashidonic acid (AA) collagen, and adenosine diphosphate (ADP) were separately used to induce platelet aggregation. The changes in the optical density were recorded, and the results are expressed as percentage of inhibition by comparison with those measured for DMSO alone (Amidi et al., 2013).

Radiolabeling of peptides with ^{99m}Tc

Peptides were radiolabeled with Tecnethium-99m using HYNIC as chelating agent, ethylenediaminediacetic acid (EDDA) and tricine as co-ligand. A solution of 250 µL of tricine (40 µg/µL of phosphate buffer pH5) and 250 µL of EDDA (20 µg/µL of NaOH 0.1 M) was added to a vial containing 20 µg peptide in ammonium acetate buffer. Stannous chloride (7 µL, 1 µg/µL of HCl 0.1 M) and sodium pertechnetate (5-10 mCi) were added to the vial. The vial was kept at 80°C for 30 min. The radiolabeled peptides were purified using activated C18 cartridge. Ethanol was used to wash the peptides from cartridge. After evaporation of ethanol, radiopeptides were dissolved in normal saline and radiochemical purity of radiolabeled peptides was determined by TLC using TLC-SG as stationary phase and methyl ethyl ketone (MEK), acetonitrile:water (1:1), and sodium citrate 0.1 M pH5 as mobile phases.

Log P determination

The solubility of radiopeptides in organic and aqueous phases (partition coefficient) was determined. To a mixture of 500 µL of PBS and 500 µL n-octanol, 50 µL of radiopeptide was added and vortexed. The radioactivity of 100 µL of each phase was measured. The ratio of radioactivity in n-octanol to PBS was determined and presented as log P.

Stability of radiopeptide in normal saline and human plasma

The solution of radiopeptides in normal saline was kept at room temperature and at different time points (2, 4, and 6 h) radiochemical purity was determined using TLC. A mixture of radiolabeled peptide (50 µL) and human plasma (450 µL) was incubated at 37 °C. At different time points (2, 4, and 6 h) 500 µL of cold acetonitrile was added to the mixture. The mixture was centrifuged for 10 min at 10000 g. The radioactivity of pellet and supernatant was measured.

Fibrin binding

A mixture of radiopeptide (100µCi) and thromboplastin reagent (5µL) was added to human plasma and incubated for 30 min at 37 °C. The pellet of fibrin was separated by centrifuge, washed with normal saline and the radioactivity was measured and compared with the total radioactivity.

Results and Discussion

Peptides synthesis and characterization

Thrombus is the main factor of life threatening diseases such as myocardial infarction, deep vein thrombosis (DVT), pulmonary embolism (PE), and stroke. Detection, location and on time treatment of thrombus is in high priority. Among the components of thrombus, fibrin is the best target for imaging of thrombus. Fibrin is resulted from fibrinogen polymerization. It is an important protein of hemostasis and has 3 protein chains, alpha, beta and gamma. There is high concentration of fibrin (20-200 µM) in all types of thrombus (acute, chronic, veins and arteries). The presence of fibrin in all types of thrombus indicates its high sensitivity and specificity for thrombus imaging (Knight, 2001; Starmans et al., 2013b). Fibrin plays roles in angiogenesis and metastasis as well. So, fibrin-targeted peptides will be good candidates for detection and imaging studies of thrombus and tumors. Imaging of fibrin provides the possibility of diagnosis of thrombus with high sensitivity and less limitations compared to other methods currently in use such as ultrasonography, MRI, CT-scan. In these methods, anatomical changes are evaluated and thrombus should be large enough to be diagnosed.

Antibodies, nanoparticles and peptides labeled with radioisotopes or fluorescents have been introduced by different researchers for imaging studies of fibrin.

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Targeted peptides for fibrin detection have been studied more than the other agents (Knight, 2001; Starmans et al., 2013b) .

In our previous studies, some fibrin-targeted peptides were designed, radiolabeled with ^{99m}Tc or ^{18}F, and evaluated in vitro and in vivo (Rezaeianpour et al., 2017; Rezaeianpour et al., 2018; Mosayebnia et al., 2018). Peptides were designed based on Pet/composer and Hex protein docking programs. The peptides that fit and dock to fibrin well like pieces of a three dimensional jigsaw puzzle are considered as good candidates for the study. Regarding molecular dynamics and binding affinities using free energy simulations, two peptides were chosen for the study. In this study, these two peptides (HYNIC-GPKGAAD and HYNIC-GPRPILE) were synthesized based on solid phase peptide synthesis and Fmoc strategy. The identity of peptides was confirmed by LC-MS. The peptides were stable in human plasma and also had no effect on platelet aggregation, which indicates that peptides do not bind to platelet receptors. Peptides (HYNIC-GPRPILE, HYNIC-GPKGAAD) were synthesized using standard Fmoc strategy, functionalized with HYNIC at the N-terminal and analyzed by LC-MS.

HYNIC-GPRPILE calculated for $C_{41}H_{65}N_{13}O_{11}$: 915.5, m/z = $[M+H]^+$ 916.5; $[M+H]^+/2$ 458.25. Analytical RP-HPLC: R_t=2.007 min; 85% (H_2O+0.1% TFA), 15% acetonitrile (Fig. 1).

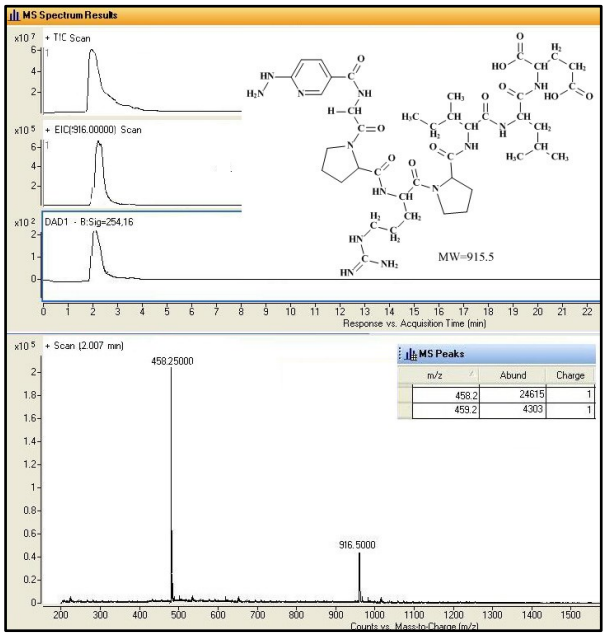

Figure 1. Liquid chromatography-mass spectrometry (LC-Ms) chromatogram of HYNIC- GPRPILE

HYNIC-GPKGAAD calculated for $C_{31}H_{47}N_{11}O_{11}$: 749.3, m/z = $[M+H]^+$ 750.3; analytical RP-HPLC: R_t=10.006 min; 85% (H_2O+0.1% TFA), 15% acetonitrile (Fig. 2).

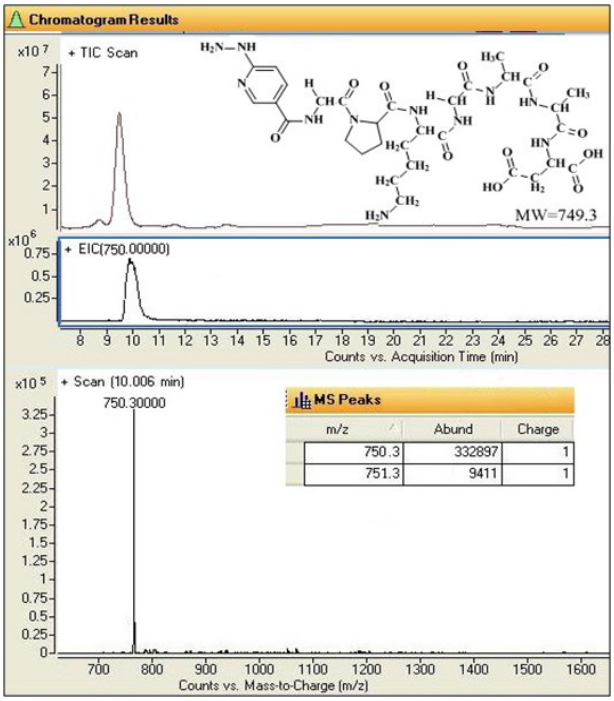

Figure 2. Liquid chromatography-mass spectrometry (LC-Ms) chromatogram of HYNIC-GPKGAAD

The stability of peptides in human plasma at 37 °C was analyzed using LC-MS and results showed that peptides were stable at least for 6 h.

The Platelet aggregation test showed that the presence of peptides had negligible effect on inhibition of platelet aggregation in all agonist induced aggregation studies.

Stability of radiopeptides and their fibrin binding

Peptides conjugated with HYNIC were radiolabeled with ^{99m}Tc using tricine and EDDA as co-ligands. The radiochemical purity was determined by TLC using 3 different mobile phases. Radiochemical purity, specific activity, log P, and fibrin binding of radiopeptides are presented in Table 2. Radiopeptides were stable in normal saline and human plasma for at least 6 h.

Technetium-99m is one of the best radioisotopes for imaging studies since it's cheap and easily available through ^{99}Mo/^{99m}Tc generator. The physical characteristics of technetium-99m is perfect for imaging studies [half-life 6 h, energy 140 keV (90%)]. The chemistry of technetium is very well known and it can form covalent-coordinate bonds with different ligands in very simple chemical reactions (Schibli and Schubiger, 2002).

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Table 2. Characteristics of radiopeptides

Peptide	% Radiochemical purity, mean ± SD	Specific activity (Ci/mmol), mean ± SD	Log P	% Fibrin binding, mean ± SD
^{99m}Tc-HYNIC-GPKGAAD	96.7 ± 5.1	374.6 ± 2.04	-2.58	71.5 ± 3.4
^{99m}Tc-HYNIC-GPRPILE	97.05 ± 6.02	457.7 ± 3.08	-1.83	72 ± 5.6

Peptides were radiolabeled with ^{99m}Tc through HYNIC as chelating agent, tricine and EDDA as co-ligands. Different concentrations of peptides (5-200 µg) in a few buffer systems (ammonium acetate, phosphate buffer saline, sodium bicarbonate, saline, citrate) at different temperatures (37, 40, 70, and 80 °C) for 10, 15, 30, and 45 min were incubated with 5-10 mCi of $Na^{99m}TcO_4$. Stannous chloride ($SnCl_2$) was used as reducing agent. The radiochemical purity over 95% was obtained with 20-50 µg peptide in ammonium acetate buffer at 80°C for 30 min.

Results showed that peptides were radiolabeled with high specific activity and radiochemical purity with ^{99m}Tc, and showed a good binding to the fibrin (70%>). Since they had no effect on platelet aggregation in the presence of inducers (AA, ADP, and collagen), their binding to fibrin is specific. The stability of these peptides in human plasma as well as log P indicate that they are good candidates for fibrin imaging studies. Further in vitro and in vivo studies on these compounds are ongoing.

Conclusion

In this study, two fibrin-targeted peptides were synthesized, radiolabeled and evaluated in vitro. Based on the data presented here, these peptides appear to be promising agents for molecular imaging of thrombus. Biological evaluation of radaiolabeled peptides in vivo is necessary for further studies.

Ethical Statement

This article does not contain human subjects and/or animals study.

Competing Interests

The authors declare that they have no conflict of interest.

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Authors' Contribution

Soraya Shahhosseini conceived of the presented idea. Maliheh Rezaeianpour designed and synthesized the peptides. Sara Sheibani and Sara Hadadi radiolabeled and evaluated the radiopeptides and have contributed equally to this work. Salimeh Amidi and Soraya Shahhosseini verified the methods and results. All authors contributed to the final manuscript.

References

Amidi, S., Kobarfard, F., Moghaddam, A.B., Tabib, K. and Z. Soleymani, (2013). ″Electrochemical synthesis of novel 1, 3-indandione derivatives and evaluation of their antiplatelet aggregation activities.″ *Iranian Journal of Pharmaceutical Research: IJPR*, **12**(Suppl): 91-103.

Aruva, M.R., Daviau, J., Sharma, S.S. and M.L. Thakur, (2006). ″Imaging thromboembolism with fibrin-avid 99mTc-peptide: evaluation in swine.″ *Journal of Nuclear Medicine*, **47**(1): 155-162.

Chung, E.J., Cheng, Y., Morshed, R., Nord, K., Han, Y., Wegscheid, M.L., Auffinger, B., Wainwright, D.A., Lesniak, M.S. and M.V. Tirrell, (2014). ″Fibrin-binding, peptide amphiphile micelles for targeting glioblastoma.″ *Biomaterials*, **35**(4): 1249-1256.

Ciesienski, K.L., Yang, Y., Ay, I., Chonde, D.B., Loving, G.S., Rietz, T.A., Catana, C. and P. Caravan, (2013). ″Fibrin-targeted PET probes for the detection of thrombi.″ *Molecular Pharmaceutics*, **10**(3): 1100-1110.

Fang, W., He, J., Kim, Y.S., Zhou, Y. and S. Liu, (2011). ″Evaluation of ^{99m}Tc-labeled cyclic RGD peptide with a PEG4 linker for thrombosis imaging: comparison with DMP444.″ *Bioconjugate Chemistry*, **22**(8): 1715-1722.

Hara, T., Bhayana, B., Thompson, B., Kessinger, C.W., Khatri, A., McCarthy, J.R., Weissleder, R., Lin, C.P., Tearney, G.J. and F.A. Jaffer, (2012). ″Molecular imaging of fibrin deposition in deep vein thrombosis using fibrin-targeted near-infrared fluorescence.″ *JACC: Cardiovascular Imaging*, **5**(6): 607-615.

Knight, L.C. (2001). ″Radiolabeled peptide ligands for imaging thrombi and emboli.″ *Nuclear Medicine and Biology*, **28**(5): 515–526.

Kongsuphol, P., Arya, S.K., Wong, C.C., Polla, L.J. and M.K. Park, (2014). ″Coiled-coil peptide based sensor for ultra-sensitive thrombin detection.″ *Biosensors and Bioelectronics*, **55:** 26-31.

Mosayebnia, M., Hajiramezanali, M., Shahhosseini, S., Bozorgi, A.H., Kobarfard, F. and S.Rezaeianpour, (2018). ″Docking, synthesis, in-vitro evaluation, and optimization of reaction conditions for direct radiolabeling of CGPRPPC with technetium-99m through the GAGG sequence.″ *Nuclear Medicine Communications*, **39**(11): 976-982.

Rezaeianpour, S., Bozorgi, A.H., Moghimi, A., Almasi, A., Balalaie, S., Ramezanpour, S., Nasoohi, S., Mazidi, S.M., Geramifar, P., Bitarafan-Rajabi, A. and S. Shahhosseini, (2017). ″Synthesis and biological evaluation of cyclic [^{99m}Tc]-hynic-cgprppc as a fibrin-binding peptide for molecular imaging of thrombosis and its comparison with [^{99m}Tc]-HYNIC-GPRPP.″ *Molecular Imaging and Biology*, **19**(2): 256-264.

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Rezaeianpour, S., Mosayebnia, M., Moghimi, A., Amidi, S., Geramifar, P., Kobarfard, F. and S. Shahhosseini, (2018). ″[18F] FDG-labeled CGPRPPC peptide serving as a small thrombotic lesions probe, including a comparison with [^{99m}Tc]-labeled form.″ *Cancer Biotherapy & Radiopharmaceuticals*, **33**(10): 438-444.

Rosebrough, S.F., McAfee, J.G., Grossman, Z.D. and L.A. Schemancik, (1989). ″Immunoreactivity of ^{111}In and ^{131}I fibrin-specific monoclonal antibody used for thrombus imaging.″ *Journal of Immunological Methods*, **116**(1): 123-129.

Schibli, R. and A.P. Schubiger, (2002). ″Current use and future potential of organometallic radiopharmaceuticals.″ *European Journal of Nuclear Medicine and Molecular Imaging*, **29**(11): 1529-1542.

Starmans, L.W., van Duijnhoven, S.M., Rossin, R., Berben, M., Aime, S., Daemen, M.J., Nicolay, K. and H. Grüll, (2013a). ″Evaluation of ^{111}In-labeled EPep and FibPep as tracers for fibrin SPECT imaging.″ *Molecular Pharmaceutics*, **10**(11): 4309-4321.

Starmans, L.W., van Duijnhoven, S.M., Rossin, R., Aime, S., Daemen, M.J., Nicolay, K. and H. Grüll, (2013b). ″SPECT imaging of fibrin using fibrin-binding peptides.″ *Contrast Media & Molecular Imaging*, **8**(3): 229-237.

Starmans, L.W., van Mourik, T., Rossin, R., Verel, I., Nicolay, K. and H. Grüll, (2015). ″Noninvasive visualization of tumoral fibrin deposition using a peptidic fibrin-binding single photon emission computed tomography tracer.″ *Molecular Pharmaceutics*, **12**(6): 1921-1928.

Thakur, M.L., Pallela, V.R., Consigny, P.M., Rao, P.S., Vessileva-Belnikolovska, D. and R. Shi, (2000). ″Imaging vascular thrombosis with ^{99m}Tc-labeled fibrin α-chain peptide.″ *Journal of Nuclear Medicine*, **41**(1): 161-168.

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