

¹⁸F-Fluoroglucosylation of an arginine-arginine-leucine peptide as a potential tumor imaging agent for positron emission tomography

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ABSTRACT

Background: Based on the principle of oxime formation, ¹⁸F labeling of polypeptides can be achieved via a reaction between an aldehyde group-containing ¹⁸F-prosthetic group and an aminooxy-modified polypeptide. The focus of this study was to investigate the one-step synthesis of 2-[¹⁸F] fluoro-2-deoxyglucose (¹⁸F-FDG)- arginine-arginine-leucine (RRL) from open-ring ¹⁸F-FDG and the aminooxy-modified RRL peptide cyclo(RRLfK)-ONH₂ and to study the biological distribution of ¹⁸F-FDG-RRL in a nude mouse model of human neuroglioma. **Materials and Methods:** The aminooxy-modified RRL peptide cyclo(RRLfK)-ONH₂ was used as the precursor to react with ¹⁸F-FDG at 100 °C and different pH values for 30 minutes to synthesize ¹⁸F-FDG-RRL. The labeling yield, radiochemical purity, and *in-vitro* stability of the product were measured, and the biological distribution of ¹⁸F-FDG-RRL in tumor-bearing nude mice was analyzed at 30 minutes, 60 minutes and 120 minutes. **Results:** The labeling yield of ¹⁸F-FDG-RRL was (25.5±5.0) % at a pH of 2.0, and its radiochemical purity was greater than 95%. ¹⁸F-FDG-RRL was mainly excreted through the kidneys, with rapid blood clearance. One hour after injection, the uptake of ¹⁸F-FDG-RRL in tumors was (1.83±0.12) injected dose per gram of tissue (%ID/g), with a tumor/muscle ratio of 7.03±0.04, a tumor/blood ratio of 4.36±0.21 and a tumor/brain ratio of 7.53±1.37. **Conclusion:** The synthesis of ¹⁸F-FDG-RRL can be achieved through oximation. This method is straightforward and easy to promote. ¹⁸F-FDG-RRL has rapid blood clearance and high uptake by tumors.

Keywords: Oxime, 2-fluoro-2-deoxyglucose, arginine-arginine-leucine, peptide.

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INTRODUCTION

2-Fluoro-2-deoxy-D-glucose (¹⁸F-FDG) is currently the most commonly used imaging agent in positron emission tomography (PET) ⁽¹⁾. Its molecular structure is similar to that of glucose, so modifications similar to glycosylation can be achieved through [¹⁸F] labeling with ¹⁸F-FDG as a prosthetic group. ¹⁸F-FDG undergoes ring opening at 100°C and exists in both the cyclic and linear states. The aldehyde group at position 1 of linear ¹⁸F-FDG undergoes an oximation reaction with an aminooxy group

under acidic conditions to achieve ¹⁸F labeling of polypeptides ⁽²⁻⁷⁾.

In 2008, Wuest *et al.* first reported the preparation of ¹⁸F labeled tracers based on ¹⁸F-FDG ⁽⁶⁾ in the production of ¹⁸F-FDG-maleimide-hexyloxime (¹⁸F-FDG-MHO) by linking ¹⁸F-FDG to aminooxy-functionalized maleimide, with a yield of approximately 45%-69%. The direct labeling of aminooxy-modified peptides with ¹⁸F-FDG was reported by Namavari *et al.* ⁽⁵⁾ and Hultsch *et al.* ⁽⁸⁾. Namavari *et al.* directly labeled linear and circular arginine-glycine-aspartic acid (RGD)

modified with an aminooxyacetyl group using ¹⁸F-FDG. The reaction was carried out at 100°C and pH 1.5-2.5 for 60 minutes, and it had a yield of approximately 41.4%. Hultsch *et al.* used ¹⁸F-FDG to label an aminooxy-modified RGD cyclic peptide in a reaction carried out at 120°C and pH 2.5 for 20 minutes, and it had a yield of approximately 56-93%. Wuest *et al.* ⁽⁹⁾ also used ¹⁸F-FDG to label the monomer, dimer and tetramer of an aminooxy-modified neurotensin (8-13) fragment (NT (8-13)). The labeling yield of the product from the monomer was the highest (80%). Wuest *et al.* also believed that the substituted fluoro group at the α-position of the carbonyl group of open-ring FDG was likely to increase the carbonyl reactivity, which made it easier for the aminooxy group to react with ¹⁸F-FDG than with glucose. Simpson *et al.* ⁽¹⁰⁾ used ¹⁸F-FDG to label aminooxy-modified and PEGylated biotin derivatives, with a product labeling yield of up to 100% and low specific activity. Jammaz *et al.* ⁽¹¹⁾ employed ¹⁸F-FDG to label aminooxy-modified folic acid and methotrexate, with a labeling yield greater than 80% and a radiochemical purity greater than 98%.

Peptides could find important applications in targeting tumors and are good candidates because of their characteristic properties ^(12,13). Many studies have shown that the peptide arginine-arginine-leucine (RRL) can target tumor vascular endothelial cells and tumor parenchyma cells ^(14,15). In this study, ¹⁸F-FDG was used as a prosthetic group to undergo a condensation reaction with an aminooxy-modified RRL peptide to synthesize ¹⁸F-FDG-RRL in one step. The ¹⁸F-FDG-RRL was evaluated for its *in-vitro* stability and biodistribution. The RRL peptide was labeled with ¹⁸F for the first time, and the ¹⁸F-labeled RRL peptide was expected to be a potential PET imaging agent for tumor targeting.

MATERIALS AND METHODS

Peptide synthesis and modification

The RRL peptide sequence cyclo(RRLfK) was designed and subjected to aminooxy

modification to obtain cyclo(RRLfK)-ONH₂. The aminooxy group was attached to the side chain of the lysine residue. Cyclo(RRLfK)-ONH₂ was synthesized using solid-phase peptide synthesis followed by manual conjugation of Bis-Boc-aminooxyacetic acid with subsequent deprotection (Shanghai Science Peptide Biological Technology Co., Ltd., China).

Peptides were purified via reversed-phase high performance liquid chromatography (RP-HPLC) with an Inertsil ODS-SP HPLC column (5μm 4.6×250mm) (Shimadzu Corporation, Japan) eluted with a gradient from 98 to 0% solvent A (0.1% trifluoroacetic in 100% water) and 2–100% solvent B (0.1% trifluoroacetic in 100% acetonitrile) at 1.0 ml/minute, with monitoring at 214 nm. The purity of the peptide was determined by RP-HPLC as listed, and its identity was confirmed via mass spectral analysis (Shimadzu Corporation, Japan).

Radiolabeling

The preparation of 120 μL of ¹⁸F-FDG (HTA Co., Ltd., China) solution was carried out with the following specifications: ¹⁸F-FDG 4-6mCi, 16% aqueous ethanol-saline solution and 0.4% trifluoroacetic acid (TFA) (Tokyo Chemical Industry Co., Ltd., Japan). To the above solution, 2 mg of cyclo(RRLfK)-ONH₂ was added. The pH was adjusted to 2.0, 3.0, 4.0 and 5.0, respectively. The mixture was kept at 100°C for 30 minutes (figure 1).

The labeling yield and radiochemical purity of the labeled product ¹⁸F-FDG-c (RRLfK) were determined using RP-HPLC. Gradient elution was performed as follows: 0-3 minutes, 5% B; 3-33 minutes, 5%-65% B; 33-36 minutes, 65%-90% B; 36-39 minutes, 90% B; 39-42 minutes, 90%-5% B (buffer A = 0.1% TFA in acetonitrile, buffer B = 0.1% aqueous TFA solution). The flow rate was 1 mL/minute.

Determination of in-vitro stability

¹⁸F-FDG-RRL (100 μL, 3.7 MBq) was mixed well with 1 mL of fresh human serum and incubated at 37°C. The radiochemical purity was measured at 30, 60, 90, 120, 180, 240, 300 and 360 minutes by radio-HPLC (LabAlliance Company, USA).

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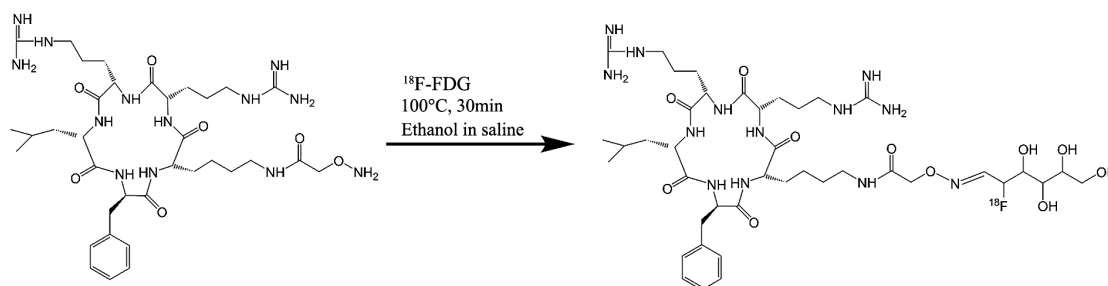


Figure 1. Schematic representation of the formation of ¹⁸F-FDG-c(RRLfK) from cyclo(RRLfK)-ONH₂ and ¹⁸F-FDG based on oxime formation at 100°C for 30 min.

Examination of biodistribution

The human malignant glioblastoma cell line U87MG was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Twenty male BALB/c nude mice, approximately 4-6 weeks of age and weighing 16-20 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The animal experiments were approved by the Animal Welfare and Ethics Committee of Qingdao University and strictly followed the standards in the "Guide for the Care and Use of Laboratory Animals" published by National Academies Press (US). A 0.2-mL single cell suspension (1×10^6 cells/mL on average) was injected subcutaneously into the right shoulder of the mice, and the long axis (a) and short axis (b) of the tumors were measured during tumor growth using a Vernier caliper. The tumor volume was calculated based on the formula $a \times b^2 / 2$. The biodistribution was examined when the tumor volume reached approximately 1.0 cm³.

The ¹⁸F-FDG-RRL solution (0.2 mL, 0.74 MBq) was injected via the tail vein into 9 tumor-bearing nude mice. At 30, 60 and 120 minutes after injection, the mice were euthanized via CO₂ inhalation. Samples of the blood, tumor, brain, heart, lungs, stomach, liver, intestines, kidneys and bone were collected, and the injected dose per gram of tissue (%ID/g) was calculated for each sample type. In addition, the tumor/blood, tumor/muscle and tumor/brain ratios were calculated.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. A *t*-test and one-way analysis of variance were employed. Data measurements

are expressed as ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was used for comparisons of variables. $P < 0.05$ was considered statistically significant.

RESULTS

Peptide synthesis and modification

Cyclo(RRLfK)-ONH₂ was successfully synthesized using solid-phase peptide synthesis followed by the manual conjugation of bis-Boc-aminoxyacetic acid with subsequent deprotection. HPLC analysis of the product indicated the formation of several compounds consisting of a major product (95.163%, retention time of 18.582 minutes) accompanied by at least 4 smaller peaks at retention times of 17.864, 18.361, 18.934 and 19.139 minutes (figure 2A). The mass spectrometry (ESI-MS) analysis of cyclo(RRLfK)-ONH₂ showed a mass peak with a *m/z* of 387.98 ([M+2H]²⁺) (figure 2B). The experimentally observed molecular weight (773.96) correlated well with the theoretical molecular weight (773.94).

Radiolabeling

¹⁸F-FDG-RRL was prepared from cyclo(RRLfK)-ONH₂ and ¹⁸F-FDG in a one-step method based on the mechanism of oximation. The reaction process is simple and easy to automate. The average labeling yields of three consecutive ¹⁸F-FDG-RRL syntheses at different pH values (2.0, 3.0, 4.0 and 5.0) were (25.5±5.0) %, (20.0±5.0) %, (20.0±4.0) %, and (2.5±2.0) %, respectively (figure 3). The radiochemical purity was above 95%.

¹⁸F-FDG-RRL was purified by HPLC, and the

radioactive trace (retention time of approximately 15.2 minutes) is basically consistent with the ultraviolet (UV) trace (retention time of approximately 14.2 minutes) (figure 4).

Analysis of in-vitro stability

The radiochemical purity was (96.1±0.39) %, (95.9±0.31) %, (95.5±1.38) %, (94.9±0.34) %, (93.9±0.61) %, (93.0±0.35) %, (91.9±0.46) % and (90.1±0.51) % at 30, 60, 90, 120, 180, 240, 300 and 360 minutes, respectively (figure 5).

In-vivo biodistribution

The biodistribution of ¹⁸F-FDG-RRL was measured in U87MG xenograft-bearing nude mice 2 hours after intravenous injection of the probe (table 1). ¹⁸F-FDG-RRL rapidly accumulated in the tumors, and its radioactivity

distribution was (2.74±0.25) % ID/g, (1.83±0.12) % ID/g and (1.24±0.16) % ID/g at 30, 60 and 120 minutes after injection, respectively. The radioactive distribution of ¹⁸F-FDG-RRL was high in the kidney, at (3.89±0.24) % ID/g, (1.95±0.15) % ID/g and (1.12±0.43) % ID/g at 30, 60 and 120 minutes after injection, respectively. ¹⁸F-FDG-RRL showed lower levels of radioactivity distribution in other tissues. The tumor/blood, tumor/muscle and tumor/brain ratios were relatively high and increased with time. The tumor/blood ratios were 3.19±0.04, 4.36±0.21 and 8.26±0.32 at 30, 60 and 120 minutes after injection, respectively; the tumor/muscle ratios were 5.07±0.10, 7.03±0.04 and 8.70±0.05, respectively; and the tumor/brain ratios were 5.71±0.08, 7.53±1.37 and 8.07±0.56, respectively (figure 6).

Figure 2. HPLC (A) and MS (B) analysis of cyclo(RRLfk)-ONH₂. HPLC analysis showed a major product (95.163%, retention time of 18.582 min) accompanied by 4 smaller peaks at retention times of 17.864, 18.361, 18.934 and 19.139 min. The MS analysis showed a mass peak with a m/z of 387.98 ([M+2H]²⁺).

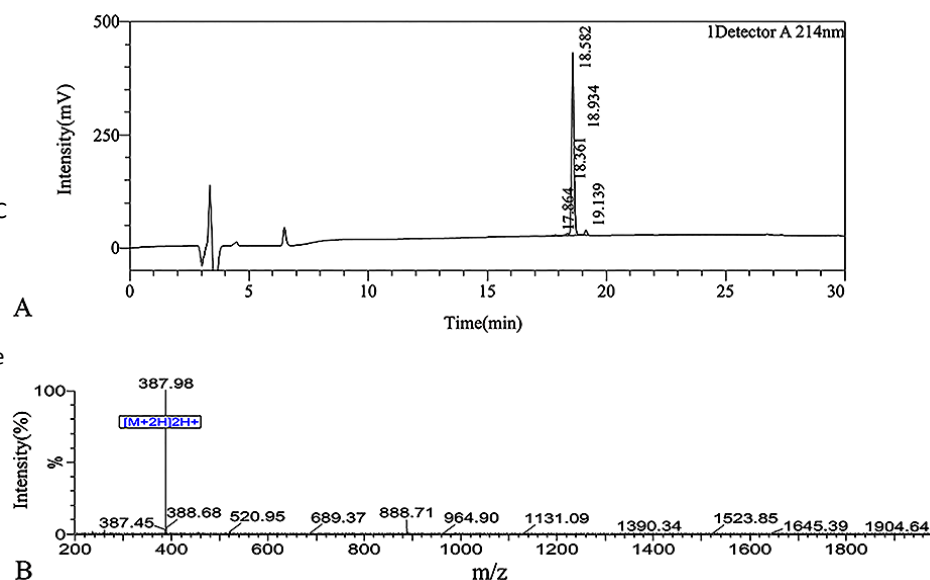
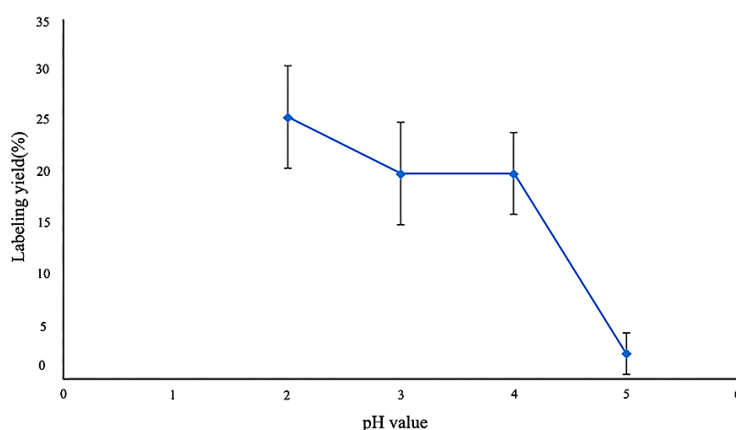


Figure 3. Labeling yield of ¹⁸F-FDG-RRL synthesized at different pH values. The labeling yield decreased significantly at pH 5.



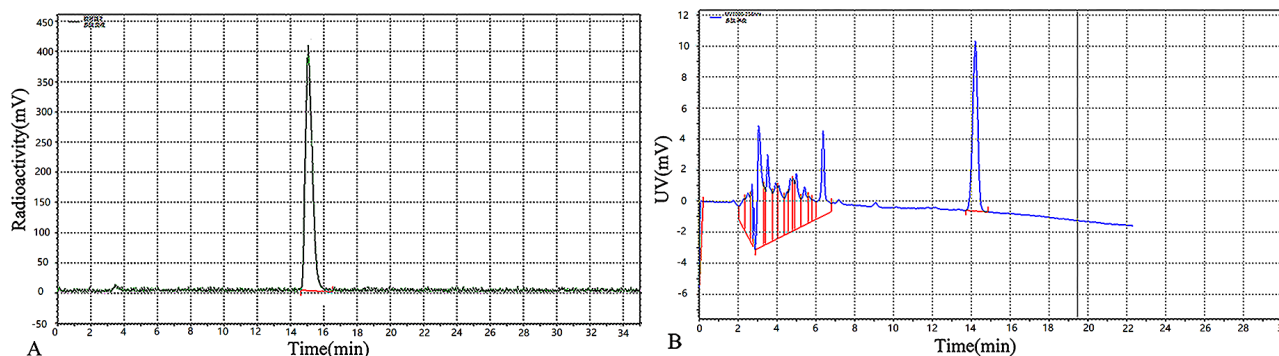


Figure 4. Analytical HPLC profiles of ¹⁸F-FDG-RRL after radio-HPLC. A is the radioactive trace, and B is the UV trace. The radioactive trace (retention time of approximately 15.2 min) is consistent with the ultraviolet (UV) trace (retention time of approximately 14.2 min).

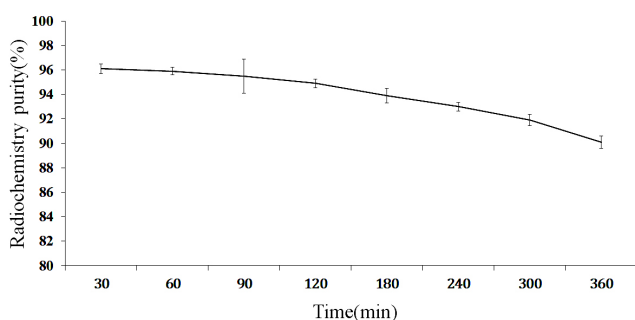


Figure 5. In-vitro stability of ¹⁸F-FDG-RRL. Means with error bars for radiochemical purity at different times; n=5. The bars show the standard deviation (SD).

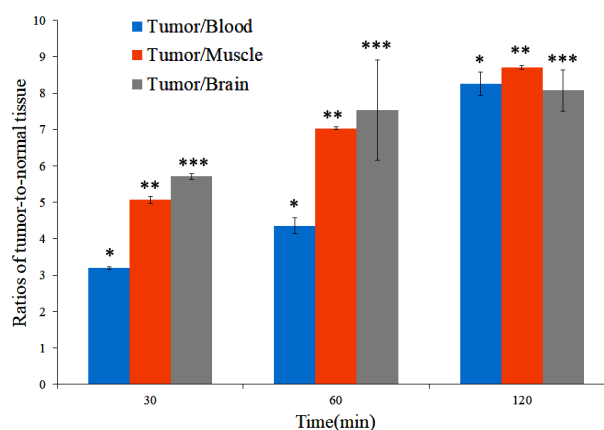


Figure 6. Tumor-to-normal tissue ratios of ¹⁸F-FDG-RRL. Means with error bars for tumor/background; n=5. The bars show the standard deviation (SD). Tumor/background increased with time (*p<0.001, **p<0.001, ***p<0.05).

DISCUSSION

¹⁸F-FDG is currently the most widely used positron imaging agent. The advantages of ¹⁸F labeling with ¹⁸F-FDG as a prosthetic group are that (1) ¹⁸F-FDG is low-cost and easy to obtain, (2) the synthesis method is a simple one-pot reaction, (3) ¹⁸F-FDG has a molecular structure similar to a monosaccharide and (4) ¹⁸F labeling with ¹⁸F-FDG as a prosthetic group can achieve effects similar to glycosylation modification. The disadvantages are that (1) ¹⁸F-FDG in clinical use contains excessive glucose and needs to be purified prior to labeling [8], (2) the labeled molecules must be specifically modified and (3) the large steric hindrance from the glycosyl group may affect the affinity of the labeled product for the target [16].

The highest labeling yield of this reaction was (25.5±5.0) % at pH 2.0, and as the pH increased, the labeling yield decreased to only (2.5 ± 2.0) % at pH 5.0, which is consistent with the study by Namavari *et al.* [5], which showed that the optimal pH of this reaction was 1.5-2.5 and that nearly no product was obtained when the pH was above 4. The possible reasons are as follows: when the pH is in the range of 1.5-2.5, (1) the pyran ring of ¹⁸F-FDG is easier to open, exposing the aldehyde group, and (2) the carbon atom of the aldehyde group of ¹⁸F-FDG is positively charged due to protonation and more susceptible to a nucleophilic substitution reaction with the aminooxy group of the polypeptide. The labeling yield may be further improved through ¹⁸F-FDG purification, RRL peptide modification and optimization of the

reaction time and temperature of the product separation. It is worth noting that clinically applied ¹⁸F-FDG will produce excessive glucose during production and that the presence of glucose will inhibit the labeling reaction. Consequently, further purification of ¹⁸F FDG is required to separate the glucose before the labeling reaction ^(8,9). The radiochemical purity of ¹⁸F-FDG-RRL in human serum was still above 90% after 6 hours, which meets the requirements for imaging.

The results of the *in-vivo* biodistribution study showed that the radioactivity uptake was highest in the kidney, suggesting that ¹⁸F-FDG-RRL is mainly excreted through the kidneys. This is likely due to the increased hydrophilicity of the RRL peptide when it is linked to ¹⁸F-FDG, which makes it easy to excrete through the kidneys, thereby reducing the background. ¹⁸F-FDG-RRL showed low levels of uptake in brain tissue, with a high tumor/brain ratio, which suggests that this imaging agent is suitable for PET imaging of brain tumors, with great advantages over the other commonly used PET imaging agent, ¹⁸F-FDG, for brain tumor imaging. The uptake of ¹⁸F-FDG-RRL was high in tumors, with increasing tumor/muscle, tumor/blood and tumor/brain ratios over time, which suggests that ¹⁸F-FDG-RRL is highly valuable for PET imaging of tumors.

CONCLUSION

In summary, ¹⁸F labeling of polypeptides with ¹⁸F-FDG as a prosthetic group is a feasible method that is simple, convenient and cost-effective. This method also improves the hydrophilicity of the labeled peptides and facilitates the clearance of the tracing agent from nontarget tissues. Further improvement of the labeling yield and reduction of the reaction time and number of precursors required will be the focus of future research.

Conflicts of interest: Declared none.

REFERENCES

1. Hamacher K, Coenen HH, Stöcklin G (1986) Efficient stereospecific synthesis of no-carrier-added 2-[¹⁸F]-fluoro-2-deoxy-D-glucose using aminopolyether supported nucleophilic substitution. *Journal of Nuclear Medicine*, **27**(2): 235-238.
2. Schottelius M, Poethko T, Herz M, Reubi JC, Kessler H, Schwaiger M, Wester HJ (2004) First (¹⁸F)-labeled tracer suitable for routine clinical imaging of sst receptor-expressing tumors using positron emission tomography. *Clinical Cancer Research*, **10**(11): 3593-3606.
3. Poethko T, Schottelius M, Thumshirn G, Hersel U, Herz M, Henriksen G, Kessler H, Schwaiger M, Wester HJ (2004) Two-step methodology for high-yield routine radiohalogenation of peptides: (¹⁸F)-labeled RGD and octreotide analogs. *Journal of Nuclear Medicine*, **45**(5): 892-902.
4. Chang YS, Jeong JM, Lee YS, Kim HW, Rai GB, Lee SJ, Lee DS, Chung JK, Lee MC (2005) Preparation of ¹⁸F-human serum albumin: a simple and efficient protein labeling method with ¹⁸F using a hydrazone-formation method. *Bioconjugate Chemistry*, **16**(5): 1329-1333.
5. Namavari M, Cheng Z, Zhang R, De A, Levi J, Hoerner JK, Yaghoubi SS, Syud FA, Gambhir SS (2009) A novel method for direct site-specific radiolabeling of peptides using [¹⁸F] FDG. *Bioconjugate Chemistry*, **20**(3): 432-436.
6. Wuest F, Berndt M, Bergmann R, van den Hoff J, Pietzsch J (2008) Synthesis and application of [¹⁸F]FDG-maleimidehexyloxime ([¹⁸F]FDG-MHO): a [¹⁸F]FDG-based prosthetic group for the chemoselective ¹⁸F-labeling of peptides and proteins. *Bioconjugate Chemistry*, **19**(6): 1202-1210.
7. Lee YS, Jeong JM, Kim HW, Chang YS, Kim YJ, Hong MK, Rai GB, Chi DY, Kang WJ, Kang JH, Lee DS, Chung JK, Lee MC, Suh YG (2006) An improved method of ¹⁸F peptide labeling: hydrazone formation with HYNIC-conjugated c (RGDyK). *Nuclear Medicine and Biology*, **33**(5): 677-683.
8. Hultsch C, Schottelius M, Auernheimer J, Alke A, Wester HJ (2009) (¹⁸F)-Fluoroglucosylation of peptides, exemplified on cyclo(RGDfK). *European Journal of Nuclear Medicine and Molecular Imaging*, **36**(9): 1469-1474.
9. Wuest F, Hultsch C, Berndt M, Bergmann R (2009) Direct labelling of peptides with 2-[¹⁸F] fluoro-2-deoxy-d-glucose ([¹⁸F]FDG). *Bioorganic and Medicinal Chemistry Letters*, **19**(18): 5426-5428.
10. Simpson M, Trembleau L, Cheyne RW, Smith TA (2011) One-pot production of ¹⁸F-biotin by conjugation with ¹⁸F-FDG for pre-targeted imaging: synthesis and radio-labelling of a PEGylated precursor. *Applied Radiation and Isotopes*, **69**(2): 418-422.
11. Al Jammaz I, Al-Otaibi B, Amer S, Al-Hokbany N, Okarvi S (2012) Novel synthesis and preclinical evaluation of folic acid derivatives labeled with (¹⁸F)-[FDG] for PET imaging of folate receptor-positive tumors. *Nuclear Medicine and Biology*, **39**(6): 864-870.

12. Shirmardi SP, Shamsaei M, Gandomkar M, Maragheh MH (2011) Synthesis and biodistribution study of a chlorotoxin derivative peptide labeled with 131- iodine for tumor therapy. *Int J Radiat Res*, **8(4)**: 243-248.
13. Yu MM, Chen YH, Wang ZG, Ding XD(2020) pHLIP(Var7)-P1AP suppresses tumor cell proliferation in MDA-MB-231 triple-negative breast cancer by targeting protease activated receptor 1. *Breast Cancer Research and Treatment*, **180(2)**: 379-384.
14. Yu M, Zhou H, Liu X, Huo Y, Zhu Y, Chen Y (2010) Study on biodistribution and imaging of radioiodinated arginine-arginine-leucine peptide in nude mice bearing human prostate carcinoma. *Annals of Nuclear Medicine*, **24(1)**: 13-19.
15. Huo Y, Kang L, Pang X, Shen H, Yan P, Zhang C, Liao X, Chen X, Wang R (2019) Noninvasive PET Imaging of a Ga-68-Radiolabeled RRL-Derived Peptide in Hepatocarcinoma Murine Models. *Molecular Imaging and Biology*, **21(2)**: 286-296.
16. Wängler C, Waser B, Alke A, Iovkova L, Buchholz HG, Niedermaier S, et al. (2010) One-step ¹⁸F-labeling of carbohydrate-conjugated octreotate-derivatives containing a silicon-fluoride-acceptor (SiFA): *in-vitro* and *in-vivo* evaluation as tumor imaging agents for positron emission tomography (PET). *Bioconjugate Chemistry*, **21(12)**: 2289-2296.

