

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**DEVELOPMENT OF PEPTIDES-BASED PROBE PRECURSORS TO IMAGE
FOLATE RECEPTOR 1 WITH POSITRON EMISSION TOMOGRAPHY
IMAGING SYSTEMS**

M.Sc. THESIS

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Department of Nanoscience and Nanoengineering

Nanoscience and Nanoengineering Programme

DECEMBER 2020

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DECEMBER 2020

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**POZİTRON EMİSYON TOMOGRAFİ GÖRÜNTÜLEME SİSTEMLERİ İLE
FOLAT RESEPTÖRÜ 1 GÖRÜNTÜLEMEK İÇİN PEPTİT TABANLI PROB
ÖNCÜLERİN GELİŞTİRİLMESİ**

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ABBREVIATIONS

^{18}F-FDG	: 2-Deoxy-2-(^{18}F) fluoro-D-glucose
ACN	: Acetonitrile
Ala (A)	: Alanine
Arg (R)	: Arginine
Asp (D)	: Aspartic Acid
CADD	: Computer-aided drug design
CT	: Computed Tomography
DCC	: <i>N, N'</i> -Dicyclohexylcarbodiimide
DCM	: Dichloromethane
DIPEA	: Diisopropylethylamine
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic acid
DOTA	: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
FDA	: Food and Drug Administration
FDG	: 2-deoxy-2-(^{18}F) fluoro-D-glucose
Fmoc	: 9H-fluorene-9-ylmethoxycarbonyl
FOLR	: Folate Receptor
FOLR1	: Folate Receptor 1
^{68}Ga	: Gallium-68
HBTU	: O-(Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HER2	: Human Epidermal Growth Factor Receptor 2
HF	: Hydrogen fluoride
ITC	: Isothermal Titration Calorimetry
LS-MS	: Liquid Chromatography–Mass Spectrometry
Lys (K)	: Lysine
MBHA	: 4-Methylbenzhydrylamine resin
MI	: Molecular imaging

MRI	: Magnetic Resonance Imaging
PD-L1	: Programmed Death Ligand-1
PET	: Positron Emission Tomography
PET-CT	: Positron Emission Tomography - Computed Tomography
Phe (F)	: Phenylalanine
PSA	: Prostate-Specific Antigen
QD	: Quantum Dots
RAM	: Rink Amide MBHA resin
RGD	: Arginine-Glycine-Aspartic
RP-HPLC	: Reversed Phase High Performance Liquid Chromatography
SPECT	: Single Photon Emission Computed Tomography
SPPS	: Solid-Phase Peptide Synthesis
SSTR	: Somatostatin Receptor
t-Boc	: Tert-butoxycarbonyl
TFA	: Trifluoroacetic Acid
TIPS	: Triisopropylsilane
Trityl	: Triphenylmethyl
Tyr (Y)	: Tyrosine
VEGF	: Vascular Endothelial Growth Factor

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DEVELOPMENT OF FOLATE RECEPTOR 1 TARGETED PEPTIDES FOR USE IN POSITRON EMISSION TOMOGRAPHY IMAGING SYSTEMS

SUMMARY

Information about cancer diagnosis and the stage of cancer can be obtained by using molecular imaging methods. This information is very vital for the early treatment plan of the disease. Molecular imaging is an imaging method that can combine patient and disease information and calculate at a cellular or molecular degree. One of the most common molecular imaging methods is Positron Emission Tomography (PET) imaging. PET imaging is a method that uses the simultaneous detection of two 511 keV-powered annihilation photons that result from a positron-electron encounter. Later, for a more advanced method, PET scanners were integrated into computed tomography (CT) and PET/CT imaging emerged. PET/CT is a nuclear medicine imaging method that displays changes in the metabolic functions of organs and tissues in the body, together with their anatomical information.

Radionuclide imaging agents are required for PET/CT imaging. Agents such as Carbon-11 (^{11}C), Fluor-18 (^{18}F) and Gallium-68 (^{68}Ga) are the most commonly used agents. Peptides are an important group for cancer imaging. It has high selectivity, sensitivity, and stability. They are used for cancer imaging by binding to specific receptors. The most important advantage of the peptides is that they have no toxicity for cancer imaging of the body of human. Folate Receptor 1 (FOLR1) is one of the most widely used receptors. The FOLR1 receptor has a high affinity. A signaling agent must be added to complete the imaging agent. The ^{68}Ga agent suitable for use in PET/CT imaging is a signaling agent suitable for marking. To provide the region suitable for the signaling agent, chelation should be added to the region with high affinity. DOTA chelates are generally used for the attachment of ^{68}Ga agents.

In this thesis, it is aimed to develop peptide-based imaging agents that can be used for PET/CT imaging and can bind to the FOLR1 receptor. RPAADK, KYAAK, RPSNK, RFSNK, KYANK, KYDAK, and KYSAK peptides were synthesized by the Solid-Phase Peptide Synthesis (SPPS) method from the peptide library that contains peptides specific to the FOLR1 receptor. These peptides are designed by Computer-Aided Drug Design (CADD) method for cancer imaging. Subsequently, the synthesized peptides were purified by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) and characterized by Liquid Chromatography-Mass Spectrometry (LC-MS).

Keywords: PET/CT, peptide, imaging agent, folate receptor 1, solid-phase peptide synthesis.



POZİTRON EMİSYON TOMOGRAFİ GÖRÜNTÜLEME SİSTEMLERİNDE KULLANIMINA YÖNELİK FOLAT RESEPTÖRÜ 1 HEDEFLİ PEPTİTLERİN GELİŞTİRİLMESİ

ÖZET

Kanser, vücudun herhangi bir organ ya da dokusundaki hücrelerin kontrolsüz çoğalması ve büyümesi sonucu ortaya çıkan bir hastalıktır. Kanser oluştuğu dokuya göre adlandırılır. 200'den fazla kanser türü tespit edilmiştir. En sık görülen ve ölüme yol açan kanser türleri kadınlarda meme, rahim ve kalın bağırsak kanserleri ve erkeklerde ise akciğer, prostat, mide ve kalın bağırsak kanserleridir. İyi huylu (benign) tümörler sınırları belirgin kitlelerdir. Ancak kötü huylu (malign) tümörler, sınırları belirsizdir ve çevre dokuya sıkıca yapışık halde bulunurlar. Komşu organ ve dokulara yayılırlar, kemik doku ile karşılaştıklarında onları dahi eritirler. Malign tümörü oluşturan hücreler o kadar farklılaşmışlardır ki orijinlerinin ne olduğunu söylemek imkânsızdır. Lenf ve kan yoluyla uzak organlara da yayılırlar. Kanser oluştuğu bölgeden başka organ ve dokulara yayılırsa, buna metastaz denir. Malign tümörler kanserdir. Bağışıklık sistemi anormal hücreleri yakalar ve yok eder. Ancak kanser hücreleri bağışıklık sisteminden kaçabilirler. Normal vücut hücrelerinde olağan olan hücre ölümü kanser hücrelerinde görülmez. Kanser oluşumunda ana etken genetik yapının yani DNA'nın doğuştan ya da edinsel etkenlerle bozulmasıdır. Hücre bölünmesi esnasında DNA replikasyonunun hatalı olması sonucu hücre farklılaşır. Ayrıca yaşam boyunca maruz kalınan radyasyon, toksik maddeler, bazı virüsler kansere yol açabilirler. Hücreler kansere neden olabilen etkenlerle uzun süre karşılaşınca, hücrelerde bazı mutasyonlar gerçekleşebilir. Laboratuvar testleri, biyopsi ve görüntüleme yöntemleri kullanarak kanser teşhis edilebilir. Teşhis edildikten sonra metastaz durumu incelenir. Buna göre kanser evrelemesi yapılır. Kanserinin erken evrelerinde tedavi ve tanı için kullanılan standart uygulamalar cerrahi operasyon, radyoterapi ve kemoterapi kullanılır. Tedavi yöntemlerinin sürekli gelişmesine rağmen kanser teşhisi için ajanlar sınırlı sayıdadır ve yeni ajanların geliştirilmesi gerekir.

Kanserin erken tanı ve teşhisinde kullanılabilecek yöntemlerden biri tümör mikroçevresinin etrafında oluşan proteinlerin biyobelirteçler olarak belirlenmesidir. Tümör oluşumu ve gelişimi sağlayan proteinler ve ligandları, tümör mikroçevresini oluşturdıkları için biyobelirteç olarak seçilmektedirler. Biyobelirteçler normal biyolojik, patolojik ve farmolojik süreçleri hastalığın belirteci gibi ölçebilir veya değerlendirebilir. En fazla öne çıkan biyobelirteç türü kanser biyobelirteçleri olarak adlandırılır. Kanser biyobelirteçleri, hastalık düzeyine göre tahmin biyobelirteçleri, tanıma biyobelirteçleri, teşhis biyobelirteçleri ve hastalık sonu tahmin biyobelirteçleri olmak üzere dört gruptan oluşmaktadır. Kanser tanısı ve kanserin hangi evrede olduğu hakkında görüntüleme yöntemleri kullanarak bilgi sahibi olunabilir. Görüntüleme yöntemleri, kanseri tarayıp teşhis koymada yetkilidirler. Bu hastalığın erken tedavi planı için çok önemlidir. Moleküler görüntüleme hastaya ve hastalığa ait bilgileri birleştirip hücresel veya moleküler derecede hesaplayabilen bir görüntüleme

yöntemidir. Moleküler görüntüleme biyolojik süreçlerin görsel sunumu, karakterizasyonu ve nicelendirilmesi bakımından teşhis edilecek parçaların daha anlamlı resmini ortaya koyar. Moleküler görüntüleme biyoloji, kimya, bilgisayar, moleküler ve mühendislik alanlarını içeren multidisipliner bir alandır. Bir çok hastalık türünde, kanser başta olmak üzere, kalp hastalıkları, mide-bağırsak rahatsızlıkları ve kemik rahatsızlıklarının tanı ve teşhisinde sıkça kullanılmaktadır. Moleküler görüntüleme teknolojisi, biyobelirteçlere bağlanacak moleküler görüntüleme ajanlarının görüntülenmesinde kullanılabilir. En yaygın moleküler görüntüleme yöntemlerinden biri Pozitron Emisyon Tomografi (PET) görüntülemesidir. PET görüntüleme, pozitron-elektron karşılaşması sonucunda ortaya çıkan iki adet 511 keV enerjili anihilasyon fotonlarının eş zamanlı deteksiyonunu kullanan bir yöntemdir. Daha sonraları, daha gelişmiş bir yöntem için PET tarayıcıları bilgisayarlı tomografiye (BT) entegre edilmiş ve PET/BT görüntüleme ortaya çıkmıştır. PET/BT, sadece anatomik bilgi değil aynı zamanda vücuttaki organ ve dokuların metabolik fonksiyonlarındaki değişiklikleri anatomik bilgileriyle birlikte görüntüleyen, onkolojik görüntüleme için kullanılan en gelişmiş nükleer tıp görüntüleme yöntemidir. Kanserin erken tanı ve doğru evreleme sayesinde tedavi planını yeniden oluşturur ve tedaviye erken yanıtı sağlar.

Moleküler görüntülemede kullanılan ajanlar hedef doku veya çevreyi görüntülemek amacıyla tasarlanırlar. Geliştirilmek istenen kanser teşhis ve görüntüleme ajanları; hedef parça ve sinyal ajanı olmak üzere iki bölümden oluşmaktadır. Sinyal ajanı olarak görüntüleme yöntemine uygun radyoizotop seçilebilirken hedef parça küçük organik moleküllerden, peptidlerden, aptamerden veya çok yönlü moleküllerden oluşabilir. En çok bilinen moleküler görüntüleme ajanı peptit temellidir. Peptitler kanser görüntüleme için önemli bir gruptur. Yüksek seçiciliğe, duyarlılığa ve kararlılığa sahiptir. Kanser görüntülenmesinde kansere özgü reseptörlere bağlanarak kanser görüntülemesi için kullanılırlar. Peptitlerin en önemli avantajı, insan vücudunun kanser görüntülemesi için hiçbir toksisiteye sahip olmamalarıdır. Folat Reseptörü 1 (FOLR1) en yaygın kullanılan reseptörlerden biridir. FOLR1 reseptörü yüksek affiniteye sahiptir. PET/BT görüntülenmesi için radyonüklid görüntüleme ajanları gereklidir. Karbon-11 (^{11}C), Flor-18 (^{18}F) ve Galyum-68 (^{68}Ga) gibi ajanlar en yaygın kullanılan ajanlardır. PET-BT görüntülemede kullanılması uygun olan ^{68}Ga ajanı işaretleme için uygun bir sinyal ajanıdır. Sinyal ajanına uygun bölgenin sağlanması için de peptite şelat eklenmesi gereklidir. ^{68}Ga ajanının takılmasında genellikle DOTA-şelatları kullanılır.

Bu tezde, PET ajanlarına alternatif olarak kullanılmak üzere tümör görüntülemesi için peptitler geliştirmek hedeflenmiştir. Bu sebeple, tümör mikroçevresindeki bulunan biyobelirteçlere yüksek bağlanma eğilimi ve seçicilikle bağlanabilen peptitler sentezlenmiştir. Kanser görüntüleme için FOLR1 reseptörüne bağlanabilen peptitlerin olduğu peptit bankasından RPAADK, KYAAK, RPSNK, RFSNK, KYANK, KYDAK ve KYSAK peptitleri Katı Fazlı Peptid Sentezi (SPPS) yöntemi ile sentezlendi. Daha sonra, sentezlenen peptitler, ters fazlı yüksek performanslı sıvı kromatografisi (RP-HPLC) ile saflaştırıldı ve sıvı kromatografi-kütle spektrometresi (LC-MS) ile karakterizasyonu elde edildi. Bu çalışma kapsamında, peptitler

moleküler görüntüleme ajanları olarak kullanılacaktır. Peptitlere bağlanma eğilimi ve stabilite analizleri yapılacaktır. Bu çalışmalardan elde edilen sonuçlara göre, yüksek bağlanma eğilimi ve seçicilik performanslarını geliştirmek için çeşitli modifikasyonlar ve değişiklikler uygulanacaktır. ITC yöntemi ile folik asit reseptörüne yüksek ilgisi olduğu tespit edilen peptitler, PET-BT cihazlarında görüntüleme amacıyla kullanılmak üzere ^{68}Ga ve DOTA-şelatları ile radyolojik olarak işaretleneceklerdir.

Anahtar Kelimeler: PET/BT, peptit, görüntüleme ajanı, folat reseptörü 1, katı fazlı peptit sentezi.



1. INTRODUCTION

1.1.Cancer

The concept of *disease* is described as the decay of regular physiology, or regular biochemistry. Cells grow and split to create novel cells for the body requirements during a lifetime. Cells die, as they grow or have harmed, and new cells occur. But, when disease occurs, some alterations in the genes of a normal cell functionally produce cell disruption. In this condition, instead of death of grown or harmed cells, these cells continue to divide [1, 2].

Cancer is a rally of more than 100 particular diseases [1]. Nearly any part of the body of a person can get cancer [2]. That's why so many different varieties of cancer are available; they can change significantly in action and reaction in cure. The three most widespread cancer cases are breast, prostate, and lung, as given in Table 1.1 [3].

Table 1.1: Most frequent cancers in the United States [3].

Cancer Site	Cases Per Year	Deaths Per Year
Breast	184,200 (15.1%)	41,200 (7.5%)
Prostate	180,400 (14.8%)	31,900 (5.8%)
Lung	164,100 (13.4%)	156,900 (28.4%)
Colon/rectum	130,200 (10.7%)	56,300 (10.2%)
Lymphomas	62,300 (5.1%)	27,500 (5%)
Bladder	53,200 (4.4%)	12,200 (2.2%)
Uterus	48,900 (4%)	11,100 (2%)
Skin	47,700 (3.9%)	7,700 (1.4%)
Kidney	31,200 (2.6%)	11,900 (2.2%)
Leukaemia	30,800 (2.5%)	12,100 (2.2%)
Subtotal	933,000 (76.5%)	368,800 (66.8%)
All sites	1,220,100 (100%)	552,200 (100%)

Cancer is a group of genetic diseases that occurs in each cell type. Cancer is caused by alterations to genes that control the function, the division, and the growth of cells. For all types of cancer, this event occurs, when the functional disruption begins to grow and divide out of control, and these cells spread into surrounding tissues. In short, these proliferating and spreading diseased cells are cancer [2].

Most cancer cells can disregard traces transmitted to cells to prevent division or start an event referred to as programmed cell death, or apoptosis, which the body uses to get rid of unnecessary cells [2]. Apoptosis, a sort of cellular death that exists in both regular and pathological tissue, is designed to annihilate undesirable host cells in an effective cell owned destruction procedure that is swayed by a special series of gene products. It is a homoeostatic mechanism to protect cell populations in tissues [1]. Although normal cells ripen to so different cell types with certain functions, cancer cells cannot. On the contrary of normal cells, this condition is one reason that cancer cells unavoidably divide [2].

When most cancer cells unavoidably divide, tumors occur. Tumors are classified as *malignant* and *benign* tumors [2]. The tumor is unavoidable reproduction of cells, whether benign or malignant (Fig. 1.1). Malignant tumors can diffuse to circumambient tissues through the circulatory or lymphatic systems, it is called metastasis. Benign tumors such as a complexion wart, stays in first tissue and not diffuse to circumambient tissues and remote body areas [3]. However, benign tumors can occasionally be rather big [2]. Just malignant tumors are called cancer. Its capability that assaults and metastasizes causes hazard [3].

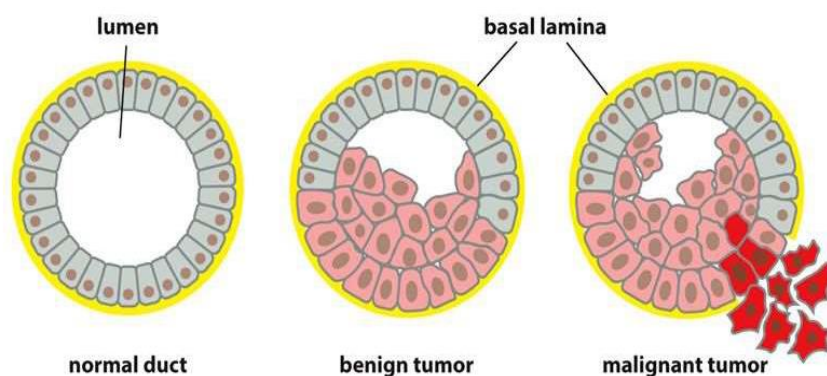


Figure 1.1: Benign versus malignant tumors [4].

The growth of cancer is a multistage procedure containing proliferation, survival, invasion, and metastasis (Fig. 1.2). *Tumor initiation*, the initial step within the procedure, is the conclusion of a genetic change that causes to unavoidably proliferation of an only cell. It is enough for only one cell to mutate. Also, *cell proliferation* causes the overgrowth of a population of clonally reproduced tumor cells. *Tumor progression* proceeds as extra mutations arise within cells of the tumor population. Some of these mutations give the cell a selective benefit, such as speedy accretion, and the descendants of a cell carrying this type of mutation can control inside the tumor population. After that, the variant cell continues to mutate with increasing growth potential. The system is known as *the clonal selection* because it was developed on the foundation of a new clone of tumor cells, raised accretion pace, or different features that provide a selective benefit. The clonal selection keeps along with tumor growth, such that tumors grow faster and become more and more malignant [3].

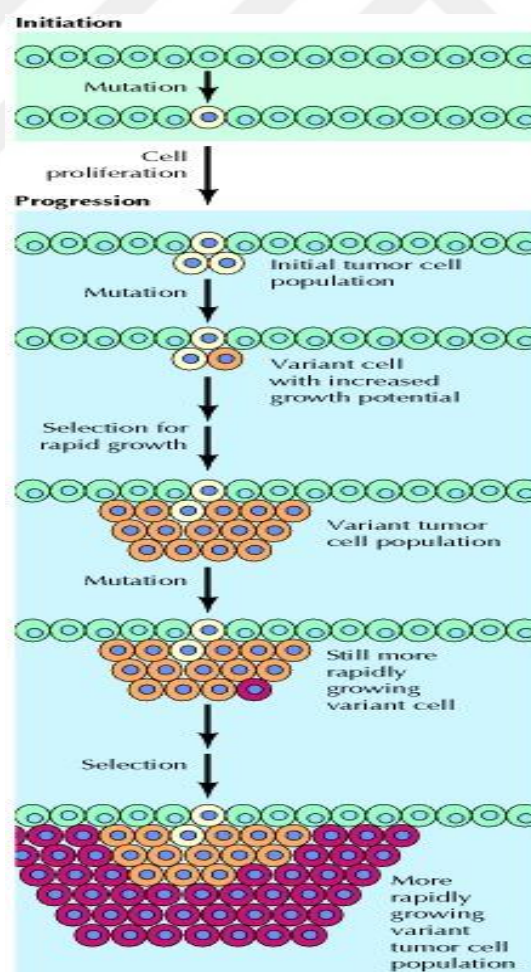


Figure 1.2: The phases of tumor growth [3].

Predominantly, cancers are divided into three primary categories according to the tissues in which it occurs. They are carcinomas, sarcomas, and leukemias, or lymphomas. *Carcinomas*, which contain about 90% of cancers, are malignancies of epithelial cells. *Sarcomas*, which are unusual, are rigid tumors of collagen tissues, which include brawn, osseous, gristle, and fibrous tissue. *Leukemias* or *lymphomas*, which occasion about 8% of malignancies, are caused by the cells composing blood and immune system cells [3].

1.2.Biomarkers

A long-standing purpose of cancer research is defining the molecular mechanisms by which cancers enhance, planning the symptomatic applications, observing those biomarkers of cancers early, and enhancing targeted significant healing plans for the cure of cancer. In the area of cancer study, the notion of precision medicine specially depends on the growth of current biomarkers that question potentially abnormal tracks that can be targeted with molecular targeted or immunological treatments [6]. Biomarkers are described as cellular, biochemical or molecular changes, such as DNA, RNA, enzyme, receptor peptide, that can be measurable in biological environments with tissues, cells, or liquids by Hulka and colleagues. Then, this description extended as a measurement as providing information regarding the health or illness statuses of an individual including the existence of an illness, a physiological alteration, response to treatment, or psychological status. Biomarkers supply to know the forecast, therapy, advancement, retreat, or the result of the cure of illness. They are used in numerous scientific ranges and are used in distinct ways at distinct phases of drug development [7]. They may be used to calculate regular biological operations in the body, illness operations inside the body, or a reply of an individual to therapy or drug. Biological tissues that include enzymes, discovered in blood or tissue samples, genetic changes, and medical images are examples of them. They are used in the diagnosis of many diseases including cardiovascular illness, infections, immunological and genetic disorders, and cancer [7, 8].

There are many benefits of the use of biomarkers. They can be early and easily measured with an advanced accuracy. They can be influenced less than other therapies. They can provide the desired pattern dimensions and quicker resolution. Several benefits and disadvantages of biomarkers are summarized in Table 1.2 [7].

Table 1.2: The benefits and drawbacks of biomarkers [7].

Benefits	Drawbacks
Practical evaluation	Sensitive timing
Measuring accuracy	Costs ahead
Reliable	Warehousing
More tendentious than survey	Irrigations in laboratory
Mechanisms of illnesses frequently observed	Hard to define normal range
Risk / disease homogeneity	Ethical Liability

Biomarker study assists to understand how fine anticipated hazards of illness of an individual, how an illness might improve as soon as it is identified, and to develop how a person will reply to a drug. So, it offers a securer and greater efficient therapy resolution. Biomarkers are used to do more effective the finding tests of drugs. Biomarkers can be used in the recent phase of cancer studies with scientific results. Only a small proportion of patients can reply to some drugs. So, it is critical for recognizing patients for clinical tests use of biomarker measures [8].

Biomarkers can improve the effectiveness of drugs. They pace up and ease medical tests. They can only be used to uncover and influence a scientific result earlier. They are used to recognize the best convenience of patients to a therapy. They evolve to know of how novel drugs operate and to direct to new ways to drug improving at both non-medical and medical stages. They can help people with moral benefit, who will not benefit from starting an unhelpful treatment. They cultivate test monitoring and halting useless tests quickly. They can resolve whether to quickly halt an experiment if the test has not a utility for patients. A drug can be prescribed earlier to patients who will benefit according to knowledge supplied by biomarkers [8].

1.2.1. Types of biomarkers

There are two main types of biomarkers according to characteristics. They are *exposure* biomarkers used in hazard estimation, and *disease* biomarkers used in screening, therapy and observation of illness development. Biomarkers utilized in hazard estimation, screening, and therapy trials are satisfactorily founded, and they have clear benefits. The grade of exposure can estimate whether an illness is caused by toxic exposure. In overall, disease biomarkers permit quick diagnosis or

specification of the result of care at the earliest phase of the illness. Blood, urinary, and cerebrospinal fluid supply the essential biological knowledge for diagnosis [7].

Cancer biomarkers are a subcategory of disease biomarkers. They are used to predict response to certain treatments, and response independently of treatment, or to observe reaction after treatment has started. Cancer biomarkers conventionally are formed from proteins or alternative matters generated by both ordinary cells and cancer cells. However, they are generated in higher quantities by cancer cells. [9].

Cancer biomarkers can be categorized according to their use. They are detective, prognostic, predictive, and diagnostic cancer biomarkers. The aim of *detective biomarkers* is to detect cancer at remediable and better result condition and even before emergence of symptoms. A *prognostic biomarker* is used to prognosticate patient result anyhow of treatment. A prognostic biomarker may not instantly depend or cause treatment; however, they aim to monitor the risks of potential clinical consequences, such as most cancer replication or progress of illness. On the opposite hand, *predictive* is used to predict reaction to a particular treatment. Predictive biomarkers predict reaction for certain healing therapies, such as HER2 positivity/activation. *Diagnostic* biomarker, a different category of cancer biomarker, is used to determine if a patient has a particular illness status [6, 9]. Diagnostic and prognostic biomarkers are measurable features that assist to primarily communicate with doubted illnesses. These especially help to know to recognize who is at hazard, therapy at a quick phase, choose the right form of care, and track patient reply. Such biomarkers are available in numerous distinct shapes; conventional biomarkers contain radiologic practices, mammograms, and other antigens grades, such as prostate-specific antigen (PSA) [10]. Predictive biomarkers have the maximal effect, as they can be used containment tests for illness cleavage. Prognostic biomarkers are used in medical applications however do not guide the use of selective treatments [9].

Intracellular and extracellular targets increase agents for imaging and therapy. These new agents are useful for the molecular description of the disease, and they are used as predictive biomarkers for therapeutic response, diagnostic agents of the disease condition, and prognostic marks of patient outcomes [9].

Widely used tumor tissue markers contain estrogen receptor and progesterone receptor, which are used to state whether hormone treatment and some targeted treatments are appropriate. EGFR gene mutation assay is used to help state cure and predict prognosis and to state whether the cure is proper for targeted treatment kinds known FOLR1, PD-L1, and Tumor Mutation Load (TML) [9].

1.2.2. Folate receptor 1 (FOLR1) for cancer diagnosis

Water-soluble vitamin B9 can be identified as *folate* or *folic acid*. Adequate folate uptake is required in quickly growing cells for the single carbon metabolism reagent and DNA biosynthesis, mend, and methylation. Folate is carried in three methods over the cellular membrane. The primary track of intake is via the reduced folate carrier (RFC), which is allocated everywhere and helps the intake of nutritional folate. The second way is the proton-coupled folate transporter (PCFT), which uses the slope of a transmembrane proton to interact with folate that carries in the cells. Lastly, folate can be carried by folate receptor that have four glycopeptide associated with molecular weight of from 38 to 40 kDa [11].

The notion of folate receptor (FR) targeting plan uses vitamin folic acid as a molecular Trojan steed to selectively transfer subjoined samples to cancer cells that expresses FR. Folic acid presents different benefits equated to other targeting agents, such as monoclonal antibodies or peptides. It is short, steady on a wide temperature row, and pH worth and is therefore suitable for site-specific chemical alteration. It is cheap, anti-immunogen, and binds to FR with high chemical attraction even after union to asymptomatic or curative load [12].

FR is important for more efficient cancer diagnosis, chemotherapy, and inflammatory illnesses. Folate conjugates bind to FR with high affinity and then move to endosomes, which can cleaves disulfide bonds (Fig. 1.3). In the endosome, the folate-disulfide-drug conjugate is released from the FR, and the prodrug is released. Since the pH of FR-containing endosomes is slightly acidic, acid-labile binders do not effectively release the attached drug [13].

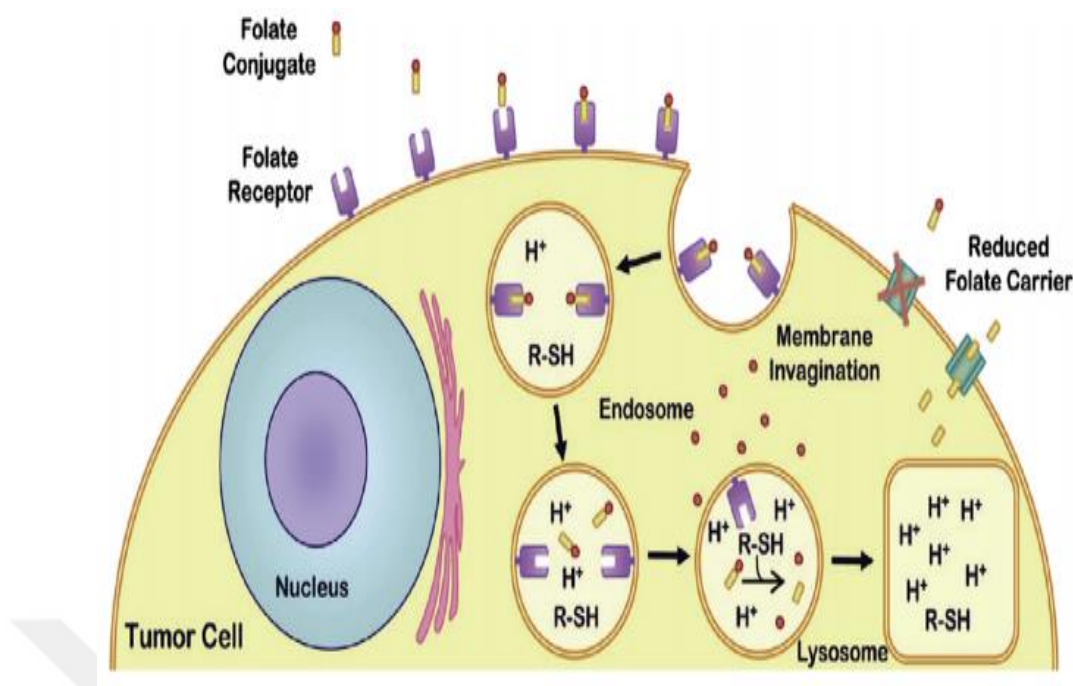


Figure 1.3. FR mediated endocytosis of the folic acid-drug conjugate [13].

The alpha isoform, *Folate Receptor α* (FRA), *folate binding protein* (FBP) or, further called *FOLR1* is a glycosylphosphatidylinositol (GPI) layer protein linked with a high affinity to attach and organize of the effective folate shape, 5-methyltetrahydrofolate (5-MTF). FRA executes the restricted regular tissue dispersion. The distribution of FRA in regular tissues is restrained to short grade finite expression on the top planes of some members of the body, for example, on the peak planes of several epithelia, such as the renal, lung and choroid network. However, it is over-expressed in a range of rigid tumors, containing gonadal cancer, non-small cell lung cancer (NSCLC), chest cancer, renal cancer, and high-grade osteogenic sarcoma. Runnings have displayed that overexpression of FRA can supply a development benefit for cancer cells to mechanisms that are relevant and free of folate entry [11, 14].

FRA, a particle of 38-40 kDa, is a fine qualified associate of the FR ancestry with a high chemical attraction for folates. FRA is attached to cell layers via a glycosylphosphatidylinositol part and carries folates through an endocytic operation. In some cancers, this overexpression of high chemical attraction FRA may have appeared to fulfill the cellular necessity for DNA synthesis and development. It can be used as molecular tumor markers for the picky delivery of anticancer drugs [11, 14].

Since not everyone who has a particular kind of cancer has a high cancer biomarker grade assembled with this cancer, evaluations of circulating cancer biomarkers are frequently knitted up according to other tests, such as biopsies or imaging for cancer diagnosis. A number of FRa targeting approaches, containing folic acid derivatives, folate drug conjugates and small molecules, vaccines, T-cell treatments, and monoclonal antibodies, are improved for clinical application for both molecular imaging and therapeutic intents [11, 14].

1.3.Molecular Imaging

Medical imaging is conventionally used to diagnose and measure degree of disease by the contrast distinctions between in tissue. Medical imaging can be separated categories as anatomic and functional. *Anatomic images* are used to diagnose, quantify, and delimit the tumor size in cancer. Advanced techniques, such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), which supply an anatomical description of the disease, are used for clinical practices. Nevertheless, such methods supply restricted information about the pathophysiology of the tumor because, the anatomical images do not involve any molecular knowledge [15]. These imaging techniques may supply basic knowledge about tumor size and dispersion at high spatial determination. The knowledge won from these imaging methods majorly helps in choosing an advantageous treatment plan for patients. When cancerous tumors are larger than one centimeter, they are identified by anatomic images. This is unfortunately one of the disadvantages [16]. Also, if the tumor has analogous properties with its circumambient tissues, CT and MRI can make it difficult to image tumors in certain situations. *Functional imaging* supplies estimations of physiological processes, such as the changes in blood flow or oxygenation at the tissue or cellular grades. Numerous cancer treatments, which can be identified by functional imaging, contain anti-angiogenic approaches contributed for blood vessel damage of cancer cells and thus tumor bulk reduces day after day [15].

A better understanding of mechanisms of disease via genomics, proteomics, and animal modeling together with progress in chemistry and molecular biology has improved a third category of medical imaging. This category is called *molecular imaging (MI)* [15]. MI is a new area purposed to combine patient-specific and

disease-specific molecular knowledge with analytical imaging. Molecular imaging records what is experienced at the cellular and molecular grade, containing knowledge about receptor quantity, signal transfer deviances, and enzymatic actions, while anatomical and functional imaging supplies knowledge about huge influences in certain situations. MI is a biological method involving scanning, characterization, and evaluation at the molecular and cellular grades in living organisms, and the other grades. The eventual aim of MI is non-invasive location and measurement of particular molecular incidents in vivo, containing internal or exterior gene phrase, point orientation, protein-protein interaction, and transcriptional organization [1, 15]. Non-invasive and real-time scanning can measure the physiological or diseased process at the cellular or molecular grade in the living organism. Therapy is usually curative when wounds are at earlier stage. Molecular imaging thus has significance in earlier and true diagnosis and drug discovery and improvement [17]. The other significance is that it provides precise monitoring of the main molecular targets and host reactions related with the first cancer stage [18]. MI briefly aims to identify illnesses situation earlier than traditional imaging processes, define underlying molecular incidents in beginning and advancement of illness, differentiate offensive and insidious illness situations, and symbolize downstream targets in defined molecular systems or pathways [1].

MI can supply more precise strategies for early cancer diagnosis and staging, medicinal response forecast and effective monitoring with the advanced cancer biology and genetics [15]. MI qualifies the pathology of diseased tissues without biopsies, and gives an impersonated care program to patients. That is the major benefit of molecular imaging. The impersonated care program describes the optimal care for patients with improved cancer processes and poor prognosis of the disease [19].

Molecular imaging needs high resolution and high precision devices to observe certain imaging agents that bind the imaging signal with the molecular incident [17]. MI supplies the definition of biological processes in tissue, cell, and intracellular elements of living organisms within anatomical imaging or functional imaging by using US, CT, MRI and optical imaging (OI). Also, MI is a straight stretch of the current methods used in nuclear medicine [1]. Radionuclide imaging containing positron emission tomography (PET), and single-photon emission computed

tomography (SPECT) is used as molecular imaging [17]. Radionuclide molecular imaging has significance in clinical and preclinical studies because of high susceptibility and measurability [17]. The most of molecular imaging in the clinic contain PET and SPECT imaging. Various PET and SPECT radiotracers are used for therapeutic imaging, containing oncology, cardiology, and neuroscience [19]. In the next section, several molecular imaging methods will be examined in more detail. In Table 1.3, a comparison of both anatomical imaging and radionuclide imaging methods can be observed.

Table 1.3: The benefits and drawbacks of imaging methods used with targeted or untargeted imaging agents [16].

Method	Benefits	Drawbacks	Contrast agents
CT	Unqualified tissue deepness influence. High spatial resolution. Can be used for entire body imaging. Short gain period. Anatomical imaging.	Radiation exposure. Bad mild tissue contrast.	X-rays
PET	Unqualified tissue deepness influence. Can be used for entire body imaging. As assembled with CT or MRI, detailed anatomical knowledge can be got.	Radiation exposure. Short spatial resolution and extended gain period. Costly.	High energy γ -rays
MRI	Unqualified tissue deepness influence. Non-ionizing radiation. High spatial resolution and perfect mild tissue contrast. Entire body imaging possible.	Costly. Extended gain period and restrained susceptibility.	Radiowaves

1.3.1. Methods of molecular imaging

1.3.1.1. Computed tomography (CT)

Computed tomography (CT) is an imaging technique that uses especial X-Ray device to examine elaborated images or scans of spaces within the body. CT examination may be used to diagnose and monitor a series of distinct statuses of the disease [20, 21]. CT imaging can be also used to identify impaired intelligence activity or

collapses in old patients with cognitive impaired estimated for the illness of Alzheimer's and other reasons of the cognitive collapse [20].

Each image examined throughout a CT process displays the members of the body, the bones, and the tissues in a slim region of the body. Throughout a CT technique, the patient lies down on a table, and the table moves towards the middle of a big donut-shaped X-Ray device bit by bit (Fig. 1.4) [19]. A series of X-rays are taken by a computer to acquire images [21].

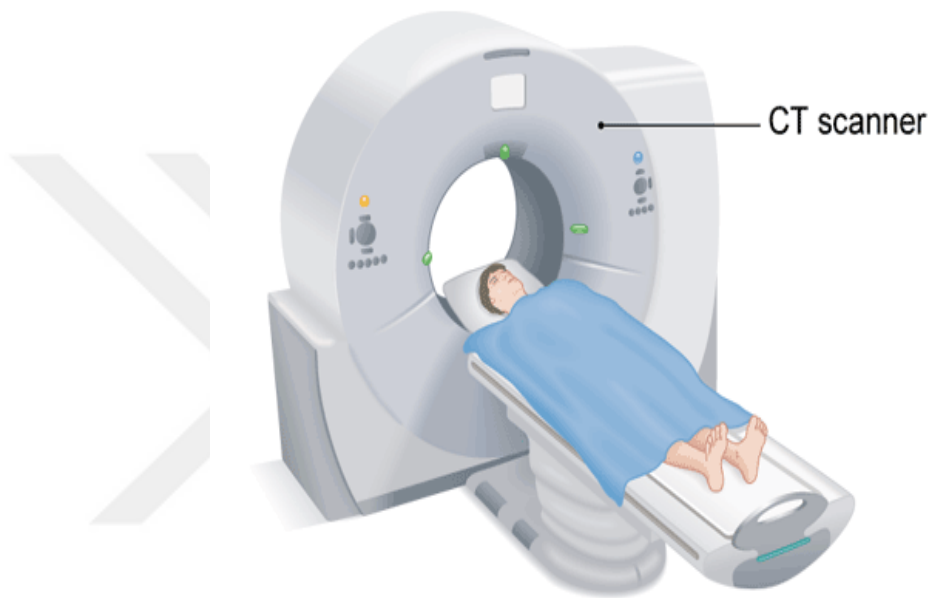


Figure 1.4: Patient at a table passing through CT device getting head X-ray images [21].

CT can occasionally contain the use of a contrast agent or dye. The dye may be given orally, syringed into a blood vessel, syringed with enema, or used all three methods. Contrast dye accentuates particular spaces within the body and supplies understandable images. Iodine and barium are two dyes frequently used in CT imaging [20].

1.3.1.2. Magnetic resonance imaging (MRI)

Magnetic Resonance Imaging (MRI) is a non-invasive imaging engineering that examines elaborated anatomical images in three dimensional. In general, it is used for identification, therapy, and follow-up of the disease [22].

MRI uses strong magnets that arrays protons in the body to a magnetic area. While a radiofrequency flow is thrown by way of the patient, protons are induced and passed from balance by force against the pull of magnetic area. Then, while the radiofrequency area is shut down and protons re-array due to the magnetic area, the MRI detectors can detect the energy released. The patient should lie down inside a big magnet to acquire an MRI image and must be motionless throughout the imaging process to avoid turbidity of the image [22].

Since MRI does not use X-Rays or other radiations, it is preferred, when frequent imaging is particularly wanted in the brain for diagnosis or treatment. The spinal cord and nerves, not only that brain, muscles and connective tissue appear so clearer in the MRI imaging than the CT imaging. Therefore, MRI is frequently used to image knee and shoulder wounds (Fig. 1.5). Though, MRI is more costly than CT examination [22].



Figure 1.5: The MRI of a knee [22].

MRI scanner can be separated into principal constituents. They are a magnet, shimming method, gradient method, transmitter and receiver of radiofrequency method, and computing method as shown in Figure 1.6 [23].

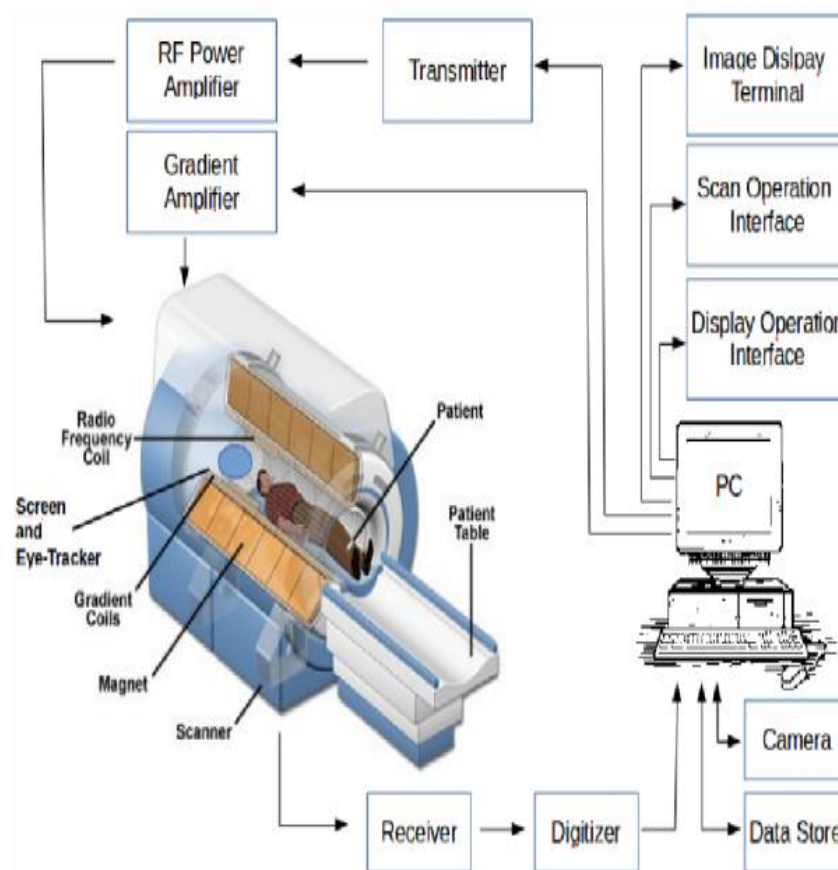


Figure 1.6: The chastened layout of practical MRI work equipment [23].

1.3.1.3. Positron emission tomography (PET)

PET is a very widely used molecular imaging method in clinical practice since 1970s. It tests the signal from the radioactive decomposition of neutron-deficient radioisotopes intravenously injected in the body and obtains in vivo images of the dissipation and uptake of them. Such isotopes radiate positrons which emerged from the nucleus as a conclusion of springless interactions with electrons in circumambient tissue (Fig. 1.7). The positrons quickly lose kinetic energy, diffuse around the tissue and crash with an electron to form two 511 keV photons taken orbit 180° side. This incident is called *annihilation*. A PET detector is planned to detect the signal and transform the occurred electrical signal to tomographic images [17]. The radioisotopes are isotopes of components that produce structural compounds in the nature, such as carbon, oxygen or nitrogen, or simply react with structural composites, such as fluorine. This provides the use of broad diversity of structural compounds in PET imaging, such as carbohydrates, amino acids, hormones, etc. [24].

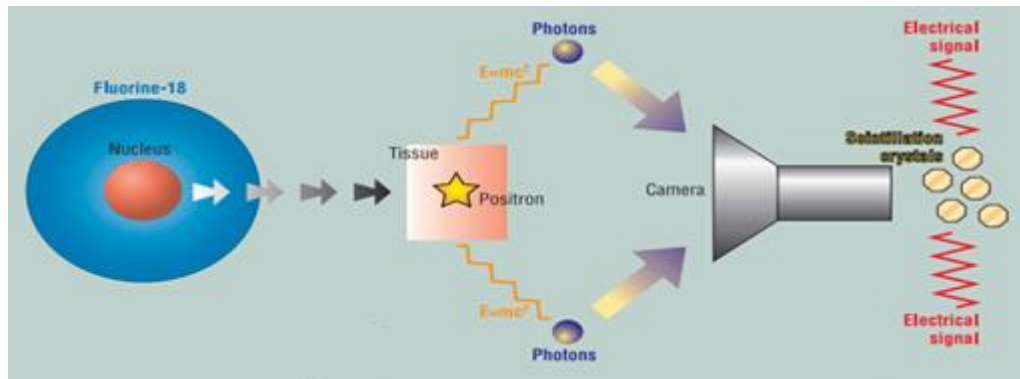


Figure 1.7: PET apparatus operation [25].

PET examinations are used to create accurate images of the body in three-dimensions. PET images frankly display the body fragments examined with unusual spaces and how well the body fragments performs as particular, instead of displaying what they see [26]. PET is a technique that tests physiological activity by looking at blood flow, metabolism, neurotransmitters, and radiolabelled drugs. Many PET systems are imaged the blood flow and oxygen absorption in distinct brain fragments [25]. PET examinations are especially useful to study cancer statuses assigned how distributed the cancer and how well it responds to therapy. PET examinations can also use to form schemes of treatments, such as coronary arterial bypass, transplantation, or neurosurgery for the treatment of the disease. These may further assist certain statuses of treatment that influence the regular functioning of the brain, such as mental illness [26]. The essential benefit of PET application is that the little probe mass and radiolabeling plans do not importantly ruin the biological procedures under search. Besides, PET has high molecular susceptibility and powerful quantitative possible. PET provides quantitative analyses to monitor comparative alterations in a period when a disease process develops or in reply to a particular stimulant. The method of PET is based on the detection of this radioactivity, after injected a little quantity of a radioactive tracer into a circumferential vein. This radioactive tracer is given as an intravenous injection labelled with oxygen, fluorine, carbon, or nitrogen. PET examinations end between 10 or 40 minutes [1, 25].

PET is developed as a method of clinical application for staging and re-staging of various malignancies. One of the clinical applications is the distinction of benign and malignant tumors. In general, all body is examined for the stage of cancer. Also, one of the widespread uses for PET is the measurement of the percentage of glucose use in distinct body fragments. ^{18}F -fluorodeoxyglucose (^{18}F -FDG) is accepted the most

commonly used PET radionuclide, when PET uses radionuclides of distinct positron such as Copper-61/64, Gallium-66/68, Zirconium-89, and Iodine-124. PET is a very susceptible and non-invasive engineering for imaging cancer biology based on ^{18}F -FDG, glucose analogue and a substrate for the hexokinase enzyme [1, 25]. FDG is a scientifically validated analogue of glucose for cells with high glucose metabolism, which is a separating property in most malignity, and acts for stage of several cancers containing breast, colorectal, oesophageal, head and neck, NSCL, melanoma and lymphoma. One of the soonest molecular imaging processes supplied by FDG provided to monitor of PET-based glucose as a precaution of the metabolic action of the tumor. Enhanced glucose metabolism in cancer cells initially observed by Otto Warburg in the 1950s called the Warburg Effect. Besides that, ^{68}Ga is used for transmission scans using a ^{68}Ga pole source in several PET plants. ^{68}Ga is an extended survived ($t_{1/2} = 278$ days) lacking neutron, usually developed by a high energy cyclotron based on spallation reaction. The practicality of using ^{68}Ga generator for more than a year is intensely cost-effectiveness negating the want for on-site cyclotron [1, 15, 18, 25].

The major limitations of PET are the exposure of patient to radiation, its poorer resolution compared with other molecular imaging systems and the absence of anatomical criteria to distinguish molecular incidents that have a correct connection with anatomical observations. It produces problems in detecting wounds in the body. Also, such anomalies in person tissues can be identified earlier with PET engineering than by CT or magnetic resonance. These limitations are overcome by combining PET with CT or MRI. These fusion systems integrate the lower resolution molecular information of PET with high-resolution anatomical details of CT [17, 27]. The combination of molecular knowledge in a tumor with anatomical or functional knowledge supplies more major susceptibility and certainty to grasp the tumor, limitly. So, multi-mode imaging is developed that assembles several imaging categories. Multi-mode imaging is a common technique in nuclear medicine. Instances of this image include PET/MRI, PET/CT, SPECT/CT, and MRI/OI. PET examinations are frequently assembled with CT examinations to obtain more accurate images. The greatest utility of PET-CT examinations is a rise in specificity facilitated by place of excessive tracer accumulation. PET/CT imaging method is composed of three principal constituents. They are a PET scanner, a CT scanner, and

a bed for the patient. Tumor staging with PET/CT is 84% effective compared to 76% PET+CT, 63% CT, and 64% PET [15, 26, 24]. The conclusion of multi-mode imaging is a combined image as shown in Figure 1.8 [24].

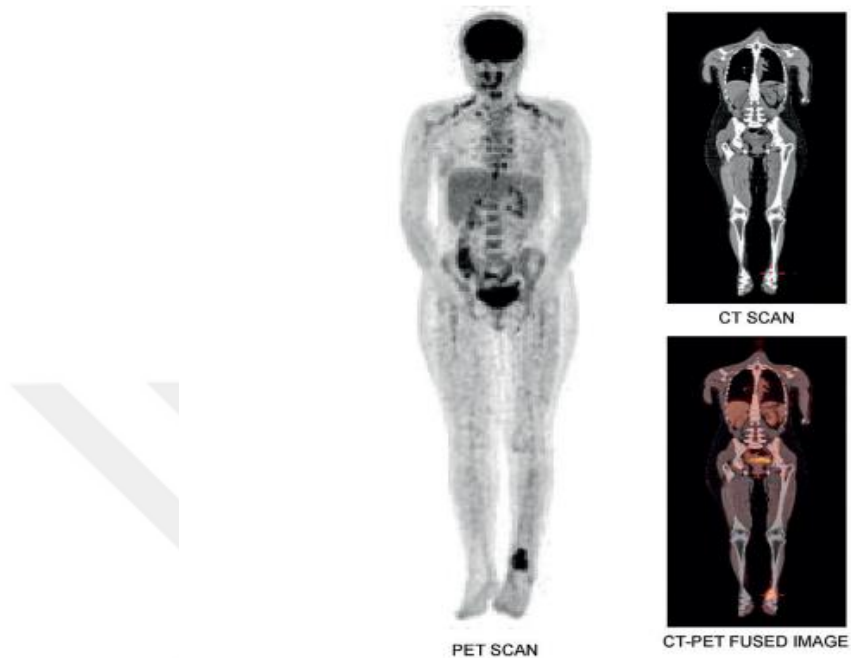


Figure 1.8: PET-CT with FDG for a melanoma patient [24].

1.3.2. Molecular imaging targets and agents

The molecular agent is a molecule that is syringed into the patient and ensures to target areas with imaging signal. Molecular imaging appliances use molecular agents to divulge biochemical procedures as an origin of contrast images. The molecular imaging area is based on the presence of appropriate biomarkers, the manufacture of selective contrast agents which are certain and discriminating for certain biomarkers, and their recovery in engineering. For such biomarkers, exogenous probes, such as small particles, antibodies, peptides, and genetically encoded probes are the two main types of probes. In extra to imaging the cancer phenotype, such as rises in metamorphosis, reproduction, angiogenesis, hypoxia, and programmed cell death, agents are improved to target certain protein markers expressed cancer cells or cancer-associated cells. These contain tumor cell receivers (EGFR, HER2/neu, ER, folate receptor, VEGFR2), integrin, peptidase, and prostate-specific membrane antigen (PSMA) [15, 19].

Extensive pre-clinical tests in theoretical establishments determine the numerous of present molecular imaging agents used in the clinic. Fig. 1.9 shows the time intense and costly preclinical steps in molecular targeting, validation, chemical labelling, cellular characterization, and animal test for new molecular imaging agents [19, 28].

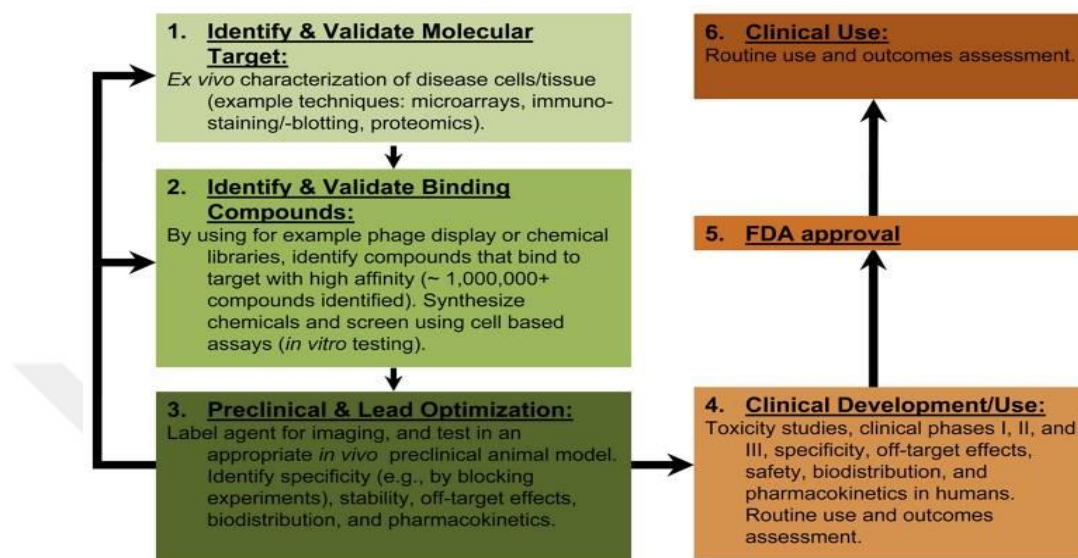


Figure 1.9: The steps of molecular agent design and clinical use [19].

MI commonly uses targeted living fluorophores and radioisotopes as agents. The agents should have extended cycle periods and the capability to achieve the target area at a sufficient absorption in vivo. Just a couple of imaging agents are present for clinical use acknowledged by the Food and Drug Administration (FDA, USA) [29].

Nuclear probes can be classified into three categories based on how the radioactive tag is added into the imaging agent as shown Figure 1.10. The radioactively tagged initial category (Class 1) probe is radionuclide, such as sodium [F^{18}] fluoride as bone-seeking PET agent used in imaging osteosarcoma. The second category (Class 2) contains a dangled radioactive tag that stretches from the molecular scaffolding, which allows biomarkers to target. This second category traditionally uses radio-metals and chelate agent for SPECT imaging; or PET agents may be used where the dangled is a radio-metal, such as Ga^{68} DOTA in [Ga^{68}]DOTATOC, or an F^{18} alkyl fluoride, such as a 3-N-(2- $[F^{18}]$ fluoroethyl) spiperone. The third probe category (Class 3) inserts the radioactive tag in the targeting particle construction. For instance, positron emitting radionuclides C-11, N-13, and O-15, which are isotopes

of endogenous components such as C, N, and O, are included in the molecules without altering their chemical characteristics for PET imaging [30].

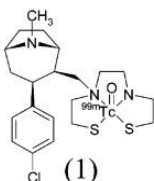
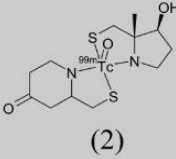
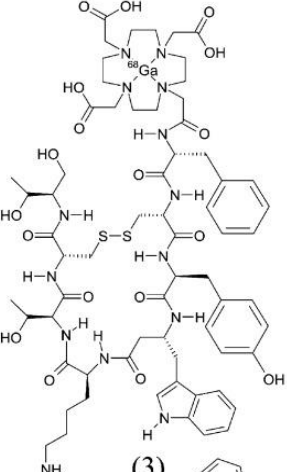
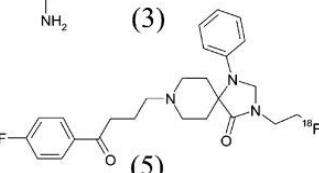
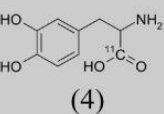
a	Class 1	Class 2	Class 3
			
b	SPECT [Tc-99m]NaTcO ₄	 (1)	 (2)
c	PET [F-18]NaF	 (3)  (5)	 (4)

Figure 1.10: Three categories of tracers. **a)** Class 1 tracers are those in which the radionuclide is the imaging agent itself. Class 2 tracers have drooping radiolabels. Class 3 knits up radionuclide into the targeting molecule. **b)** (1) [Tc-99m] TRODAT-1, (2) Experimental [Tc-99m] estradiol. **c)** (3) [Ga-68] DOTATOC, (4) [C-11] DOPA, (5) 3-N- (2 [F-18] Fluoroethyl) spiperon [30].

A perfect imaging probe should have high binding affinity and certainty for a given receptor and should be quickly removed from non-targeted substances to provide a suitable target-to-background proportion. In addition this, high steady and unity under physiological status, low immunogenicity and toxicity for a patient observation, and simply manufacture are essential in the clinic [29]. Thus, the

numerous of targeting fragments, such as small molecule, peptide, antibody, and aptamer, are used to equip ligand-oriented imaging agents for acknowledge certain diseased tissues [28].

1.3.2.1.Targeted molecular imaging agents based on small molecules

In molecular imaging, small molecules, normally lesser than 500 Da, have a significant part. Small molecules have a large implementation containing both intracellular and inner neurological methods because of their small dimension. ^{18}F -FDG is the most largely used imaging agent based on small molecules clinically added for cancer imaging [31].

1.3.2.2.Targeted molecular imaging agents based on antibodies

Antibodies, qualified by the capability to bind to their target with high certainty and affinity and simpler synthesis, are implemented for identification and treatment. More than eight FDA-acknowledged radio-labeled antibodies for SPECT molecular imaging and approximately twenty antibodies acknowledged for treatment are present [31].

1.3.2.3.Targeted molecular imaging agents based on aptamer

Aptamer, single-chain DNA or RNA oligonucleotides, may bind to their target with high particularity and certainty. Their advantages include high affinity and specificity, low immunogenicity, small size, stable structures, and ease of production [31].

1.3.2.4.Multifunctional molecular imaging agent

Due to distinct imaging methods can just observe matching contrast agents, the patient who wants to conduct multimodal imaging may need distinct contrast media. This can display flank influence with the common intervention between distinct contrast agents. Small molecules are not convenient for multifunctional imaging, because of their restrained binding spots. By comparison, nanoparticles are nominees for multifunctional imaging probe. Nanoparticles have high surface area/volume ratio, numerous alterations in ligands and distinct imaging agents inside or on them [31].

1.3.2.5.Nanoparticle based agents

Nanotechnology is the science and engineering of the control of manufacture of products at atomic, molecular, or macromolecular scales. At least one dimension of the nano-product is between 1 and 100 nano-meters according to the US National Nanotechnology Initiative (NNI). The most remarkable benefit of nanotechnology is the bridge constituted between the worlds of macroscopic and microscopic, where nanomaterials demonstrate to be the perfect environment for interfacing with the biological methods. Their large surface area/volume ratio supply quicker reaction. The porousness of permeable granules can control to raise adsorption and their surfaces can be chemically applied for the production of picky adsorbents [32, 34]. They have simple and steerable surface chemistry targeting for binding to small molecules of drugs, imaging tags, and targeting ligands, such as antibodies, peptides, nucleic acids, etc. [33]. The nanoscale construction can be also used as singular assets or combined into bigger substance constituents, methods, or builds [34].

The big surface area can be actualized with distinct targeting pieces and a multifunctional nano-platform can be constituted for targeted observation of distinct diseases. The ability to be multimodality is a great benefit of using nanoprobe on the conventional biological parts [33].

Biological processes, containing the things essential for the existence and caused cancer actualize at the nanoscale. Thus, everything consists of numerous of biological nano-machines. Nanotechnology offers the chance to investigate and control macromolecules in the real time and the first phases of cancer advancement. It can alter the method of diagnosing and removing cancer. It can ensure that cancer cells and their molecular alterations are observed quickly and precisely, even when they include just a little percent of all cells. Also, it can produce new and very efficient curative agents in molecular diagnostics and imaging [34]. Nanomaterials, which contain nanoscale granules, nanotubes, and nano-arrays, ease biomarker observation in medical applications [32].

Nanoparticles can be used as contrast agents for the quick observation of cancer (Fig. 1.11). Nanoparticles enable attained high certainty for a target by removing the threat of flank influences as both imaging agents and therapeutics at the identical period. Nanoparticle-based contrast agents (NCAs) have remarkable benefits as per the small

molecules. They are in modular constitution and their surface changes supply several tasks. When NCAs are only necessary, they can answer to their microenvironment or outer stimulants to supply treatment and contrast. NCAs can come through biological hurdles and achieve to tumors with active or inactive targeting processes. Their small dimension permits single intracellular and extracellular interactions, such as extravasation through endothelial cells and enhanced permeability and retention (EPR) in tumor tissues. Many tumors can be passively targeted with the influence of EPR in certain tissues. In passive targeting, a NCA gather in the tumor and hold on through EPR action. By comparison, active targeting joins targeting ligands to the surface of the NCA and allows the NCA to insert the cancer cell through receptor-mediated endocytosis. Also, distinct sorts of treatment can be revealed by the NCAs [16, 33, 35]. Many distinct radionuclide-nanoparticle mixtures have been described; however, less and quickly clearing nanoparticles and quickly degradable radioactive signals are aimed for possible clinical conversion. Radioisotope-nanoparticle mixtures are used for distinct imaging objects, containing ^{68}Ga , ^{89}Zr , ^{111}In , ^{198}Au [35].

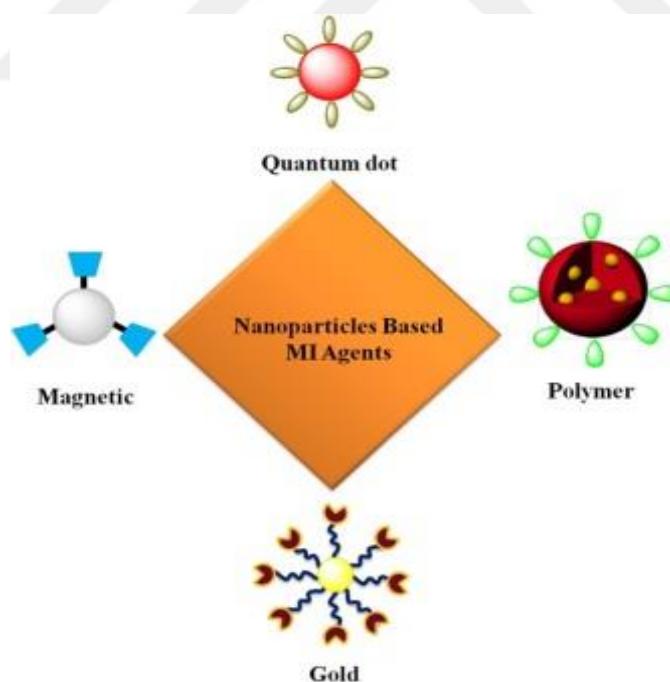


Figure 1.11: Nanoparticles targeted for imaging [16].

Semi-conductive radiant nanocrystals, called *quantum dots (QDs)*, are used as probes for biomedical imaging and tagging because of their single optical characteristics. As per conventional fluorophores, QDs have wide absorption spectra, high quantum

efficiencies, less photo-bleaching and chemical impairment strength, such as structural dyes. The surface of QDs is normally changed with biomolecules in implementations of cancer-related molecular and cellular imaging, such as antibodies, peptides, or small molecules, which have the capable of sticking and replying to receptors or antigens on target cells [16].

Superparamagnetic iron oxide nanoparticles (SPIOs) are the most frequently used contrast agent for clinical cancer diagnosis. SPIOs are clinically used to display tumors and metastases with acute contrast, and supply an understandable difference between normal and cancer tissues [16].

Gold nanoparticles (AuNPs) in biomedical implementations have single size-dependent characteristics. AuNPs are very biocompatible, simple to arrange, and usually non-toxic [16].

Polymeric nanoparticles (PN) have single characteristics for imaging probes. PN-based imaging agents have different benefits, which are long cycle periods, enhanced steadiness and target certainty, inaction in the immune system, and small or no toxicity [16].

1.3.2.6. Peptide-based molecular imaging agents

Peptides are innate ligands which oppose significant biomarkers. The peptide is a significant ligand category that is used for molecular imaging. As per small molecules, the peptide has very benefited, for example, high particularity, certainty, and simpler change without altering their binding or dispersion characteristics. In extra, the peptide is steadier than antibodies at ambient temperature and has shorter immunogenicity as per antibodies to bind biomarkers. The use of peptides as an agent for cancer imaging is very practical due to its high binding affinity and specificity for cancer cells, and high stability. Also, a molecular imaging probe should have minimal or acceptable level of toxicity before it can be safely employed in human. The major advantage of using peptides as imaging agents is that the peptides are non-toxic. Thus, they can be used easily without the need for additional toxic removal [28].

The peptide biological actions are adjusted by binding with the suitable receivers. Those with receivers over-expressed on tumor cells instead of regular cells are perfect nominees for tumor imaging in vivo [29].

Targeting peptide sequences can mostly be picked in three distinct methods. They are reproduction from native proteins, chemical synthesis and structure-based reasonable development, and the sifting of peptide bookcases [29].

Peptides perform essential cellular purposes and intercellular transmission actions. They consist of amino acid monomers that are bounded by amide bonds and characteristically have a low molecular weight which allows them to be rapidly cleared of both the blood and non-target tissue. Peptides usually appear in superior chemical attractions and certainties for their receivers and are effective up to nanomolar grade. A quantity of peptides, for example, somatostatin (SST) peptide, vasoactive intestinal peptide (VIP), Arg-Gly-Asp (RGD) peptide, and bombesin/gastrin-releasing peptide (BBN/GRP) are ably qualified for tumor receiver imaging [29].

Tripeptide RGD particularly binds to integrin receivers. Integrins form two subunits. The integrin stirpes, particularly $\alpha V\beta 3$, are important for angiogenesis and metastasis of tumor. *Amphibian BBNs* and their regarded peptides are formed of stirpes of neuropeptides that execute diversified physiological influences, for example, exocrine and endocrine discharges, thermoregulation, sucrose regulations, and cell development. The bombesin-like peptide receivers are overexpressed in very tumors, for example, chest cancer, gonadal cancer, and gastrointestinal stromal tumors. *VIP* is a neuropeptide containing 28 amino acids. It cannot just encourage vasodilation. However, it further encourages cell development and reproduction with cell-surface receiver-intervened development. *SSTs* are inherently cyclopeptide hormones containing 14 or 28 amino acids. They can prevent the excretion of insulin, glucagon, and certain other hormones. The biological influences are intervened by binding to certain high-affinity somatostatin receptors (SSTRs), which are overexpressed in very tumors, containing gliomas, neuroendocrine tumors, and chest tumors. SST peptide and its analogs, DOTATOC, DOTATATE and DOTANOC have all been tagged with ^{68}Ga and evaluated to find the symptomatic potential in patients with neuroendocrine tumors [1, 29].

The FDA characteristically treats peptides as traditional drugs rather than biological results that focalize their complex construction. Peptides are ordinarily sensitive to chemical alterations. Once the amino acid residues for certain targeting have been identified, chemical modifications are used to design the peptides for increased

metabolic steadies and proper pharmacokinetics. Imaging tags are immediately or mediate merged to the peptides for use in vivo imaging [29].

Three phases are commonly needed to configure a peptide-based imaging probe. They are recognition of the receiver and targeting peptide, peptide design and arrangement of the peptide parallels to maximize the biological action and metabolic attitude, and peptide chemical union of an imaging feature as shown in Figure 1.12. The clinical implementation of peptide-based agents may largely depend on the appraisal of in vivo particularity, the in vivo steady, the pharmacokinetic outline, and the toxicological surveys [29].

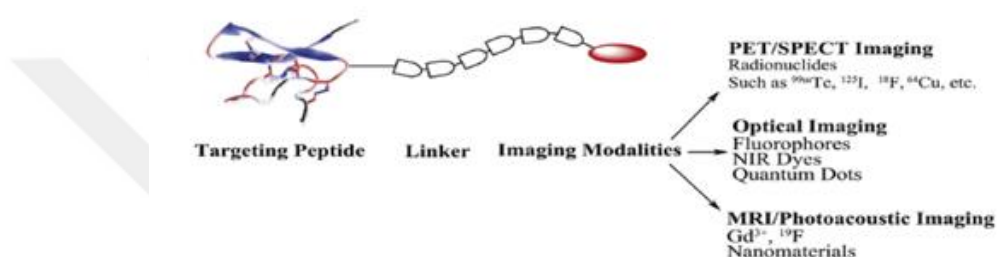


Figure 1.12: The graph of peptide-based imaging probes [29].

Peptides require to be tagged with imaging portions to function as imaging probes in vivo. Some common imaging components contain structural paints for OI, radionuclides for PET and SPECT imaging, and Gd^{3+} chelators for MRI. Useful peptide categories present for union contain, however, are not restrained to, the ϵ -amino category in the lysine chains, the guanidinium category in the arginine side chains, the carboxyl groups on aspartic acid or glutamic acid, cysteine thiol, and phenol on tyrosine. Carbodiimide / *N*-hydroxysuccinimidyl (EDC / NHS) intervened carboxyl and amine binding, maleimide union to thiol categories and diazonium changes of phenol on tyrosine are the most widespread union reagents [29].

^{68}Ga -DOTA-peptides bind particularly to somatostatin receivers (SST), which are over-expressed on neuroendocrine tumor (NET) cells. The organization of ^{68}Ga -DOTA-peptides contains an effective part that binds to SST (TOC, NOC, and TATE), a chelated (DOTA), and a beta ejecting isotope. From a handy spot of outlook, ^{68}Ga -DOTA-peptides offer different benefits, containing a simple tagging and synthesis operation, not being dependent on cell metabolism and supplying non-invasive knowledge regarding SST receiver phrase with straight therapeutical containment. It is used not just to appraise the spread of the illness, however further

for the choice of situations that will utilize from therapy with chill or fervent somatostatin parallels and take the forecast of the patient [36].

A few examples of folate-peptide conjugates are described. In literature, approximation to chemical stability and water solubility questions has been to form a drug-bound camptothecin (CPT) folate conjugate at 20-OH by means of a hydrophilic peptide spacer including a disulfide-releasable carbonate binder. Folate-peptide structure with terminal cysteine residue, Pte- γ -Glu-Asp-Arg-Asp-Asp-Cys, was synthesized on an H-Cys(Trt)-2-Cl-Trt resin using normal Fmoc-protected amino acids and N10-trifluoroacetylpteroic acid [37].

Figure 1.13 shows the conjugation of the folate peptide to the pyridyldithioethyl carbonate camptote, which constitutes the finished folate-peptide-CPT prodrug. The disulfide carbonate-peptide spacer adds water solubility to the prodrug without sacrificing FR fastening affinity and supplies an extrication mechanism for quick release of camptothecin in FR+cancer cells [37].

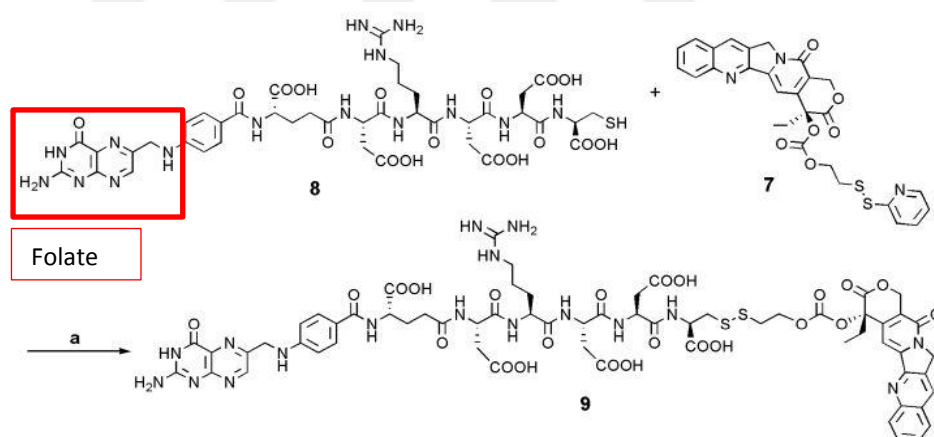


Figure 1.13: The synthesis of folate-peptide-CPT. (a) DMSO, (7) pyridyldithioethyl carbonate camptothecin, (8) folate-peptide, (9) folate-peptide-CPT prodrug [37].

In other literature, arginine and lysine linked cationic peptides ease intracellular input of macromolecular loads through the lipid bilayer. Two linear peptides Lys-Gly-His (Pep-1) and Arg-Arg-Arg-Arg-Lys-Gly-His (Pep-2) were synthesized based on arginine or lysine including peptides to enhance tumor accumulation. Peptides adorned with folate (FA-Pep-1 and FA-Pep-2) were radioactively marked with $[(99\text{mTc}(\text{I}(\text{CO})_3\text{I}(\text{H}_2\text{O})_3)]^+$ precursor (Fig. 1.14). In vivo, tumor-targeting abilities were judged by performing biodistribution, and scintigraphic runnings in mice. Lastly, the most promising targeting part (FA-Pep-1) was used to decorate PLGA

nanoparticles charges with 5-fluorouracil (5-FU) to target the over-expressed folate receptor on the surface of FR-positive tumor cells [38].

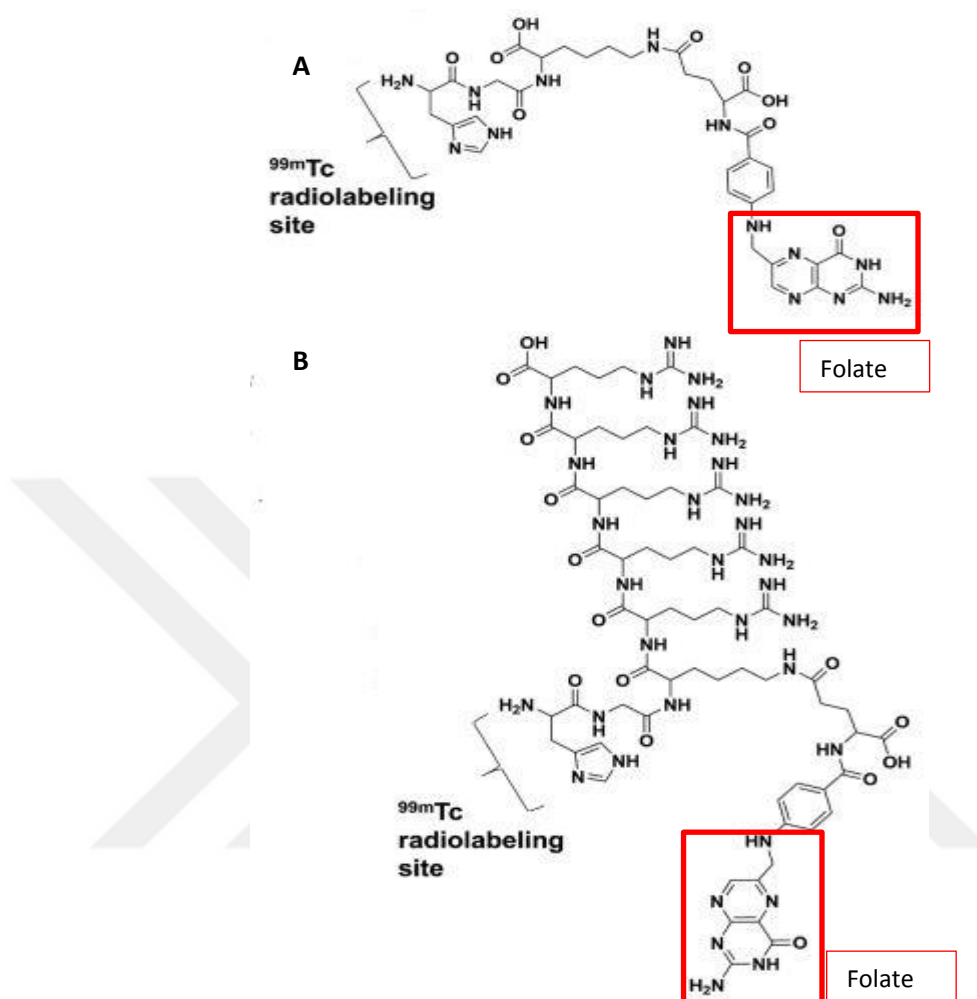


Figure 1.14: The chemical structure of folate conjugated peptides. **(A)** FA-Pep-1, **(B)** FA-Pep-2 [38].

In another literature, ^{99m}Tc -EC20 is indicated as the most promising nominee for ^{99m}Tc -folate, ^{99m}Tc (V) (Technetium-99m) is complexed with a brief folate-linked peptide (Cys-Asp-Dap-D-Glu-Pte) (Fig. 1.15). ^{99m}Tc -EC20 imaging is defined as a confident, non-invasive procedure that can define FRs in repetitive or metastatic maladies without the necessity for a biopsy to define ill who may benefit from a cure with FR targeted treatment [39].

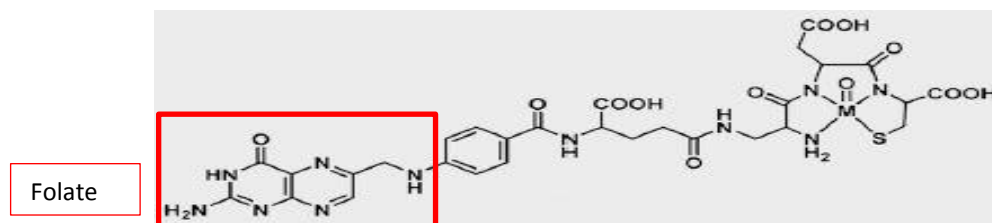


Figure 1.15: The chemical structures of folic acid radioconjugates: $^{99\text{m}}\text{Tc}$ -EC20 ($\text{M} = ^{99\text{m}}\text{Tc}$) [39].

2. PURPOSE OF THE THESIS

The goal of this study is to synthesize, characterize and purify peptide-based imaging agents selected from the peptide library that were designed with Computer-Aided Drug Design (CADD) for Folate Receptor 1 (FOLR1) targeting and capable of strongly binding to reactive cancer cells with affinity as an alternative to conventional PET agents to monitor tumor microenvironment. The peptides which can bind to FOLR1 receptor with high sensitivity and affinity have been synthesized by Solid-Phase Peptide Synthesis (SPPS) method, purified with Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) and characterized with Liquid Chromatography-Mass Spectrometry (LC-MS) for utilizing as an imaging agent in PET to diagnose cancer. It will examine and evaluate the potential use of the peptides synthesized by the Solid-Phase Peptide Synthesis (SPPS) method in PET imaging of cancerous regions in the body. After biologically active compounds are identified, they will be combined with radio-labeling of DOTA-chelates with Ga⁶⁸ for PET imaging. So, pharmaceutical sciences and biochemistry come together to produce new molecules for PET imaging.



3. EXPERIMENTAL PART

3.1. The Materials and Chemicals

4-(2', 4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl- (Rink amide MBHA resin), $N\alpha$ -Fmoc- $N\omega$ -(2, 2, 4, 6, 7-pentamethyldihydro-benzofuran-5-sulfonyl)-L-arginine, *N*-(9-Fluorenylmethoxycarbonyl)-L-alanine, $N\alpha$ -Fmoc- $N\epsilon$ -Boc-L-lysine, Fmoc-L-phenylalanine, *N*-(9-Fluorenylmethoxycarbonyl)-L-proline, Fmoc-L-aspartic acid 4-tert-butyl ester, *N*-(9-Fluorenylmethoxycarbonyl)-O-tert-butyl-L-tyrosine, Fmoc-O-tert-butyl-L-serine, $N\alpha$ -(9-Fluorenylmethoxycarbonyl)- $N\gamma$ -trityl-L-asparagine and Fmoc-L-glutamic acid 5-tert-butyl ester were purchased from Chem-Impex International. O-(1H-Benzotriazol-1-yl)-*N*, *N*, *N'*, *N'*-tetramethyluronium hexafluorophosphate (HBTU) and *N*, *N*-Diisopropylethylamine (DIPEA) were purchased from Alfa Aesar. Piperidine, triisopropylsilane (TIPS), trifluoroacetic acid (TFA), Acetonitrile (ACN), dichloromethane (DCM), and *N*, *N*-Dimethylformamide (DMF) were purchased from Sigma-Aldrich.

Isolab brand vortex and Bandelin brand sonicator were used for agitation of a solution. Millipore brand pure water device was used for supplying pure water. Heidolph brand rotary evaporator was used to remove solvents. Martin Christ brand lyophilizer was used for the freeze-drying of samples. Shimadzu brand analytical and preparative Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) and Waters brand preparative RP-HPLC were used purification of peptides (analytical column was GL Science Inertsil ODS-3 C18 column (150 mm x 4.6 mm, 5 μ m) and preparative column was Thermo Scientific Hypersil GOLD C18 column (250mm x 10.0 mm, 12 μ m)). Thermo Fisher Scientific Q Exactive Orbitrap Liquid Chromatography-Mass Spectrometry (LC-MS) was used for the characterization of peptides.

3.2. The Solid-Phase Peptide Synthesis (SPPS)

Solid-phase peptide synthesis (SPPS) is based on the solid support, linker, properly protected amino acids, procedure for coupling and cleaving the peptide from the solid support. The efficient solving of the peptide and the resin is important for effective chain assembly throughout solid-phase synthesis. Swollen resin is packed in columns and used in a continuous-flow mode by pumping reagents and solvents through the resin [40].

The technique of solid-phase peptide synthesis protects chemistry in solution and has a covalent binding step wherein the nascent peptide chain to an insoluble polymeric support. Then, the synthesis is continued by a series of cycles (Fig. 3.1). When the synthesis is finished, the crude peptide is released from the support. In this methodology, the solid-phase technique provides that reactions are occurred by using of excess soluble reagents, which are simply removed by filtration and washing without losing any product [40].

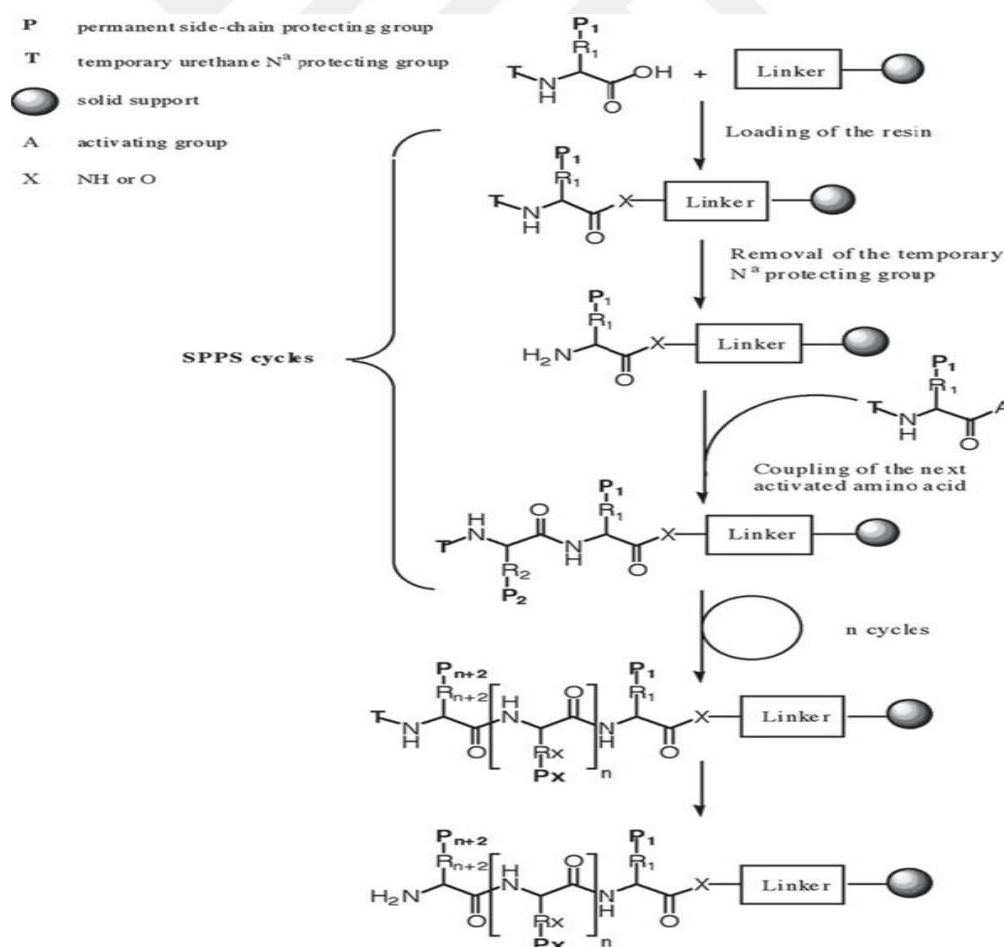


Figure 3.1.: Generalized access to solid-phase peptide synthesis [41].

In this thesis, Fmoc-mediated peptide synthesis was utilized. Table 4 shows the mass of amino acids in each sequence.

Table 3.1: The mass of amino acid in coupling reactions.

Amino Acid Code	Amino Acid Name	Weight (mg)
A	Alanine	37.68
R	Arginine	78.50
N	Asparagine	72.20
D	Aspartic acid	49.79
F	Phenylalanine	46.88
K	Lysine	56.70
P	Proline	40.83
S	Serine	46.39
Y	Tyrosine	55.60

Peptides exhibiting high affinity towards the FOLR1 protein by Computer-Aided Drug Design (CADD) by Assoc. Dr. Atilla Akdemir and his team at Bezmialem Vakıf University. Following to *in silico* studies, seven peptides, whose sequences are shown below, were selected for synthesis:

- H₂N-RFAADK-CONH₂
- H₂N-KYAAK-CONH₂
- H₂N-RFSNK-CONH₂
- H₂N-RPSNK-CONH₂
- H₂N-KYANK-CONH₂
- H₂N-KYDAK-CONH₂
- H₂N-KYSAK-CONH₂

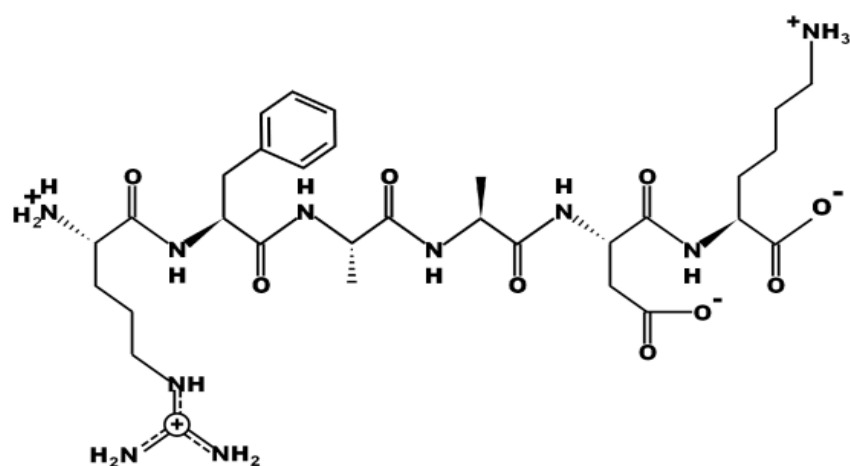


Figure 3.2: The structure of $\text{H}_2\text{N-RFAADK-CONH}_2$ [42].

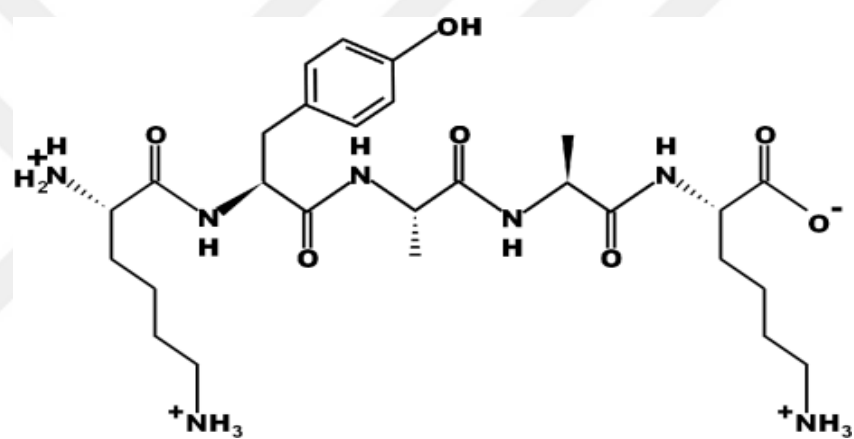


Figure 3.3: The structure of $\text{H}_2\text{N-KYAARK-CONH}_2$ [42].

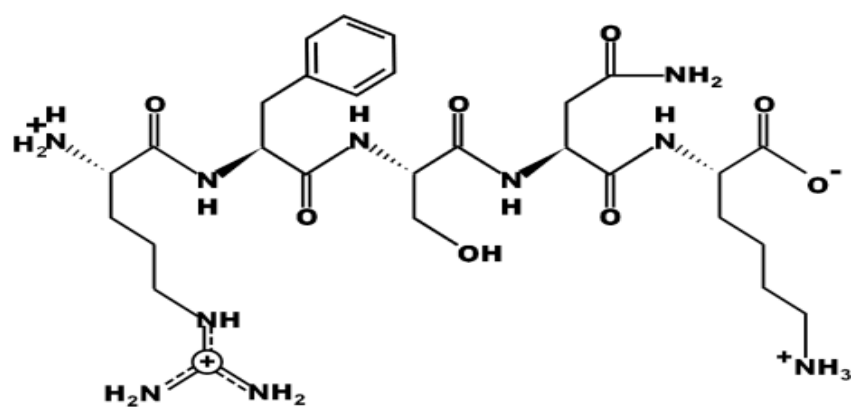


Figure 3.4: The structure of $\text{H}_2\text{N-RFSNK-CONH}_2$ [42].

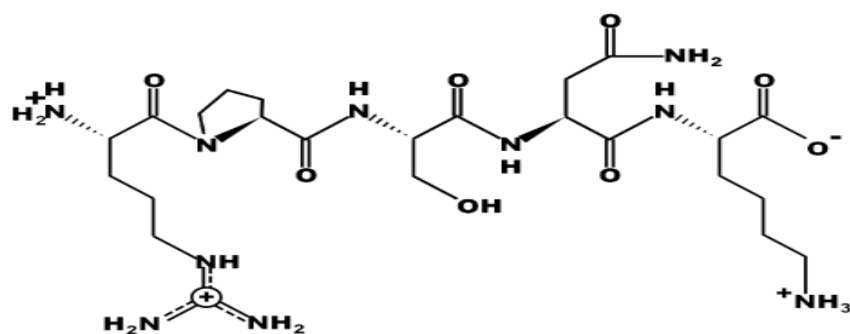


Figure 3.5: The structure of $\text{H}_2\text{N-RPSNK-CONH}_2$ [42]

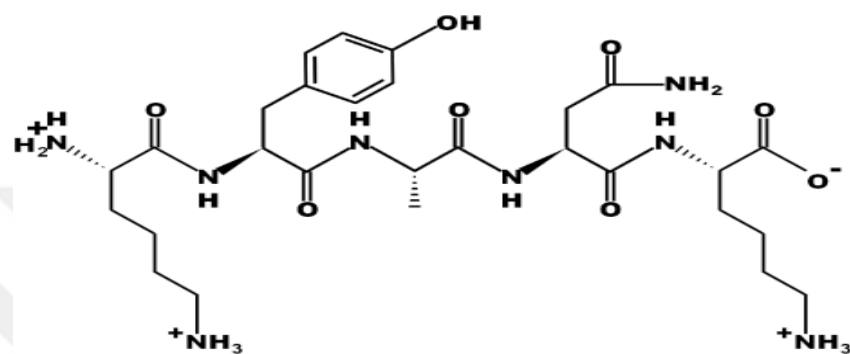


Figure 3.6: The structure of $\text{H}_2\text{N-KYANK-CONH}_2$ [42].

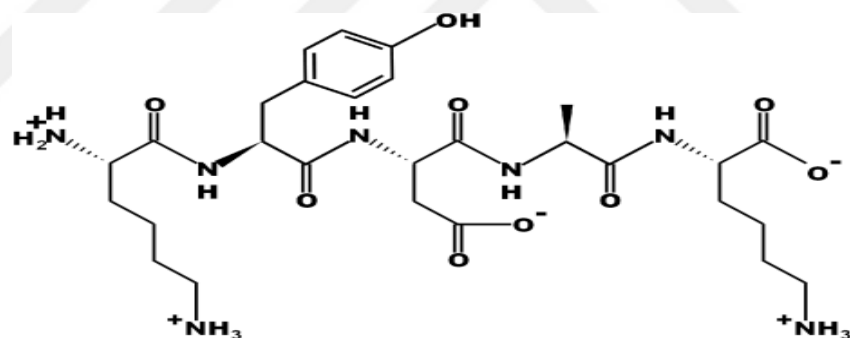


Figure 3.7: The structure of $\text{H}_2\text{N-KYDAK-CONH}_2$ [42].

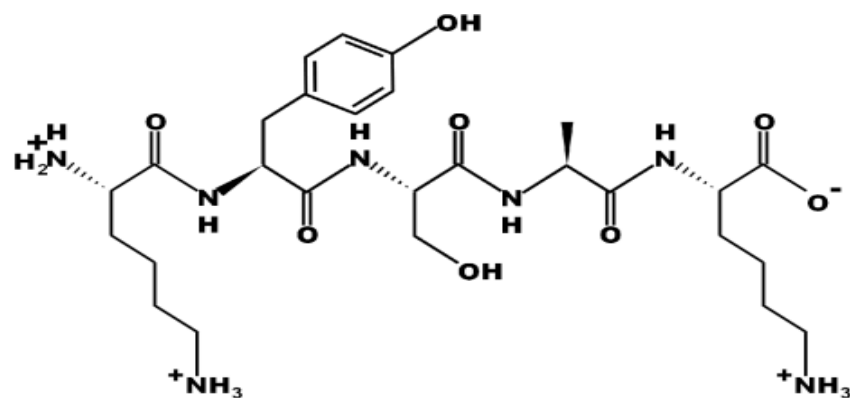


Figure 3.8: The structure of $\text{H}_2\text{N-KYSAK-CONH}_2$ [42].

3.2.1. The Synthesis of RFAADK Sequence with Fmoc-Chemistry

3.2.1.1. The Swelling of the Rink Amide MBHA Resin

48 mg (0.022 mmol, 0.44 meq/g) rink amide MBHA resin (RAM) was weighed. The resin was applied into a peptide reactor and was swollen in 10 mL DMF in a reaction column for 30 minutes. Then, the solvent was streamed with vacuum system.

3.2.1.2. The Removal of Fmoc Group of the Rink Amide MBHA Resin

The Fmoc protecting group was removed by treating the pre-swollen resin with 20% piperidine solution in DMF for 10 minutes (2x855 μ L). Then, the solution was streamed with vacuum system and washed with DMF (10x5 mL).

3.2.1.3. The Coupling of Amino Acids

Firstly, 56.70 mg K (0.121 mmol, 5,5 eq.) with the Fmoc protected group and HBTU (42 mg, 5 eq.) dissolved in DMF (1,5 mL). Then, DIPEA (50 μ L) was added. After the addition of DIPEA, solution is mixed. After 30 seconds, it was added to the resin. Amino acid cocktail that was added to the resin was stood within 1 hour for binding of amino acid and the resin. Then, the solution was filtered off and the resin was washed with DMF (2x5 mL). The Fmoc protecting group of K was removed by treating the resin with 20% piperidine solution in DMF for 10 minutes (2x855 μ L). Then, the solution was streamed with vacuum system and washed with DMF (10x5 mL).

The amino acids are activated according to the following procedure.

- 49.79 mg D,
- 37.68 mg A,
- 46.88 mg F,
- 78.50 mg R.

Each amino acid (0.121 mmol, 5,5 eq.) and HBTU (42 mg, 5 eq.) are dissolved in DMF (1,5 mL). Then, DIPEA (50 μ L) was added. After addition of DIPEA, solution is mixed. For 30 seconds, it was applied to the resin. Then, amino acid was reacted with the resin for 1 hour. Then, the solution was filtered off and the resin was washed with DMF (2x5 mL). The Fmoc group on each amino acid was removed by treating

the resin with 20% piperidine solution in DMF for 10 minutes (2x855 μ L). Then, the solution was streamed with vacuum system and washed with DMF (10x5 mL).

3.2.1.8. The Removal of the Last Fmoc Group

The Fmoc group of last amino acid was removed by treating the resin with 20% piperidine in DMF for 10 minutes (2x855 μ L). Then, the solution was streamed with vacuum system. Then, the resin was washed with 5 mL DMF. The resin was washed with DCM (25 x 5 mL) to remove DMF. Then, the resin was dried with vacuum system.

3.2.1.9. The Cleavage of Peptide

The cleavage cocktail was prepared with 98% TFA, %1 DCM and %1 TIPS (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS) to cleave the peptide from the resin. 1.1 mL of the cocktail (2 x 550 μ L) was reacted with for 1 hour and the solution was collected. The solvent was removed under reduced pressure. The crude material was lyophilized for 48 hours. The peptide was purified with HPLC and characterized with LC-MS.

3.2.2. The Synthesis of Other Peptide Sequences

Other peptides are synthesized according to the same procedure. The amounts of amino acids and the other chemicals are shown in the following tables.

Table 3.2: The synthesis of KYAAK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	DMF-Piperidine	2x0.855 μ L
HBTU	42 mg	Alanine	37.68 mg
DMF	1.5 mL	Lysine	56.70 mg
DIEA	50 μ L	Tyrosine	55.60 mg

Table 3.3: The synthesis of RFSNK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	Arginine	78.50 mg
HBTU	42 mg	Lysine	56.70 mg
DMF	1.5 mL	Phenylalanine	46.88 mg
DIEA	50 μ L	Asparagine	72.20 mg
DMF-Piperidine	2x0.855 μ L	Serine	46.39 mg

Table 3.4: The synthesis of RPSNK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	Arginine	78.50 mg
HBTU	42 mg	Lysine	56.70 mg
DMF	1.5 mL	Proline	40.83 mg
DIEA	50 μ L	Asparagine	72.20 mg
DMF-Piperidine	2x0.855 μ L	Serine	46.39 mg

Table 3.5: The Synthesis of KYANK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	Tyrosine	55.60 mg
HBTU	42 mg	Lysine	113.4 mg
DMF	1.5 mL	Alanine	37.68 mg
DIEA	50 μ L	Asparagine	72.20 mg
DMF-Piperidine	2x0.855 μ L		

Table 3.6: The Synthesis of KYDAK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	Tyrosine	55.60 mg
HBTU	42 mg	Lysine	113.4 mg
DMF	1.5 mL	Alanine	37.68 mg
DIEA	50 μ L	Aspartic acid	49.79 mg
DMF-Piperidine	2x0.855 μ L		

Table 3.7: The Synthesis of KYSAK.

Reagent	Amount	Reagent	Amount
Resin	48 mg	Tyrosine	55.60 mg
HBTU	42 mg	Lysine	113.4 mg
DMF	1.5 mL	Alanine	37.68 mg
DIEA	50 μ L	Serine	46.39 mg
DMF-Piperidine	2x0.855 μ L		

3.3. The Purification and Characterization of the Peptides

3.3.1. The Analysis of Peptides by Analytical RP-HPLC

After synthesis, the peptides were purified with RP-HPLC. The peptides were dissolved at concentration ranging from 0.3 mg/mL to 1.3 mg/mL. The analytical HPLC was used first to form the HPLC method. The samples were passed through the filters (0.45 μ m), transferred to vials, and injected to HPLC. The analysis method is given below.

Table 3.8: The analysis method of peptides by analytical HPLC.

Flow rate (mL/min)	Mobile Phase A	Mobile Phase B	UV Detector (nm)	Injection Volume (μ L)	Column Temperature ($^{\circ}$ C)	Duration Time (min)	Solvent gradient
1.0	Water with 0.08 % TFA	Acetonitrile with 0.08 % TFA	214	20	40	70	From 5% ACN to 100% ACN

3.3.2. The Purification of Peptides by Semi-Preparative RP-HPLC

RFAADK, KYAAK, RPSNK, KYANK, KYDAK, and KYSAK peptides were purified with Waters brand preparative RP-HPLC device after analytical HPLC according to the following method.

Table 3.9: The purification method of peptides by semi-preparative HPLC.

Flow rate (mL/min)	Mobile Phase A	Mobile Phase B	UV Detector (nm)	Injection Volume (μ L)	Column Temperature ($^{\circ}$ C)	Duration Time (min)	Solvent gradient
5.0	Water with 0.08 % TFA	Acetonitrile with 0.08 % TFA	214	3500	25	70	From 5% ACN to 100% ACN

RFAADK, KYAAK, RPSNK, KYANK, KYDAK, and KYSAK peptides were prepared by dissolving 2 mL of acetonitrile and 2 mL of distilled water. The prepared samples were passed through the filters (0.45 μ m) and transferred to a clean HPLC vial and placed. Then, collected fractions were lyophilized for 48 hours.

RFSNK peptide was analysed and purified on Shimadzu brand preparative RP-HPLC device after analytical HPLC with the following method.

Table 3.10: The purification method of RFSNK by semi-preparative HPLC.

Flow rate (mL/min)	Mobile Phase A	Mobile Phase B	UV Detector (nm)	Injection Volume (μ L)	Column Temperature ($^{\circ}$ C)	Duration Time (min)	Solvent gradient
2.0	Water with 0.08 % TFA	Acetonitrile with 0.08 % TFA	214	1700	40	40	From 5% ACN to 90% ACN

RFSNK peptide was prepared by dissolving 1 mL of acetonitrile and 1 mL of distilled water. The prepared sample was passed through the filters (0.45 μ m) and transferred to a clean HPLC vial and placed in the device. During elution, all peaks observed in the detector were collected in glass test tubes. Then, it was placed in clean glass containers and lyophilized for 48 hours.

3.3.3. The Analysis of Purified Peptides by Analytical RP-HPLC

Purified RFAADK, KYAAK, RPSNK, KYANK, KYDAK, and KYSAC peptides were analysed on Waters brand preparative RP-HPLC device after purification with the following method.

Table 3.11: The analysis method of purified peptides by analytical HPLC.

Flow rate (mL/min)	Mobile Phase A	Mobile Phase B	UV Detector (nm)	Injection Volume (μ L)	Column Temperature ($^{\circ}$ C)	Duration Time (min)	Solvent gradient
5.0	Water with 0.08 % TFA	Acetonitrile with 0.08 % TFA	214	400	25	40	From %5 to %20 for 30 min and from %20 to %100 for 10 min

RFAADK, KYAAK and RPSNK peptides that were taken from lyophilizer were prepared by dissolving 1.5 mL of acetonitrile and 1.5 mL of distilled water. The prepared samples were passed through the filters (0.45 μ m) and transferred to a clean HPLC vial and placed in the device.

Purified RFSNK peptide was analysed on Shimadzu brand analytical RP-HPLC device after purification with the following method.

Table 3.12: The analysis method of purified RFSNK by analytical HPLC.

Flow rate (mL/min)	Mobile Phase A	Mobile Phase B	UV Detector (nm)	Injection Volume (μ L)	Column Temperature ($^{\circ}$ C)	Duration Time (min)	Solvent gradient
1.0	Water with 0.08 % TFA	Acetonitrile with 0.08 % TFA	214	50	40	40	From %5 to 90%

RFSNK peptide was prepared by dissolving 500 μ L of acetonitrile and 500 μ L of distilled water at low concentrations ranging from 0.3 mg/mL and 1 mg/mL. The prepared sample was passed through the filters (0.45 μ m) and transferred to a clean HPLC vial and placed in the device.

3.3.4. The Characterization of Peptides by LC-MS/MS

The molecular weight of all four peptides considered to be purified was performed on the Thermo Scientific Q Exactive Orbitrap LC-MS/MS device. 0.02 mg of the samples were prepared by dissolving 500 μ L of acetonitrile and 500 μ L of distilled water and then passing through the filters (0.45 μ m) and transferred to a clean device vial and placed in the device.



4. RESULTS AND DISCUSSION

After the synthesis and purification of peptides, they are characterized by LC-MS. The sequences of certain peptides were synthesized, purified with RP-HPLC, and characterized with LC-MS. These results are given below:

4.1. HPLC and LC-MS Analysis of Peptides

4.1.1. RFAADK

The HPLC and mass spectroscopy chromatogram of RFAADK are given below in the following figures, respectively.

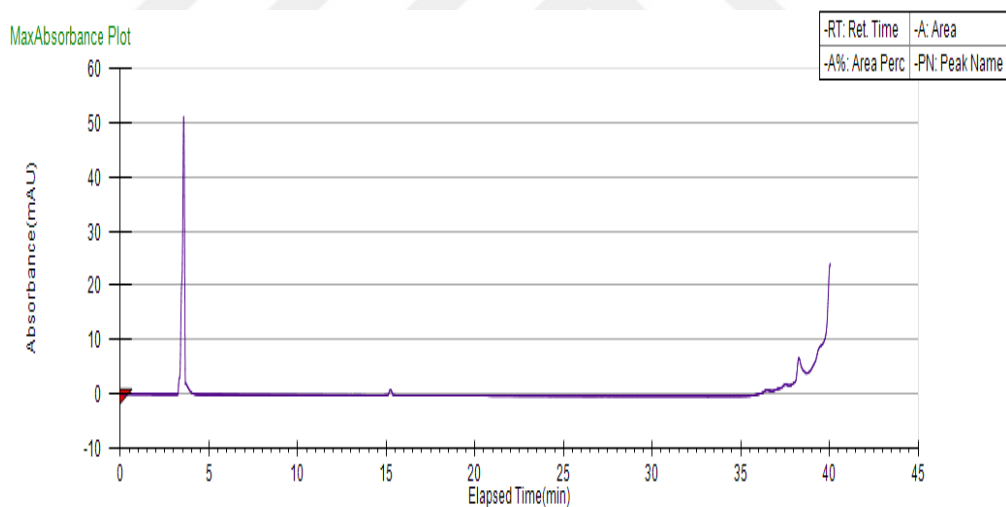


Figure 4.1: The HPLC chromatogram of purified RFAADK.

Table 4.1: The HPLC chromatogram of purified RFAADK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	98.3628	456428.1439	3.57	50.1392
Peak 2	1.6372	7597.087	15.24	0.8218

RFAADK1+ #1179 RT: 6.37 AV: 1 NL: 5.41E7
T: FTMS + p ESI Full ms [80.0000-1200.0000]

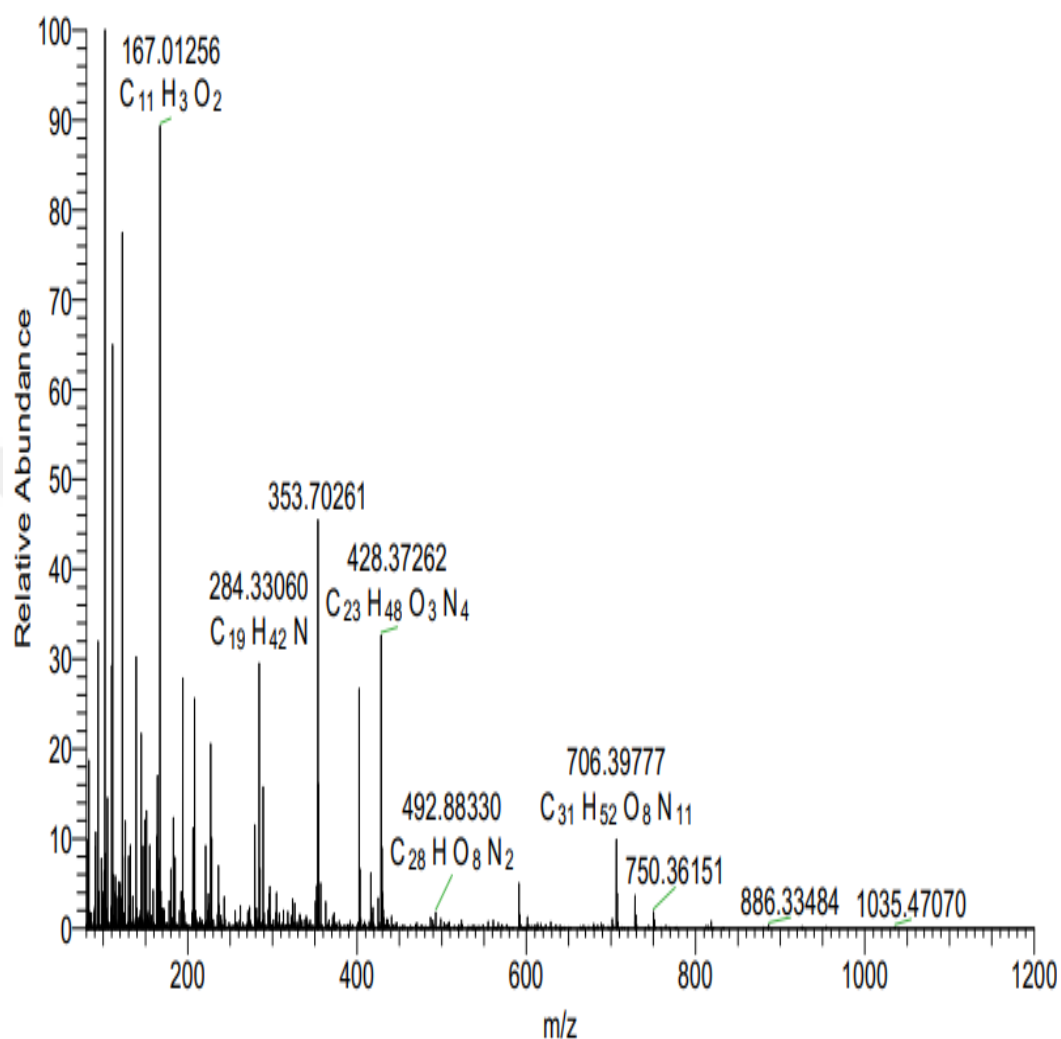


Figure 4.2: The mass chromatogram of RFAADK.

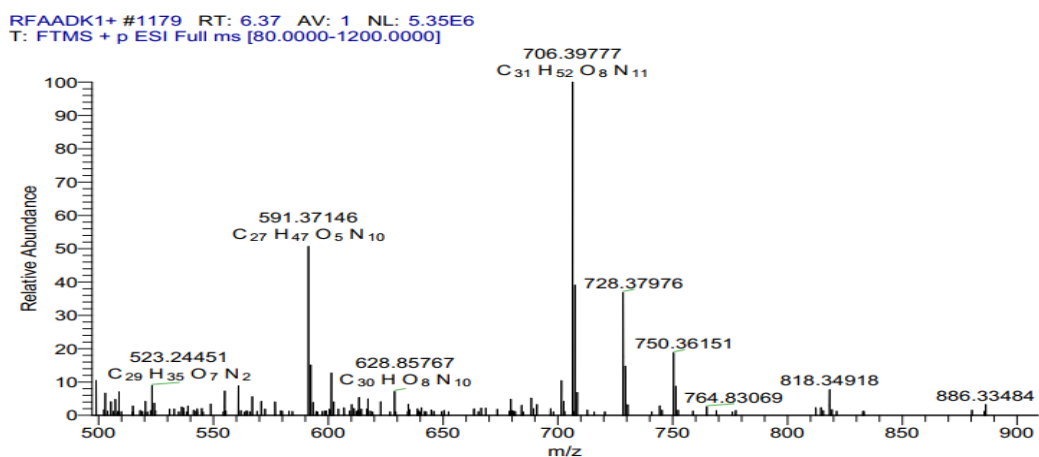


Figure 4.3: The expansion of mass chromatogram of RFAADK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted RFAADK sequence has a purity of 98, 36 % in light of integration of the corresponding signal. The molecular mass of RFAADK sequence with C-term amide is 705, 81 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 706, 39 g/mol in the mass chromatogram.

$$[M + H]^+ = 706,39 \rightarrow M_w = 705,39 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 705,81 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

4.1.2. KYAAK

The HPLC and mass spectroscopy chromatogram of KYAAK are given below in the following figures, respectively.

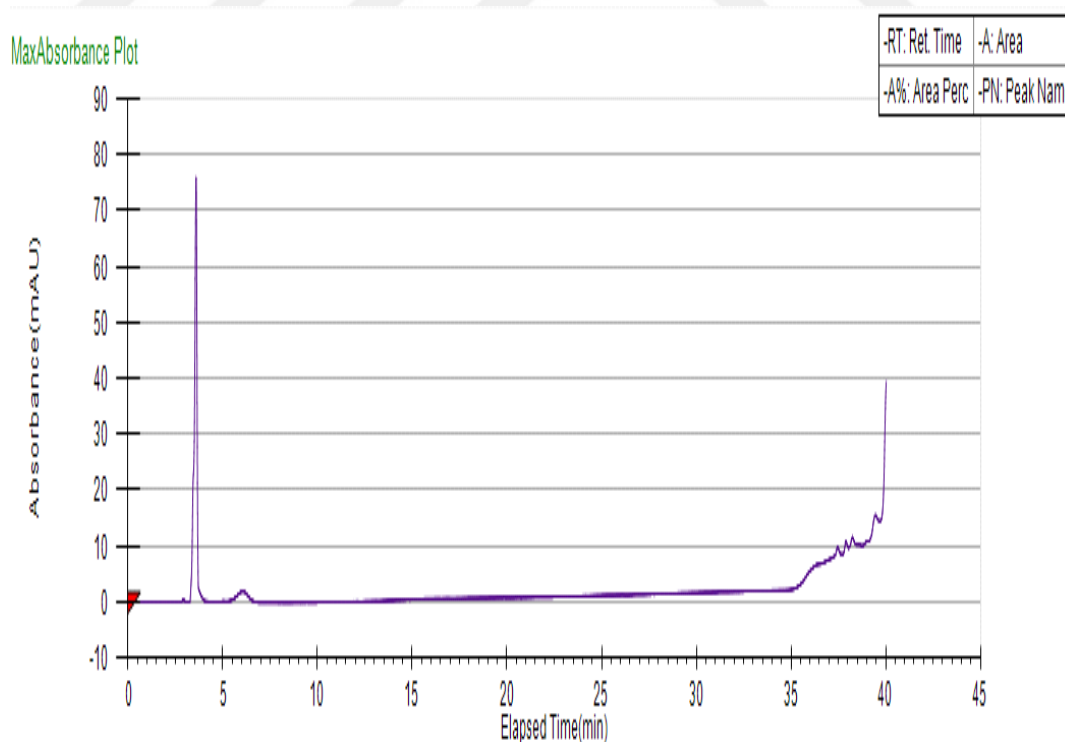


Figure 4.4: The HPLC chromatogram of purified KYAAK.

Table 4.2: The HPLC chromatogram of purified KYAAK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	93.7932	730707.8335	3.58	75.6569
Peak 2	6.2068	48354.6888	6.06	1.4861

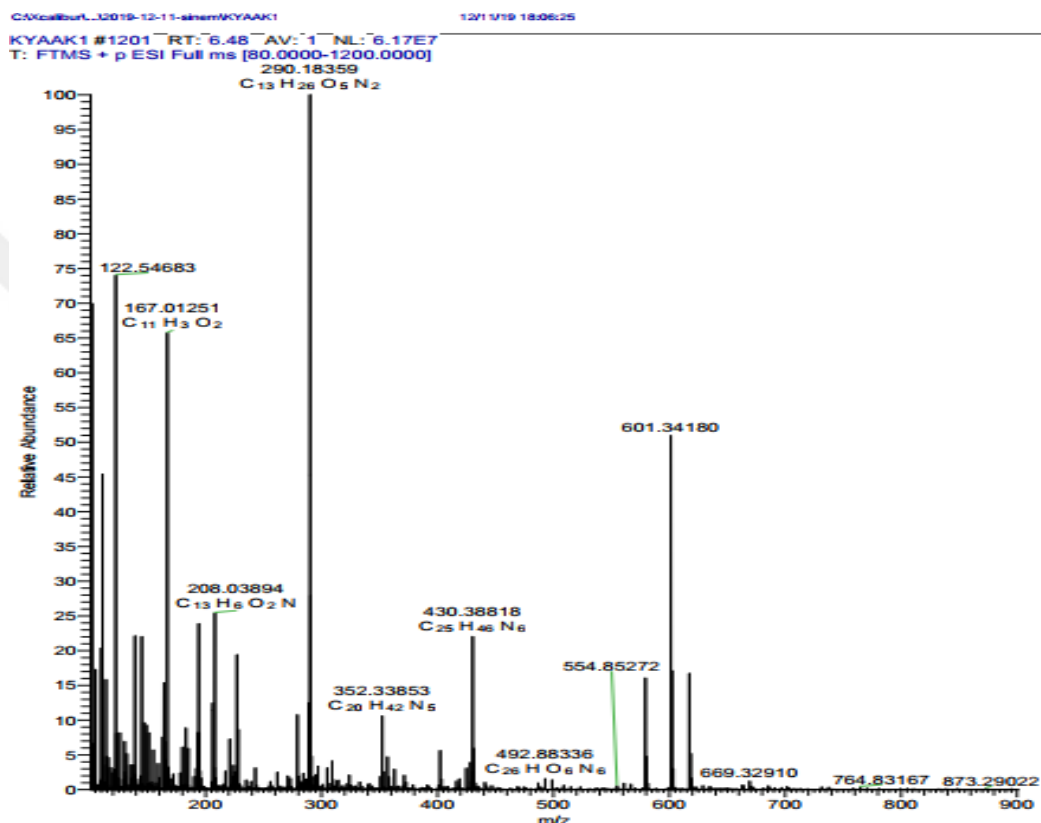


Figure 4.5: The mass chromatogram of KYAAK.

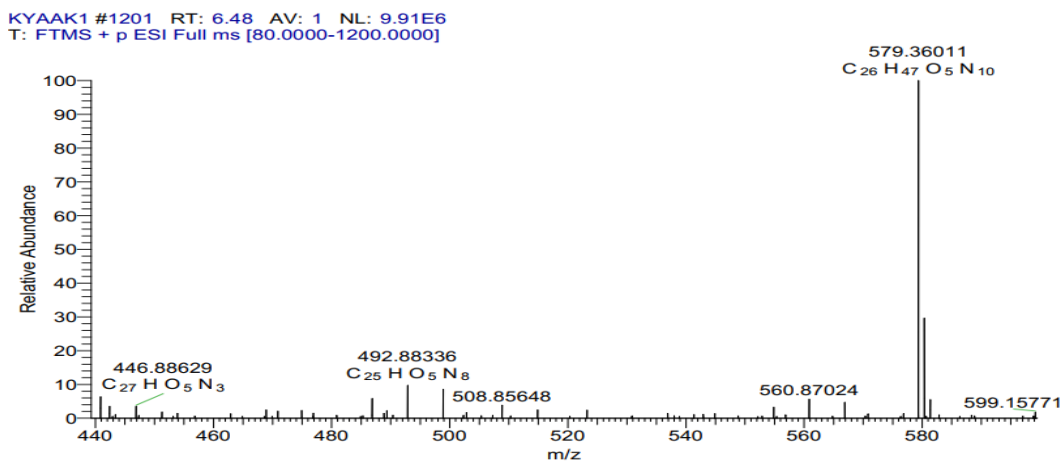


Figure 4.6: The expansion of mass chromatogram of KYAAK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted KYAAK sequence has a purity of 93, 79 % in light of integration of the corresponding signal. The molecular mass of KYAAK sequence with C-term amide is 578, 71 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 579, 36 g/mol in the mass chromatogram.

$$[M + H^+]^+ = 579,36 \rightarrow M_w = 578,36 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 578,71 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

4.1.3. RFSNK

The HPLC and mass spectroscopy chromatogram of RFSNK are given below in the following figures, respectively.

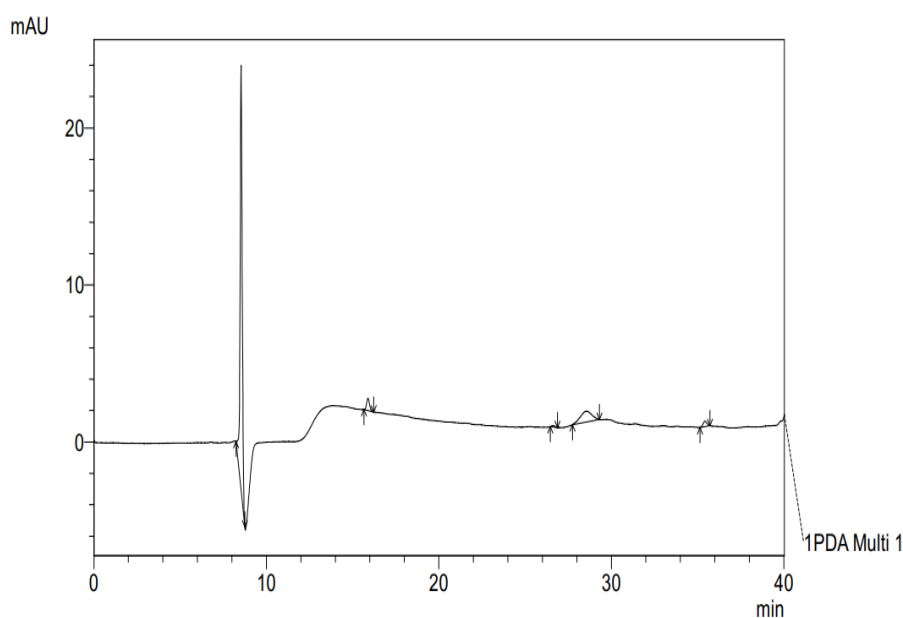


Figure 4.7: The HPLC chromatogram of purified RFSNK.

Table 4.3: The HPLC chromatogram of purified RFSNK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	82.9481	232429	8.527	26993
Peak 2	3.3462	9376	15.881	786
Peak 3	0.4107	1151	26.594	89
Peak 4	11.3931	31925	28.541	690
Peak 5	1.9019	5329	35.419	345

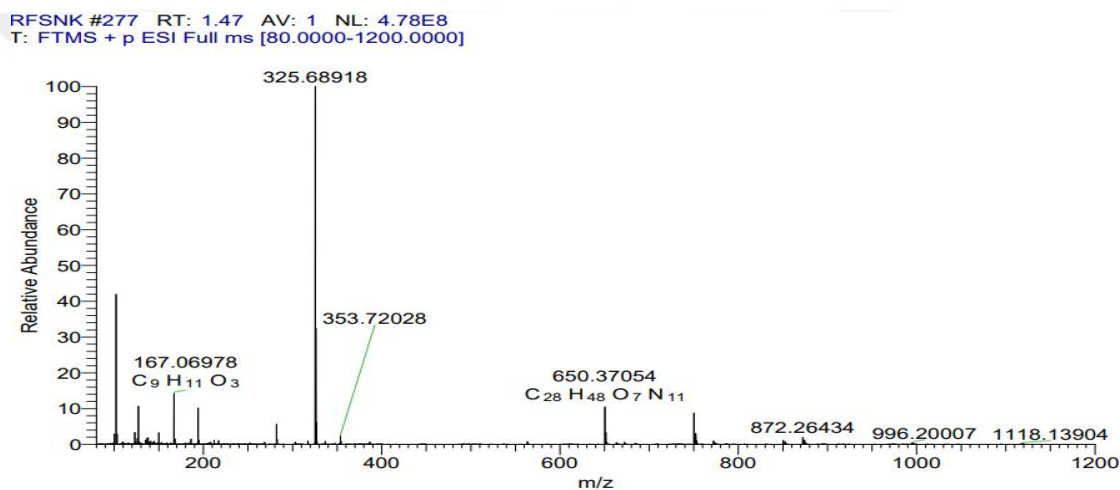


Figure 4.8: The mass chromatogram of RFSNK.

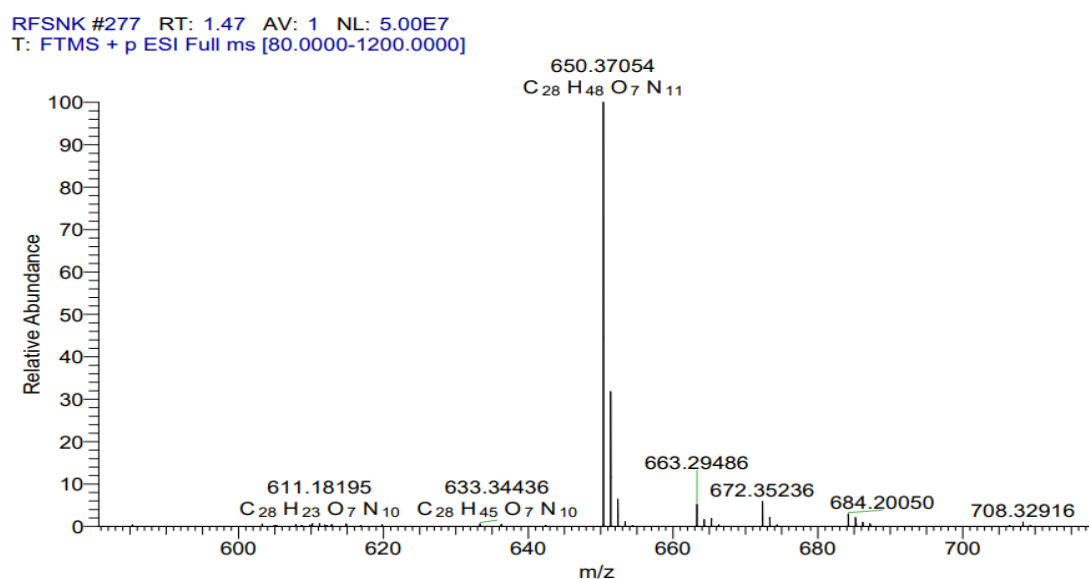


Figure 4.9: The expansion of mass chromatogram RFSNK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 8 minutes in the first chromatogram indicates that targeted RFSNK sequence has a purity of 82, 94 % in light of integration of the corresponding signal. The molecular mass of RFSNK sequence with C-term amide is 649, 75 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 650, 37 g/mol in the mass chromatogram.

$$[M + H^+]^+ = 650,37 \rightarrow M_w = 649,37 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 649,75 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

The reason why the HPLC chart of the RFSNK differs from the HPLC chart of the other sequences is that the chart of the RFSNK was obtained in the Shimadzu preparative device and the others were obtained in the Waters preparative device.

4.1.4. RPSNK

The HPLC and mass spectroscopy chromatogram of RPSNK are given below in the following figures, respectively.

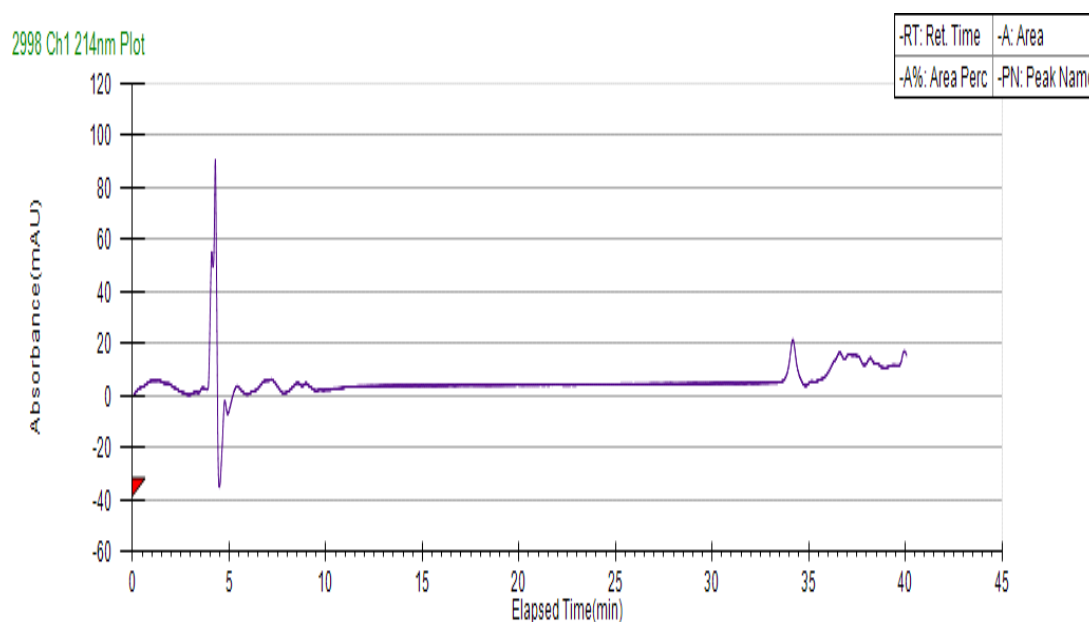


Figure 4.10: The HPLC chromatogram of purified RPSNK.

Table 4.4: The HPLC chromatogram of purified RPSNK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	79.8563	4274041.5926	4.28	295.1578
Peak 2	3.2765	175363.6638	5.42	8.9683
Peak 3	14.3062	765690.6921	7.18	16.3627
Peak 4	2.2775	121895.4313	8.54	5.5068
Peak 5	0.2835	15176.0101	8.97	1.7344

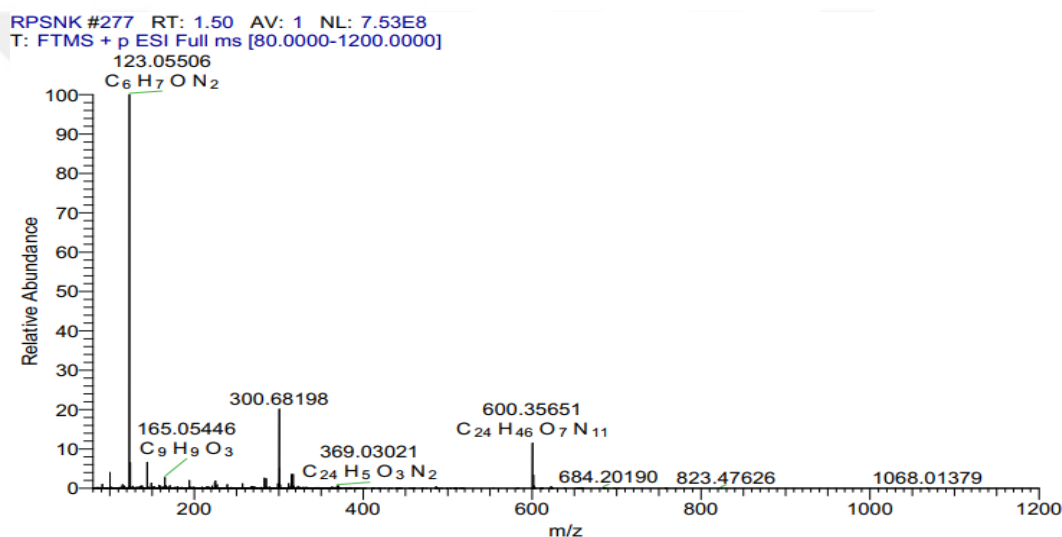


Figure 4.11: The mass chromatogram of RPSNK.

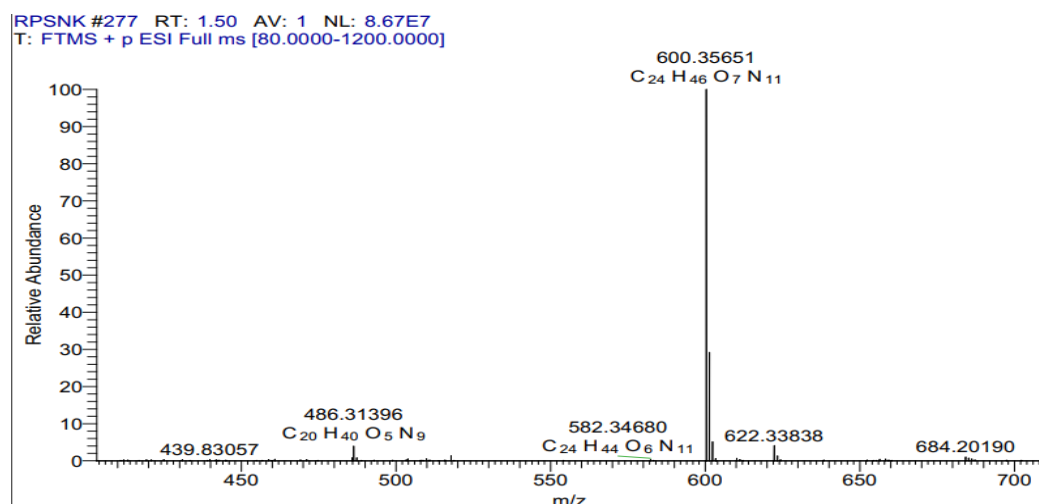


Figure 4.12: The expansion of mass chromatogram of RPSNK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted RPSNK sequence has a purity of 79, 85 % in light of integration of the corresponding signal. The molecular mass of RPSNK sequence with C-term amide is 599, 69 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 600, 35 g/mol in the mass chromatogram.

$$[M + H^+]^+ = 600,35 \rightarrow M_w = 599,35 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 599,69 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

4.1.5. KYANK

The HPLC and mass spectroscopy chromatogram of KYANK are given below in the following figures, respectively.

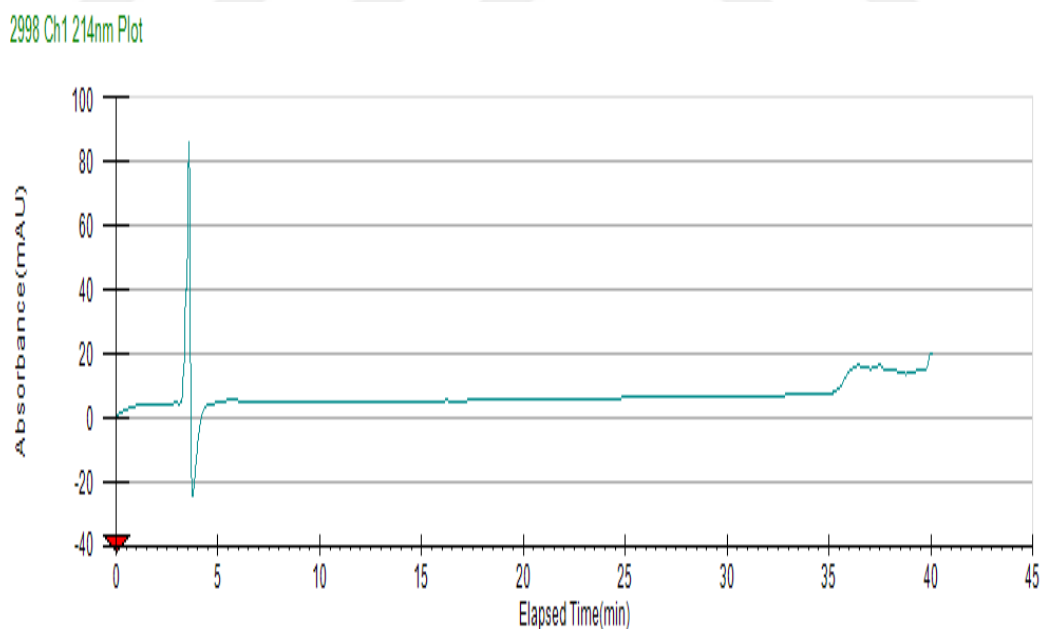


Figure 4.13: The HPLC chromatogram of purified KYANK.

Table 4.5: The HPLC chromatogram of purified KYANK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	98.924	968478.9766	3.58	92.6572
Peak 2	1.076	10534.0817	5.7	0.6208

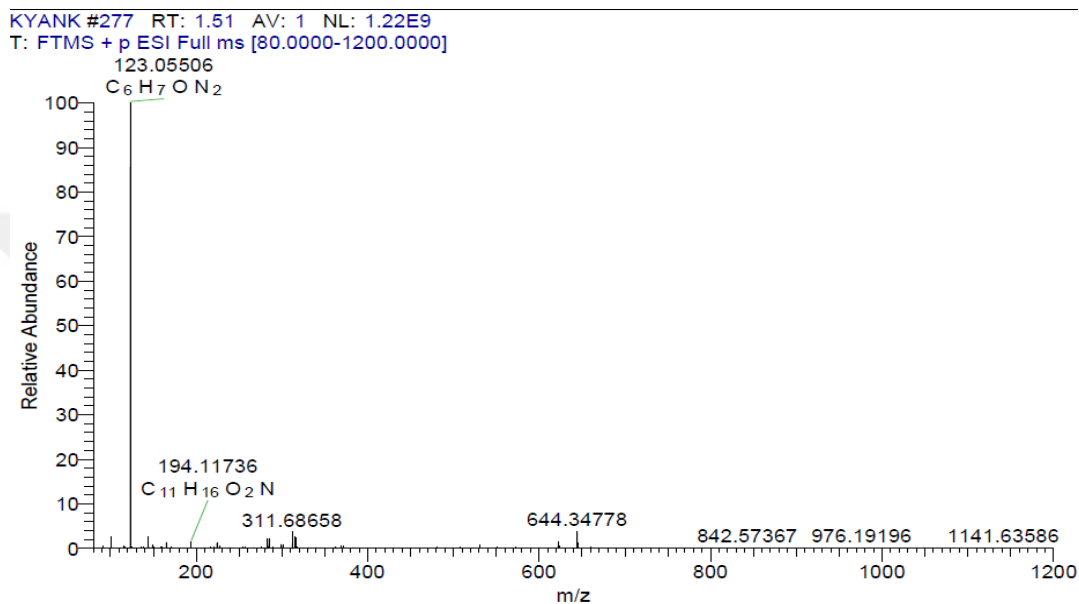


Figure 4.14: The mass chromatogram of KYANK.

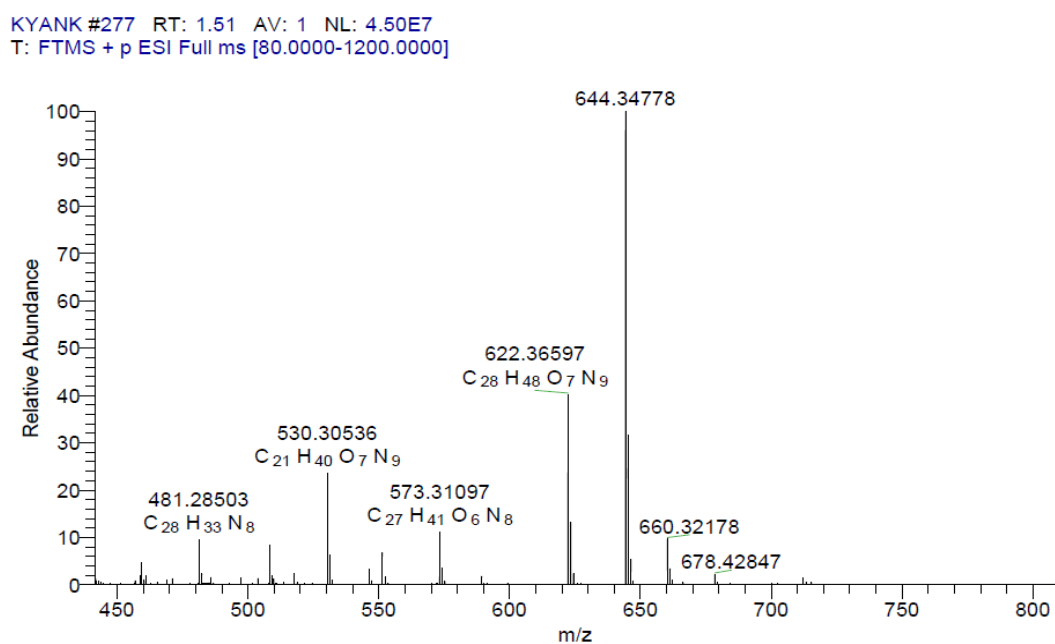


Figure 4.15: The expansion of mass chromatogram of KYANK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted KYANK sequence has a purity of 98, 92 % in light of integration of the corresponding signal. The molecular mass of KYANK sequence with C-term amide is 621, 74 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 622, 37 g/mol in the mass chromatogram.

$$[M + H]^+ = 622,37 \rightarrow M_w = 621,37 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 621,74 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

4.1.6. KYDAK

The HPLC and mass spectroscopy chromatogram of KYDAK are given below in the following figures, respectively.

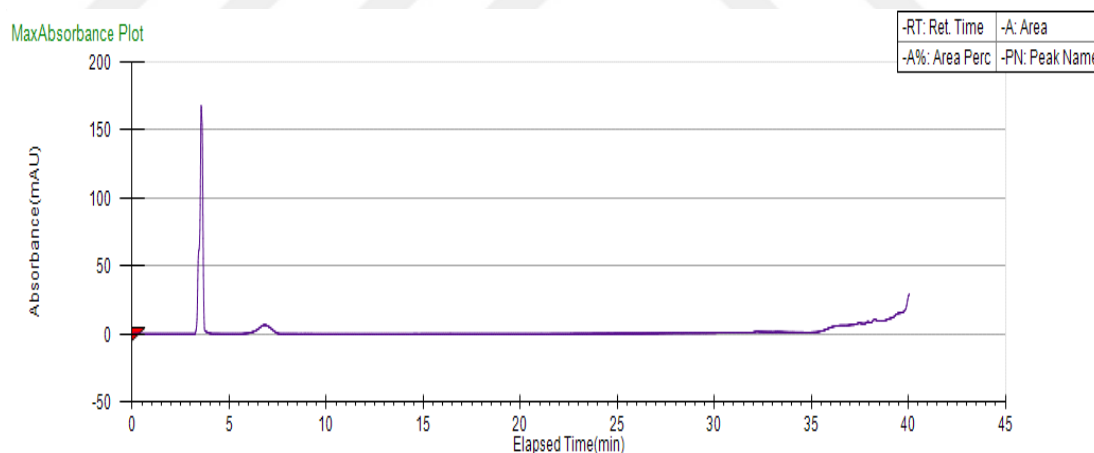


Figure 4.16: The HPLC chromatogram of purified KYDAK.

Table 4.6: The HPLC chromatogram of purified KYDAK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	91.4685	1868796.9569	3.55	166.7092
Peak 2	8.5315	174307.6131	6.82	4.8295

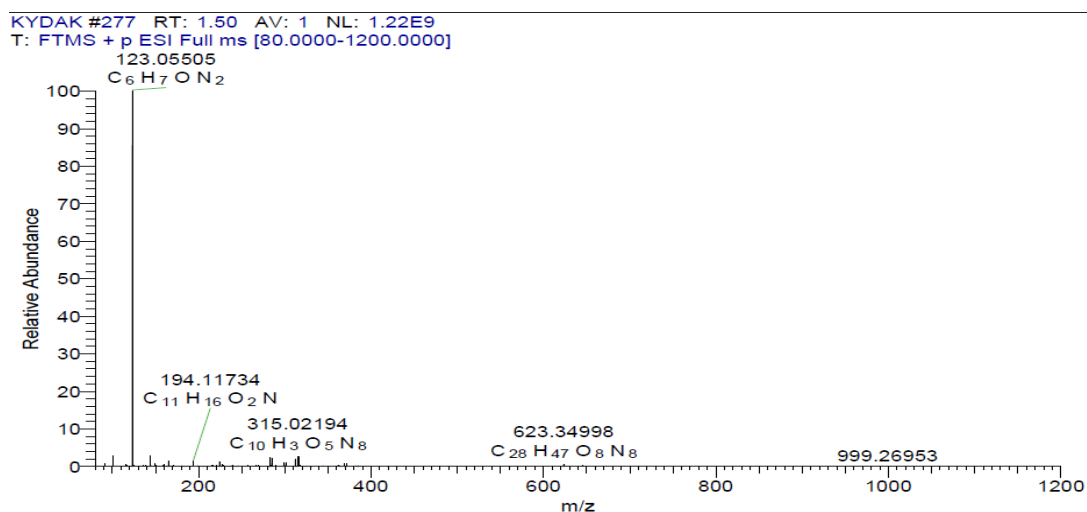


Figure 4.17: The mass chromatogram of KYDAK.

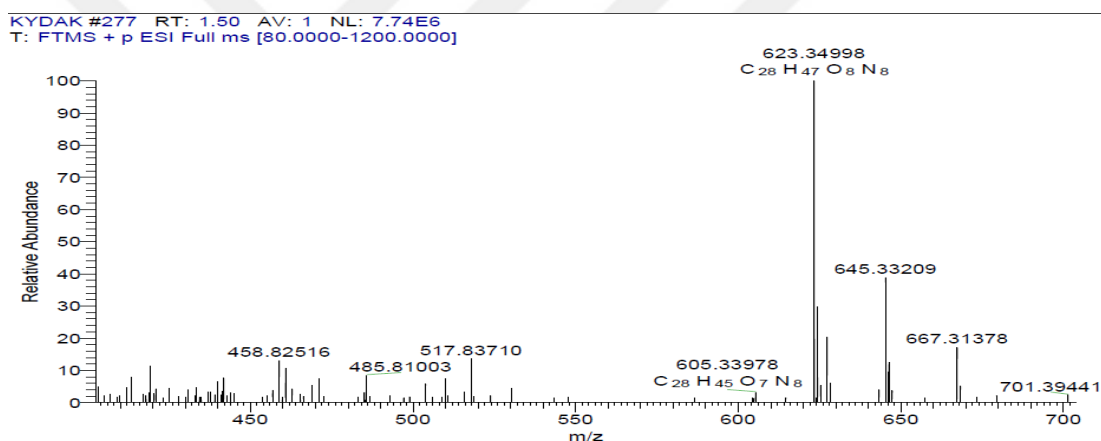


Figure 4.18: The expansion of mass chromatogram of KYDAK.

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted KYDAK sequence has a purity of 91, 47 % in light of integration of the corresponding signal. The molecular mass of KYDAK sequence with C-term amide is 622, 72 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 623, 35 g/mol in the mass chromatogram.

$$[M + H]^+ = 623,35 \rightarrow M_w = 622,35 \text{ g/mol}$$

$$MW_{\text{teoritical}} = 622,72 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.

4.1.7. KYSAK

The HPLC and mass spectroscopy chromatogram of KYSAK are given below in the following figures, respectively.

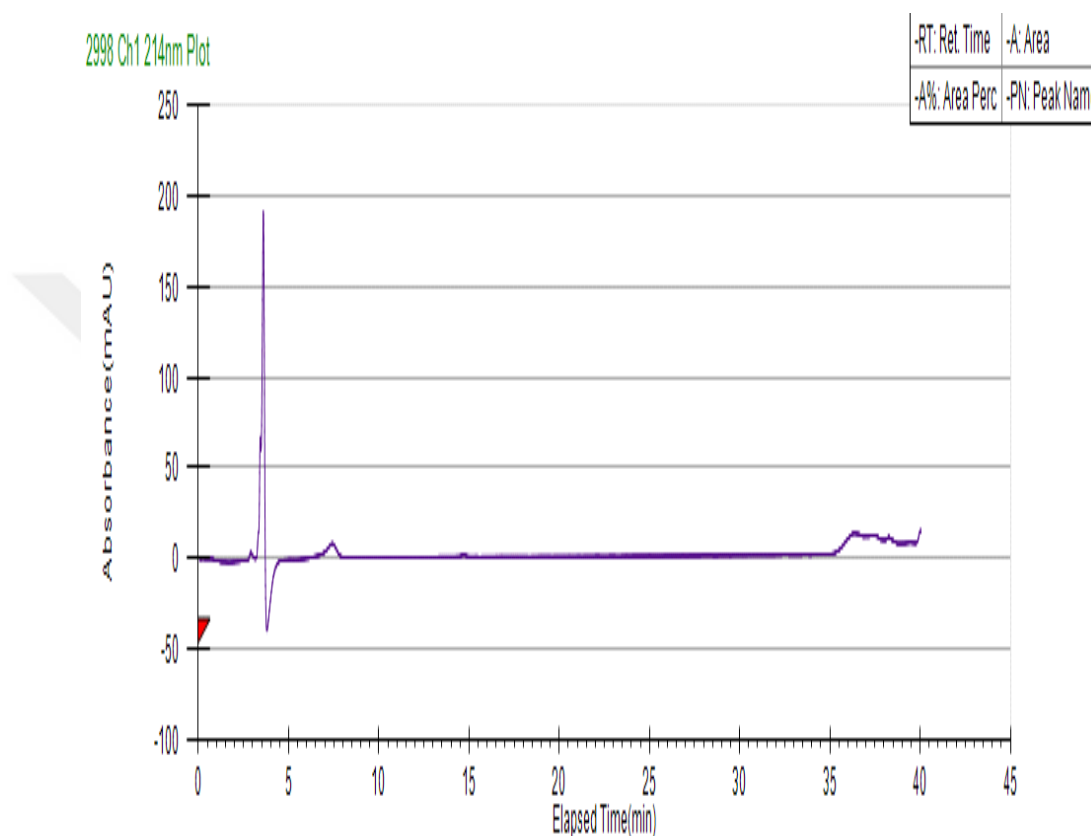


Figure 4.19: The HPLC chromatogram of purified KYSAK.

Table 4.7: The HPLC chromatogram of purified KYSAK.

	Area Percent	Area	Retention Time (min)	Height
Peak 1	92.8947	2110915.0712	3.59	205.0544
Peak 2	7.1053	161459.8342	7.42	5.7604

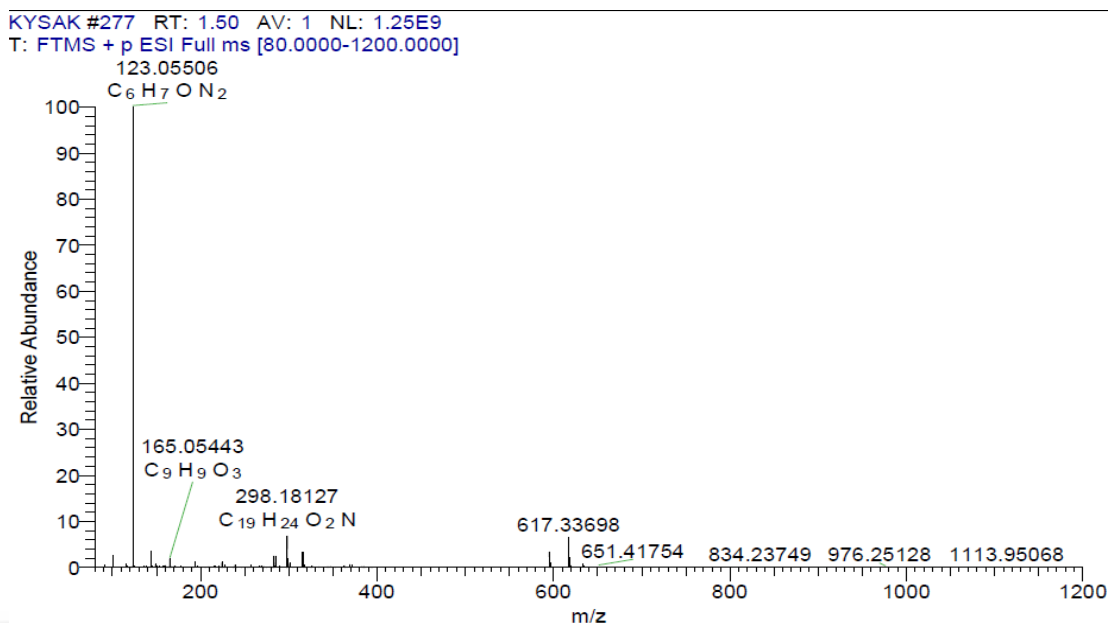


Figure 4.20: The mass chromatogram of KYSAK

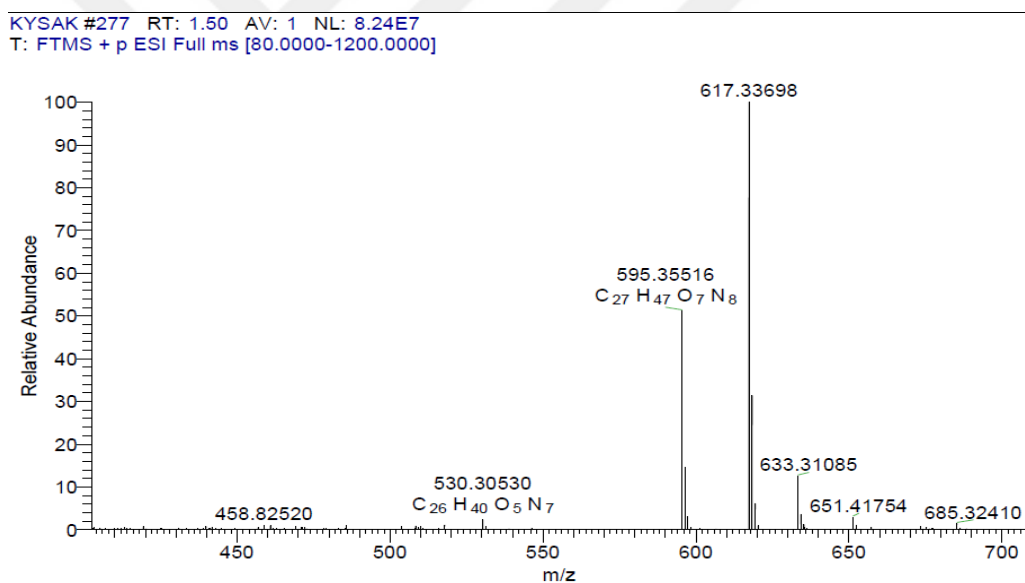


Figure 4.21: The expansion of mass chromatogram of KYSAK

According to these results, the mass chromatogram shows that the peptide sequence is at the expected weight, as a result, it can be said that the peptide has been successfully synthesized. The peak obtained between retention time of 3 minutes in the first chromatogram indicates that targeted KYSAK sequence has a purity of 92, 89 % in light of integration of the corresponding signal. The molecular mass of KYSAK sequence with C-term amide is 594, 71 g/mol. Because mass chromatogram shows with extra hydrogen, molecular mass is seen 595, 35 g/mol in the mass chromatogram.

$$[M + H^+]^+ = 595,35 \rightarrow M_w = 594,35 \text{ g/mol}$$

$$MW_{\text{teoretical}} = 594,71 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS is shown to be similar to the molecular weight calculated from literature.





5. FUTURE AIMS

Isothermal titration calorimetry (ITC) peptides will be performed to test binding affinity to the FOLR1 protein for future studies. In in vitro and in vivo studies, these peptides will be labeled with fluorescent dye and radionuclides, respectively, after collection of peptides. Those exhibiting high selectivity will be radiologically labeled with ^{68}Ga and DOTA-chelates for use in PET/CT system.



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