

REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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

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MASTER OF PHILOSOPHY THESIS

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Cem ÖZEL

Signature



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*Dedicated to my family
and my best friends*



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LIST OF SYMBOLS

A	Alanine
R	Arginine
Br ₂	Bromine
¹¹ C	Carbon-11
Cl ⁻	Chlorine
Cu	Copper
°C	Degree Celsius
F ₂	Fluorine
Gd	Gadolinium
⁶⁸ Ga	Gallium-68
γ	Gamma Rays
g	Gram
H	Histidine
¹¹¹ In	Indium-111
I ⁻	Iodine
keV	Kiloelectron Volts
kHz	KiloHertz
K	Lysine
Mn	Manganese
MeV	Mega (Million) Electron-Volts
μ	Micro
mm	Milimeter
nm	Nanometer
nM	Nanomolar
¹³ N	Nitrogen-13
Nα	N-terminus of the Peptide
¹⁵ O	Oxygen-15
F	Phenylalanine
β	Positron Radiation
P	Prolin
rpm	Revolutions per minute
Sc	Scandium
s	Second
R	Side Chains of the Peptide
^{99m} Tc	Technetium-99m
⁸⁹ Zr	Zirconium-89

LIST OF ABBREVIATIONS

¹⁸ F-FDG	2-Deoxy-2-(¹⁸ F) fluoro-D-glucose
ACN	Acetonitrile
Arg(Pbf)-OH	N ^ω -(2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl)-L-arginine
BLI	Bioluminescence Imaging
CADD	Computer-aided drug design
CAT	Computed Axial Tomography
Cbz	Carboxybenzyl
CCK-B	Cholecystokinin-B
CD206	Macrophage Mannose Receptor
CD61	Integrin beta 3
CEA	Carcinoembryonic Antigen
CT	Computed Tomography
Cy5	Cyanine 5
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	N,N'-Diisopropylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
DNA	DeoxyriboNucleic Acid
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DVB	Divinylbenzene
ECM	Extracellular Matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
Eq	Equivalent
FDA	Food and Drug Administration
FLI	Fluorescence Imaging
FLT	3'-deoxy-3'- ¹⁸ F-fluorothymidine
Fmoc	9-fluorenylmethoxycarbonyl
FOLR	Folate Receptor
FOLR1	Folate Receptor 1
GITR	Glucocorticoid-Induced TNFR-Related Protein
GLUT	Glucose-Transporter
Glut	Glucose Transporter Proteins
HATU	N-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-b]-pyridino-1-ylmethylene]-Nmethylmethan-aminium hexa fluorophosphate)
HBTU	O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HER2	Human Epidermal Growth Factor Receptor 2
HF	Hydrogen fluoride
His(Trt)-OH	N ^{im} -trityl-L-histidine
HPV	Human Papillomavirus
IARC	International Agency for Research on Cancer
IO	Iron oxide

ITC	Isothermal Titration Calorimetry
JAMA	The Journal of the American Medical Association
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homologue
LAG-3	Lymphocyte-Activation Gene 3
LC-MS	Liquid Chromatography-Mass Spectrometry
MBHA	4-Methylbenzhydramine hydrochloride
MI	Molecular imaging
MMP	Matrix Metalloproteinases
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NCI	National Cancer Institute
NET	Neuroendocrine Tumours
NHS	N-hydroxysuccinimide
NIH	National Institute of Health
NIR	Near-Infrared
NIRF	Near-Infrared Fluorescent
NMP	N-Methylpyrrolidone
NP	Nanoparticles
NSCLC	Non-Small-Cell Lung Cancer
PA	Photoacoustic
PASP	Polyaspartic Acid
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PD-1	Programmed Death-1
PD-L1	PD-1 ligand 1
PDMA	Poly-N,N-dimethylacrylamide
PEG	Polyethylene Glycols
PEG-p(L-His)	Ethylene glycol-b-poly(L-histidine)
PET	Positron Emission Tomography
PET/CT	Positron Emission Tomography-Computed Tomography
PG	Protecting groups on the amino acids
Phe-OH	Phenylalanine
PNAM	Poly(N-acryloylmorpholine)
Poly(APr)	Poly(N-acryloylpyrrolidine)
PPIs	Protein-protein interactions
Pro-OH	Pyrrolidine-2-carboxylic acid
PS	Polystyrene
PSA	Prostate-Specific Antigen
PyBOP	Benzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
QD	Quantum Dots
RAM	Rink Amide
RGD	Arginine-Glycine-Aspartic
RP-HPLC	Reverse Phase High-Performance Liquid Chromatography
SCLC	Small Cell Lung Cancer
SGK	Social Security Institution
SPECT	Single Photon Emission Computed Tomography
SPNs	Solitary Pulmonary Nodules
SPPS	Solid-Phase Peptide Synthesis
SPS	Solution Phase Synthesis

SSTR	Somatostatin Receptor
SUV	Standardized Uptake Value
<i>t</i> -Boc	<i>tert</i> -Butyloxycarbonyl
<i>t</i> -Bu	<i>tert</i> -Butyl
TAPMG	Turkish Thoracic Society Lung and Pleural Malignancies Study Group
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIE2	TEK Receptor Tyrosine Kinase 2
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TIPS	Triisopropylsilane
Trityl	Triphenylmethyl
TUIK	Turkey Statistical Institute
USG	Ultrasonography
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
X	Leaving group on the non-full-length peptide

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Development of Folate Receptor 1 Targeted Peptides for Use in Positron Emission Tomography Imaging Systems

Cem ÖZEL

Master of Philosophy Thesis

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Cancer is a fatal disease that affects many people not only in our country but also in the whole world. During cancer formation, development and maturation, there are some changes in the metabolism, surface and microenvironment of cancer cells. These changes allow precise diagnosis of cancer. Diagnosis of cancer can be determined by imaging these changes in cancer cells. Positron emission tomography-computed tomography (PET/CT) is among the methods used for oncologic imaging in today's health area. PET/CT is used for diagnosis, staging, detection of recurrence, and evaluation of response to treatment of many cancer types. Imaging of cancer cells in the PET/CT device is provided by radionuclide imaging agents. Food and Drug Administration (FDA)-approved 2-deoxy-2-(^{18}F) fluoro-D-glucose (^{18}F -FDG) is the most widely used radionuclide imaging agent nowadays for cancer diagnosis in PET/CT. However, ^{18}F -FDG is known to lose its selectivity and sensitivity when glucose consumption increased, for example, inflammation and infection situations. Therefore, the development of new imaging agents for cancer diagnosis has become inevitable.

Peptides can be preferred for cancer imaging by binding to specific receptors of cancer cells because of its high sensitivity and selectivity to cancer cell. Folate Receptor 1 (FOLR1) is known a very common cancer receptor. The aim of this thesis is to develop peptide-based imaging agents for tumour imaging to use as an alternative to conventional PET agents. The peptides produced during these studies were designed with a pharmacophore approach. The designed peptides by Computer-Aided Drug Design (CADD) which can bind to FOLR1 receptor with high sensitivity and affinity have been synthesized for utilizing as an imaging agent in

PET/CT to diagnose cancer. For the FOLR1 receptor, peptides have not been designed in the literature by the CADD method. Within the scope of this thesis, synthesis of RPAAK, RFAAK, HRAFAK and KRAFAK peptides designed by CADD method for the first time was performed for cancer diagnosis. The designed four peptides were synthesized by Solid-Phase Peptide Synthesis (SPPS) method. The synthesized peptides were purified with Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) and characterized with Liquid Chromatography-Mass Spectrometry (LC-MS). It was observed that all peptides were successfully synthesized and purified.

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



Pozitron Emisyon Tomografi Görüntüleme Sistemlerinde Kullanılacak Folat Reseptörü 1 Hedefli Peptitlerin Geliştirilmesi

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Kanser, sadece ülkemizde değil, tüm dünyada birçok insanı etkileyen ölümcül bir hastalıktır. Kanser oluşumu, gelişimi ve olgunlaşması sırasında, kanser hücrelerinin metabolizmasında, yüzeyinde ve mikroçevresinde bazı değişiklikler olmaktadır. Bu değişiklikler kanserin kesin teşhisine izin verir. Kanser teşhisi, kanser hücrelerinde bu değişikliklerin görüntülenmesiyle yapılabilir. Pozitron emisyon tomografisi-Bilgisayarlı tomografi (PET/BT) günümüzde sağlık alanında onkolojik görüntülemelerde kullanılan yöntemler arasındadır. PET/BT, birçok kanser türünün tanısı, evrelendirilmesi, nüksün tespiti ve tedavisine yanıtın değerlendirilmesinde kullanılır. PET/BT cihazında kanserli hücrelerin görüntülenmesi radyonüklid görüntüleme ajanları ile sağlanır. Gıda ve İlaç İdaresi (FDA) onaylı 2-deoksi-2-(¹⁸F) floro-D-glikoz (¹⁸F-FDG), günümüzde PET/BT'de kanser diagnozu için en yaygın kullanılan radyonüklid görüntüleme ajanıdır. Bununla birlikte, ¹⁸F-FDG'nin iltihaplanma ve enfeksiyon durumları gibi artan glikoz tüketimi olduğunda seçiciliğini ve hassasiyetini kaybettiği bilinmektedir. Bu nedenle, kanser diagnozu için yeni görüntüleme ajanlarının geliştirilmesi kaçınılmaz hale gelmiştir.

Peptitler, kanser hücresine yüksek duyarlılığı ve seçiciliği nedeniyle kanser hücrelerinin spesifik reseptörlerine bağlanarak kanser görüntüleme için kullanılmıştır. Folat Reseptörü 1 (FOLR1) çok yaygın bir kanser reseptörü olarak bilinir. Bu tezin amacı, geleneksel PET ajanlarına alternatif olarak kullanılmak üzere tümör görüntülenmesi için peptit bazlı görüntüleme ajanları geliştirmektir. Bu çalışmalar sırasında üretilen peptitler bir farmakofor yaklaşımı ile tasarlanmıştır.

Bilgisayar Destekli İlaç Tasarımı (CADD) tekniđi ile yüksek hassasiyet ve afinite ile FOLR1 reseptörüne bağlanabilen peptitler tasarlanmış, kanser teşhisi için PET/BT'de görüntüleme ajanı olarak kullanılmak üzere sentezlenmiştir. FOLR1 reseptörü için literatürde daha önce CADD yöntem ile peptitlerin dizaynı yapılmamıştır. Bu tez kapsamında ilk kez CADD yöntemi ile tasarlanan RPAAK, RFAAK, HRAFAK ve KRAFAK peptitlerinin sentezi kanser teşhisi için gerçekleştirildi. Tasarlanan dört peptit, Katı Fazlı Peptid Sentezi (SPPS) yöntemi ile sentezlenmiştir. Sentezlenen peptitler, Ters Fazlı Yüksek Performanslı Sıvı Kromatografisi (RP-HPLC) ile saflaştırılmış ve Sıvı Kromatografi-Kütle Spektrometresi (LC-MS) ile karakterize edilmiştir. Tüm peptitlerin başarıyla sentezlendiđi ve saflaştırıldıđı gözlemlenmiştir.

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



1.1 Literature Review

Today, cancer is the second leading cause of death after heart disease in the world and Turkey. Cancer is a group of diseases characterized by uncontrolled cell proliferation as a result of genetic changes in cells. In this way, metabolism change in cancer cells shows because of forming new intracellular arrangements. These cells continue to grow, divide and re-divide instead of dying and spread into surrounding tissues [1]–[4]. Known causes of cancer with more than two hundred species are environmental, genetic and individual factors [5].

In 2018, 18.1 million people were diagnosed with cancer and 9.6 million in death. [6]. It is estimated that in 2030, new cancer levels will be 21.4 million and approximately 13.2 million people (13.6% of all deaths) will die from cancer. In Turkey, in a similar way to other countries, the incidence of cancer is increasing. Approximately 160,000 people are diagnosed with cancer each year, while approximately 100,000 people die because of cancer [7], [8].

Molecular imaging (MI) provides a method of collecting tremendous functional information about cancer by targeting the changing metabolism rate or surface molecules in cancer cells. For this purpose, some chemical biomolecules specific to the targeted pathway are given to the organism by labelled radioactive isotopes [4]. Nowadays, Positron Emission Tomography-Computed Tomography (PET/CT), is among the methods used for oncologic imaging in today's health sector in clinical and preclinical applications, is used for staging, detection of recurrence and evaluation of response to treatment of many cancer types [9]. 2-deoxy-2-(^{18}F) fluoro-D-glucose (^{18}F -FDG) is used as a cancer imaging agent for targeting cancer cells recently. However, ^{18}F -FDG and similar imaging agents such as 3'-deoxy-3'- ^{18}F -fluorothymidine (FLT), ^{68}Ga -1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-Tyr³-octreotide can lose its selectivity and sensitivity to cancer diagnosis [10]. Therefore, the development of new cancer cell recognition agents has become inevitable.

Peptides which are smaller versions of proteins are involved in many biological, metabolic and physiological processes within organisms. They play an incontrovertible role in substances transporting, and intercellular communication [11]–[13]. Utilizing peptides as a targeting ligand/agent in cancer imaging is very functional thanks to its high binding affinity and specificity for cancer cells, high stability, and low immunogenicity [14]. Radiochemically-labelled synthetic peptides became very useful in PET/CT for clinical applications, especially cancer disease [15]. In the literature, surface receptors of cancer cells were targeted. One of these receptors is Folate Receptor 1 (FOLR1). As far as we were aware, no peptide-based imaging agent for cancer has been previously developed in the literature against the FOLR1 receptor.

1.2 Objective of the Thesis

The aim of this study was to synthesize, characterize and purify peptides which designed with Computer-Aided Drug Design (CADD) for Folate Receptor 1 (FOLR1) targeting. The potential use of the peptides synthesized by Solid-Phase Peptide Synthesis (SPPS) method in PET/CT imaging of cancerous regions in the body will be examined and evaluated. The widespread impact of this thesis is developing a peptide-based imaging agent which is capable of binding to cancer cells with high affinity and sensitivity.

1.3 Hypothesis

The hypothesis of this thesis is to synthesize, characterize and purify FOLR1 targeted peptides for evaluating in PET imaging systems. Use of these candidate cancer-targeted peptides as an imaging agent in order to avoid erroneous results due to the loss of selectivity and sensitivity of ^{18}F -FDG would lead to the use of the PET device in cancer imaging systems. As a result of this thesis, preliminary studies will be carried out to develop imaging agents with different sensitivity, interest, and selectivity to determine the distribution of cancer foci in the body thanks to peptides.

2.1 Cancer Leads to Death

Today, cancer is one of the major problems for all countries in the world. Cancer remains one of the world's most devastating diseases, with more than 10 million new cases every year [16]–[18]. According to the Turkey Statistical Institute (TUIK) data, Turkey has about 400 thousand cancer patients and this number accounts for 20% of all deaths in Turkey. Cancer is the second cause of death after cardiovascular diseases both in the world and in our country with a rate of 22% [19], [20]. The prediction of the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) for 2030 is that cancer will be the leading cause of death [21].

Share of the total population with any form of cancer in Turkey and the world are dramatically increasing in the last 30 years (Figure 2.1). While the share of the population with cancer cases was 0.58% in 1990, it increased regularly every year and at the end of 2017, Turkey has reached the alarm level with 1%. The increasing tendency is similar worldwide and the number of cases is even greater [16].

The cancer continues its killing pace. According to the latest numbers, it does not seem to have any mercy [22]. Globally one of every 6 deaths is caused by cancer and these numbers are the highest point of all time [17]. According to the statistical data of 2017 published by the TUIK in 2018, 83.163 people in Turkey died of benign and malignant tumours, comprising of the 19,7% of all death cases in Turkey. Deaths from cancer have increased in industrial and population-intensive regions such as İstanbul, İzmir, Bursa [23].

Share of population with cancer

Share of total population with any form of cancer, measured as the age-standardized percentage. This share has been age-standardized assuming a constant age structure to compare prevalence between countries and through time.

Our World
in Data

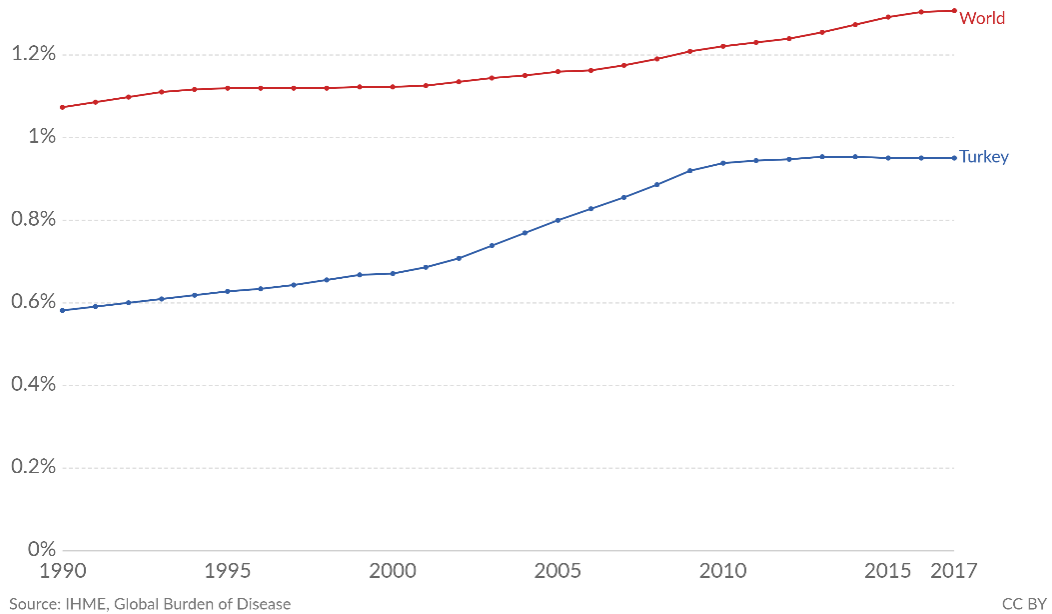


Figure 2.1 Share of the population with cancer is increasing every year [16]

2.2 The Story of Cancer

There are over 200 types of cancer while in all types of cancer, the same scenario has occurred; normal cells in a part of the body begin to grow out of control. These cells continue to grow, divide and re-divide instead of dying and spread into surrounding tissues (a spreading process known as metastasis) [1]–[3]. Some cells, such as neurons and muscle cells, remain stable after a certain age. They may grow in size or increase the number of connections with other cells, but cell numbers do not change. Cancer formations generally do not develop in such stable tissues but in dynamic tissues where cells are constantly renewed [24]. Mitotic cell divisions are those that occur in somatic cells (body cells), where two new cells are formed with the same genetic information as the parent cell. DNA duplication occurs in the S phase of the interphase stage of the mitotic division, doubling the genetic material [25], [26]. Mutations and errors can occur for various reasons during DNA duplication and recombination. Most mutations and errors are eliminated by instant cell death (apoptosis). However, in some rare cases, these mutations provide vital advantages to somatic cells and these mutations are transferred to new (pyrogen) cells by mitotic divisions. Some of the pyrogen cells allow a better adaptation to the

surrounding conditions thanks to the mutation and multiply the mutation it carries with mitotic divisions. If these mutated genes cause abnormal proliferation of cells, they are called cancer genes [27].

Uncontrolled cells can lead to benign or malignant cancer. During the early stages of cancer, mostly benign cancer cells are observed. Benign cancer cells keep in normal boundaries of tissue and do not spread other tissues. If the cancer cells grow, spread and invade other tissues, then these cancer cells call as malignant cancer cells (metastasis) (Figure 2.2)[28]. The malignant cells are the main life-threatening cancer cells. The metastasis is the main element of cancer morbidity and death rate and is responsible for about 90% of cancer deaths [29], [30].

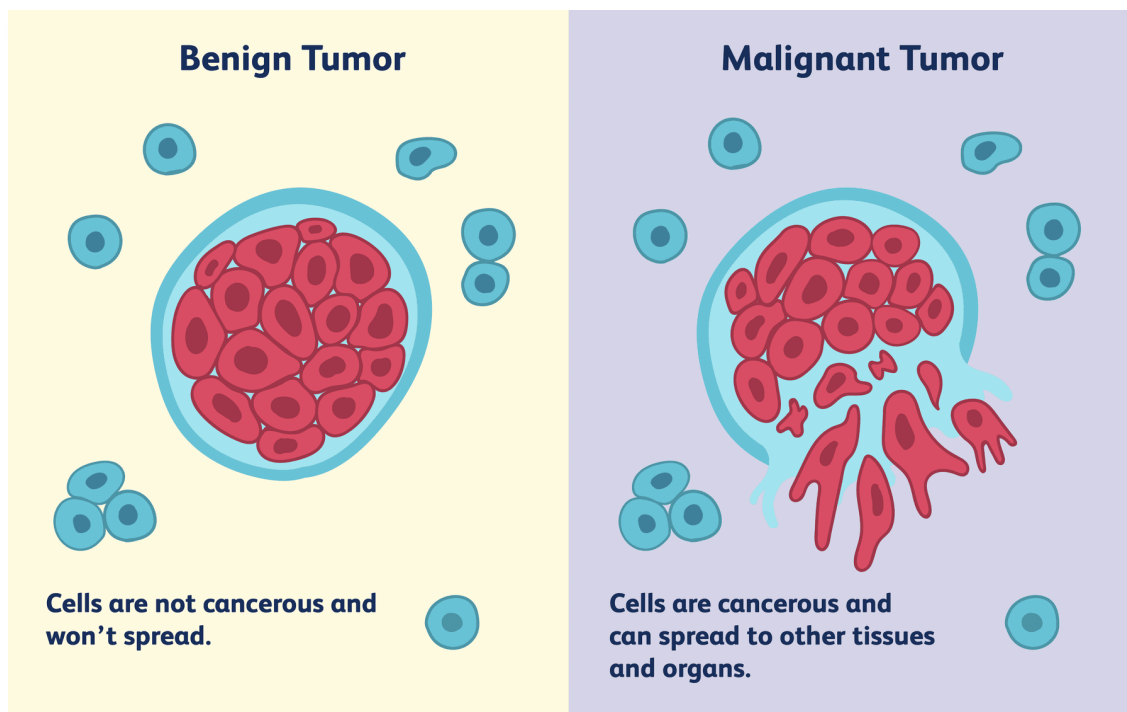


Figure 2.2 Difference between a malignant and benign cancer cell [31]

Cancer cells may be caused by different factors, but the main reason is always the same: damage to a particular gene that is responsible for regulating cell division. This phenome is called carcinogenesis [1], [32]. These factors may also vary from country to country [26]. For example, the rate of gastric cancer in Japanese people is higher than the rate of colon cancer whereas the rate of colon cancer in Hawaii is higher than the rate of gastric cancer. The presence of Hawaiian cancer characteristics (colon cancer rate, higher than stomach cancer rate in Japanese

immigrants) in the second generation Japanese immigrants living in Hawaii shows that environmental factors are dominant in Hawaii [24].

The cancer factors may be internal and environmental/acquired [33]. The internal factors are inherited mutations, hormones (testosterone, insulin-like growth factors, estrogen, and progesterone) [34], and immune conditions [22]. The environmental factors comprise 90-95% of the total cancer risks. The environmental factors are the tobacco, some carcinogenetic materials (ethylene oxide, arsenic, crystalline silica, etc.) [35], lifestyle (alcohol, diet, obesity) [36], [37], radiation [38], and infectious organisms (viruses such as human papillomavirus (HPV), hepatitis B virus (HBV) and hepatitis C virus (HCV), inflammation, bacteria and parasite such as *Helicobacter pylori*, *M. tuberculosis*, *Schistosoma haematobium*, etc.) [39], [40].

Treatment of cancer is a highly costly disease. In the study conducted by Çakır Edis and Karlıkaya, direct cancer expenditures accounted for 41% of the total expenditure. 56% of direct costs were treatment costs, while only 11% of costs were diagnostic costs (Figure 2.3). Chemotherapy and radiotherapy accounted for 51% and 18% of treatment costs, respectively while Surgical operation constitutes only 3% of the costs [41]. While lung cancer treatment costs diagnosed at an early stage are approximately \$ 30,000 per year by surgical methods, these costs go up to \$ 45,000-70,000 in the advanced stage [42]–[44].

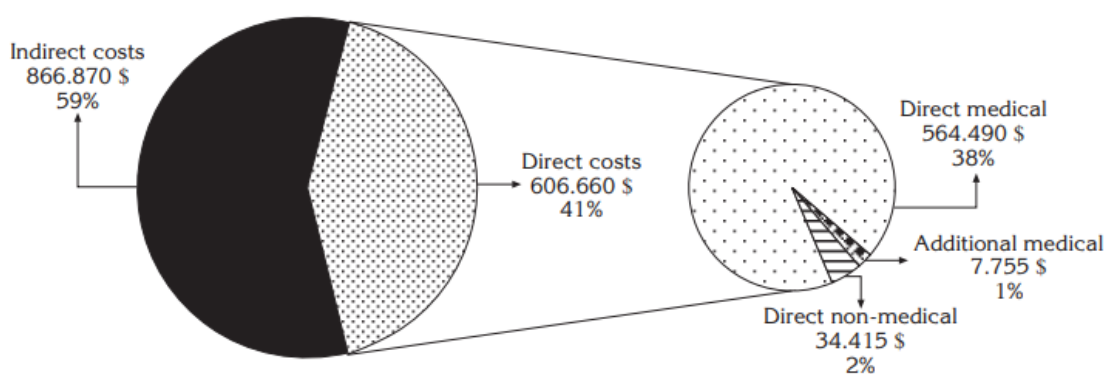


Figure 2.3 The cost of lung cancer [41]

In 2016, lung cancer treatment in the US was calculated to be \$ 203,217 a year if there is social security [45]. In the study conducted in Turkey early stage in per capita medical expenses for \$ 3986 ± 2.683 while Stage IIIB and Stage IV treatment costs were found to be \$5.644 ± 3.974 and \$ 6.240 ± 4.716 \$, respectively [41]. Here,

the expensive drugs used for chemotherapy play an active role. In pemetrexed/cisplatin treatment used for Non-Small-Cell Lung Cancer (NSCLC) treatment, 69.86% of the total cost is composed of the cost of chemotherapy drugs and 8.70% of the cost of the drugs used in combination with chemotherapy [46]. As can be seen, a great deal of money is lost during cancer treatment in the view of both the state and patients and the diagnosis of cancer in Stage I and Stage II is known to reduce the cost of cancer while greatly improving patients' chances of survival.



MOLECULAR IMAGING TECHNOLOGY IN MEDICINE

3.1 Molecular Imaging Technologies

Molecular Imaging (MI) has provided to characterize biological processes in tissue, cell and intracellular components of living organisms within anatomy-based imaging or structural imaging, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI) in the first years of diagnosis era. Using this technique, using specific molecular probes or contrast agents, diseases can be diagnosed at an early stage and the efficacy of treatment can be quickly assessed. Molecular imaging is rooted in nuclear medicine, and in many ways, it is a direct extension of the present method [47], [48]. Nowadays, different techniques such as USG, CT, and MRI, for anatomical imaging, single photon emission computed tomography (SPECT), positron emission tomography (PET) and optical imaging of molecular level diseases, especially cancer, imaging are possible [49]. The used MI applications in preclinical and clinical are shown in Figure 3.1.

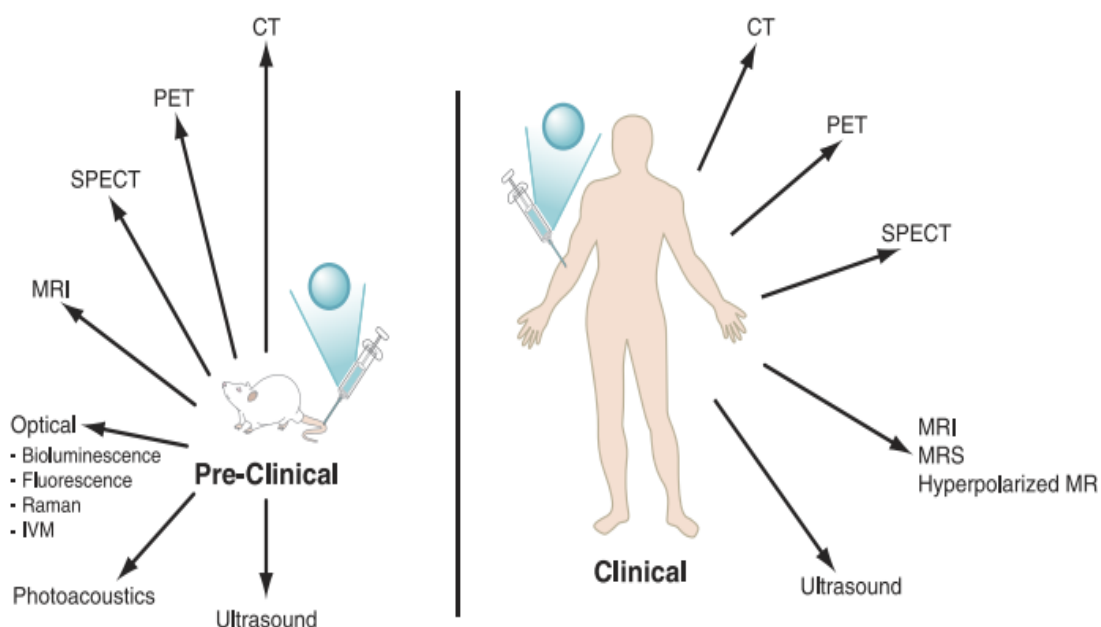


Figure 3.1 MI methods used for preclinical and/or clinical applications [50]

MI is a rising area derived from diagnostic imaging studies that aim to merge patient and disease-specific molecular information. The aim of MI is the non-invasive or minimally invasive localization, quantification of tumour mass, quantification of

certain molecular events *in vivo*, including gene and protein expression, better clinical risk stratification, evaluation of tumour microenvironment, signal transduction, protein-protein interaction, better disease treatment choice, and follow-up of treatment response [51]. The MI is not just useful for imaging of disease both anatomically and molecular level and also the development of new molecular targeted drugs and design and implementation of improved patient-tailored therapies [47], [52]. According to Jaffer and Weissleder, there are four crucial questions for a certain disease to the evolution of a proper MI strategy [51];

- “Is there a molecular target relevant to the disease of interest?”
- “Once a target is selected, is there a high-affinity ligand that will bind to the target?”
- “What is the appropriate MI system to provide the required spatial resolution, sensitivity, and depth penetration for the disease?”
- “For a given imaging system, can an agent be synthesized to detect the desired molecular target?”

MI probes can be developed by rapidly increasing knowledge and techniques of cellular/molecular targets currently available. The combination of molecular biology and medical imaging is the reason for the rapid development of this new field and offers alternative methods for the observation of cellular/molecular phenomena, such as “reporter gene” analysis, derived from conventional molecular analysis. Advances in biotechnology and functional genome science result in progress in parallel with molecular imaging innovations and applications [48], [53]. As the development, validation, and application of these new imaging techniques increases in living individuals, the mechanisms of diseases will be better understood with the development of molecular medicine. So, the treatment of diseases will be easier [54]. For all these imaging methods, a certain number of “reporter” markers are required. Since the chemical structure of the reporters and the sensitivity of each technology are different, the tissue concentration required to obtain the signal varies considerably from method to method [48].

MI has already participated in a crucial role in defining many cellular and biochemical mechanisms as well as varies disease diagnosis is *in vitro* and *in vivo*. One of the most essential application fields of MI is in clinical oncology over the past

few decades [55], [56]. The National Cancer Institute (NCI) [57], [58] and National Institute of Health (NIH) [59], [60] promote the improvement and training of MI in radiology [61]. The MI technology may detect the location and extent of the tumour by characterization of the tumour surface and biochemical properties [62], [63]. The MI does not only help the where a tumour is located and but also the activity of specific biological processes of those tumours which give us information of tumour specific behaviours and contribute the response to therapy are imagined in the tumour tissue as well as individualized treatment, and drug development [56], [63].

3.2 Molecular Imaging Technologies in Cancer

Molecular imaging methods such as MRI, CT, US, optical imaging (bioluminescence and fluorescence), SPECT and PET are a wide range of technologies applied non-invasive *in vivo* MI studies in many diseases including cancer [51], [64]. Early cancer detection has become more important than before with developing technologies due to that cancer stage effects dramatically cancer survival rate [65]. Stage 1 cancer patients have a >90% survival rate in 5-year in many cancer cases [66]. If the cancer stage is earlier than this stage (such as at the premalignant stage), the therapy of the patient is more curative [56]. Lung cancer is a good example of this case. 86% of lung cancer patients die in five years after being diagnosed. Lung cancer is very fatal because patients are usually diagnosed with cancer in Stage IV, where cancer cells are now spreading to other organs and tissues. In lung cancer detected in the early stage, this number decreased to 30% [67]. In the study performed by the Turkish Thoracic Society Lung and Pleural Malignancies Study Group (TAPMG), according to the stage of initial lung cancer, patients were diagnosed with stage I 5.6%, stage II 7.7%, stage IIIA 14.2%, stage IIIB 32.1% and stage IV 40.4% [68]. In the studies of Yurdakul *et al.* and Özlü *et al.*, they took this delay in diagnosis of lung cancer similar to that in operation in Turkey and over 50% of patients, the diagnosis revealed that these delays have occurred and have an average delay of 88 days [69], [70]. In lung cancer, delays in diagnosis can lead to a progression of the tumour stage in many patients, especially since the time it takes for the tumour to double its volume is between 4 and 56 weeks. This change may cause cancer to progress to the next stage. This may lead to a loss of chance of surgical intervention [70], [71]. According to the stages of lung cancer, treatment, survival rate, and patient comfort vary

(Figure 3.2). In the early diagnosis of lung cancer, such as Stage I and Stage II, patients are not only treated with operations but also have a better chance of survival [72], [73]. Since the patients with the diagnosis of stage IIIA, stage IIIB and stage IV lung cancer spread to other organs and tissues, the surgical operation is not enough, and chemotherapy (chemical therapy) and radiotherapy must be applied. Patients who can be diagnosed in these stages have a low chance of survival. Patients with stage IV NSCLC have a 1-year and 5-year survival rate of 17% and 1-13%, respectively. This rate increases to 83% and 49-73% for stage I NSCLC [74]–[76].

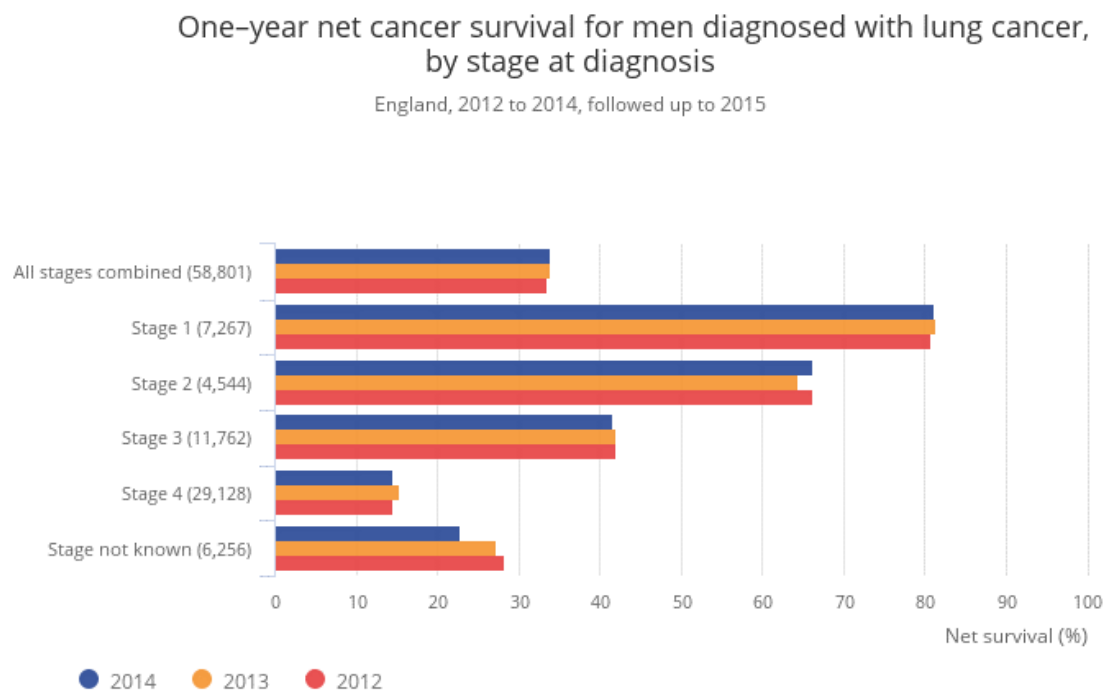


Figure 3.2 One-year net cancer survival for men diagnosed with lung cancer, by stage at diagnosis [77]

The conventional imaging techniques can only locate a centimetre or greater size in diameter (approximately $>10^9$ cancer cells) without information about specific genomic and proteomic cancer characteristics (Is it Kirsten Rat Sarcoma Viral Oncogene Homologue (KRAS) or Human Epidermal Growth Factor Receptor 2 (HER2) in lung cancer?). It will become more clear with recently developed MI technologies [56].

According to the applied area and aim, there are some situations in which each technology is at the forefront, and these technologies are shown in Figure 3.3 and Table 3.1 which represent the different technical properties of the non-invasive *in vivo* MI technologies. Each imaging method will be discussed in detail in the following sections.

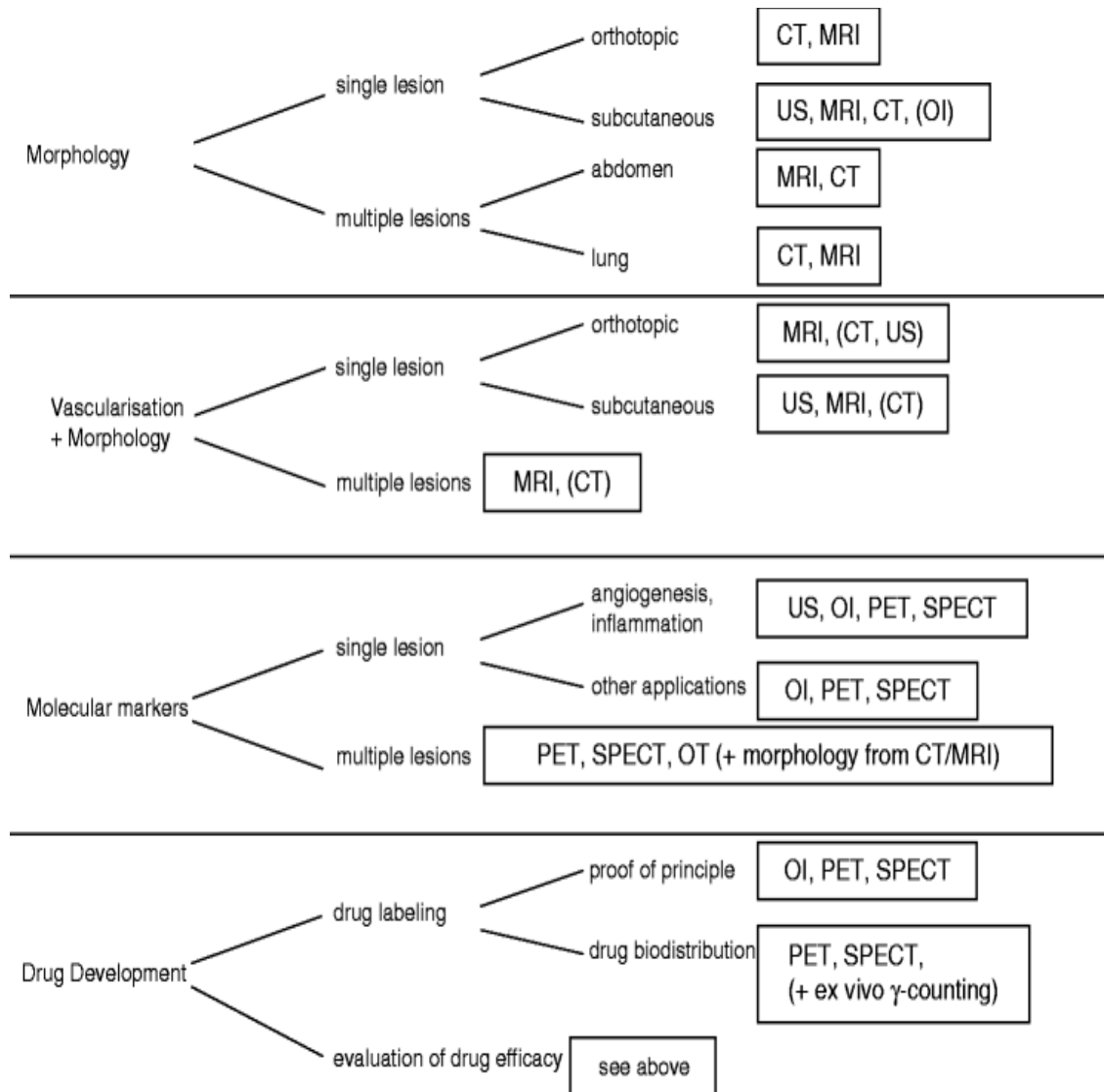


Figure 3.3 Major indications for disease imaging and the recommended imaging modality is represented. The order of imaging methods takes precedence over certain imaging indications. The proposal is based on the author's experience and focuses on the broad and highly efficient implementation of imaging [78]

Table 3.1 The MI technology in cancer diagnosis [47], [78]

Imaging technology	Energy form	Clinical spatial resolution (mm)	Acquisition time (s)	Penetration depth (m)	Probe mass (μg)
CT	X-rays	0.5–2	1–300	>0.3	-
MRI	Radio frequency waves	0.1–1	50–3000	>0.3	1–1000
US	High frequency sound waves	0.1–2	0.1–100	10^{-3} –0.2	1–1000
PET	Annihilation photons	3–8	1–300	>0.3	10^{-3} – 10^{-1}
SPECT	γ -photons	5–1000	60–2000	>0.3	10^{-3} –1
BLI and FLI	Visible to infrared light	-	10–2000	10^{-3} – 10^{-2}	1–1000

3.2.1 Optical Imaging

Optical imaging is one of the most accepted MI applications usage in the research area. Optical imaging uses photons emitted by bioluminescence, is called bioluminescence imaging (BLI) or fluorescent probes, called fluorescence imaging (FLI) [79]. It is established the detection of light photons after photons interact with the cell and tissue [47]. Probes used for optical imaging may be synthetic fluorophores, semiconductor fluorescent crystals and lanthanide-based probes [48], [80].

BLI is used with luciferase (cellular expression of an enzyme from jellyfish (*Aequorea*), some insects fireflies (*Photinus pyralis*), coral (*Renilla*), and bacteria glow) for the source of the bioluminescence [79], [81]. When luciferin interacts with its natural substrate, D-luciferin, a visible light (between 400 and 700 nm wavelength) is radiated with 1.5– 3.0 eV energy from the cell. Since the depth of the BLI technology is low, it is generally used *in vivo* imaging experiments especially in small animals (Figure 3.4) (in these animals the depth is no more than 1–2 cm). The generation of bioluminescence occurs only in the cells that carry a luciferase

reporter gene in its genetic material. This feature restrains its application area for studying only genetically manipulated cells, transgenic mice, or infectious agents [47].

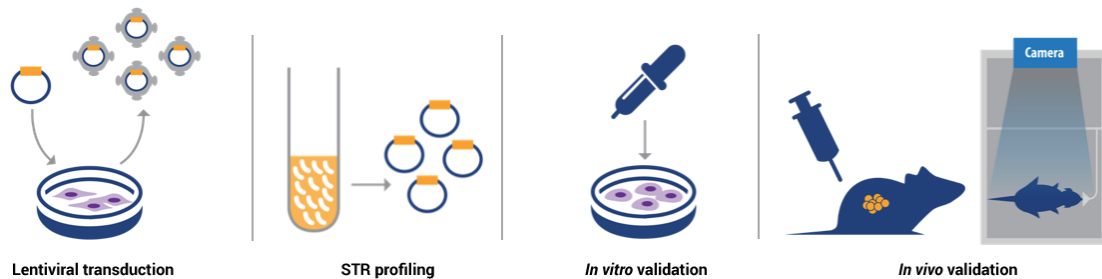


Figure 3.4 Luciferase-labelled model in BLI system allows to monitor the of tumour cell survival, expansion, and homing, and evaluate tumour response to cancer therapy as well as the expression of therapeutic genes in real-time [82], [83]

FLI utilizes fluorescent molecules or proteins with a surface distribution of fluorescent signals for imaging genes and proteins related to certain diseases [47], [81]. The fluorescence molecules can be bond with carbohydrates, peptides, proteins, and antibodies. Those designed structures may help to be activated by the presence or absence of some specific molecules or molecular events in the cell or tissue [47], [84], [85]. Fukumura *et al.* reported that vascular endothelial growth factor (VEGF) promoter activity results in a high amount of green fluorescent protein in the tissue. A huge amount of VEGF in tumours is hugely provided by stromal cells surrounding the tumour cells [86].

Optical imaging is the most sensitive method for detecting tumour tissue near/on the body surface. Optical imaging is used to evaluate tissue perfusion, cancer cell vascularization, and metabolism. The signal intensity on imaging is consistent with the live tumour mass. Optical imaging allows the separation of viable and necrotic tissue. The sensitivity and resolution of the method decrease as the localization of the lesion deepens. Optical imaging is accurate enough to allow a small number of cells to be displayed up to a minimum of 1000 cells. Fractional vascular volume using intravascular staining is assessed by fluorescence tomography, an optical imaging method [49], [52].

In all of these optical imaging technologies, tissue penetration has always been a limitation to this application [51]. The main disadvantage of this method is its failure

to reach deep tissues (0-2 cm depth) [48]. More depth sensitivity can be achieved by debilitation and autofluorescence is decreased in the near-infrared (NIR) window, adding a red and near-infrared portion to the camera system, permitting deep tissue imaging up to 10 cm [47], [87]. It has more advantageous compared to other imaging methods. Because it has the ability to detect low energy photons, relatively inexpensive, does not expose the broad spectrum from visible light to NIR light, does not expose to ionizing radiation, and has a high spatial resolution [48]. Also, Optical imaging provides a suitable way to record surface anatomical information with cellular and molecular information [47].

Choi *et al.* have able to selectively target, treat and monitor colon tumours *in vivo* using PEGylated hyaluronic acid nanoparticles together with a near-infrared fluorescent (NIRF) dye, Cyanine 5 (Cy5), with irinotecan added as a theragnostic agent [88]. Quantum dots (QD) are also commonly used in optical imaging. This is because they have fluorescent properties that can be adjusted in many ways. Tan *et al.* synthesized quantum dots encapsulated in chitosan nanoparticles as a contrast agent and achieved targeted delivery to SK-BR-3 human breast cancer cells with excessive HER2 expression using HER2/neus gene, and showed that these cells could be targeted and quantified by “gene silencing” [89]. However, concerns about cytotoxicity seem to disrupt the use of quantum dots despite these advantages [90].

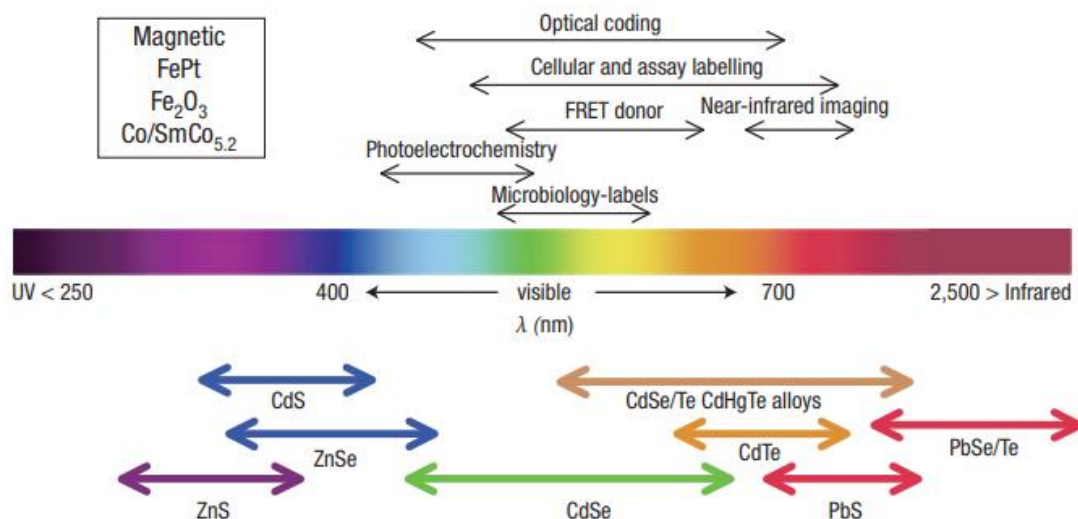


Figure 3.5 Variation of emission wavelengths of QD particles according to their size and material [91]

3.2.2 Ultrasound Imaging

Ultrasound Imaging (sonography) is one of the most widely used clinical imaging methods thanks to its low cost, speed, simplicity, safety results in a short time, and applicable even under laboratory conditions [48], [49]. In this imaging technique, a transducer that produces high-frequency sound waves (N20 kHz, above human-hearing threshold) is placed on the skin and ultrasound images are obtained relative to the sound waves reflected from the internal organs [81], [92]–[94]. Unlike other methods, ultrasound images are displayed in real-time. In this way, the movement of the internal organs of the body and blood flowing through the blood vessels can show in real-time [95]. Ultrasound Imaging is an easy, reliable, non-invasive and radiation-free method for subcutaneous tumour evaluation (Figure 3.6). Ultrasound Imaging may also be preferred for MRI and CT for deeply located tumour size follow-up [49]. Ultrasound offers a high spatial resolution ($<1\text{mm}$), like an MRI, and can also work together with molecular information to offer outstanding anatomical information. But despite this important feature, it has some limitations [51].

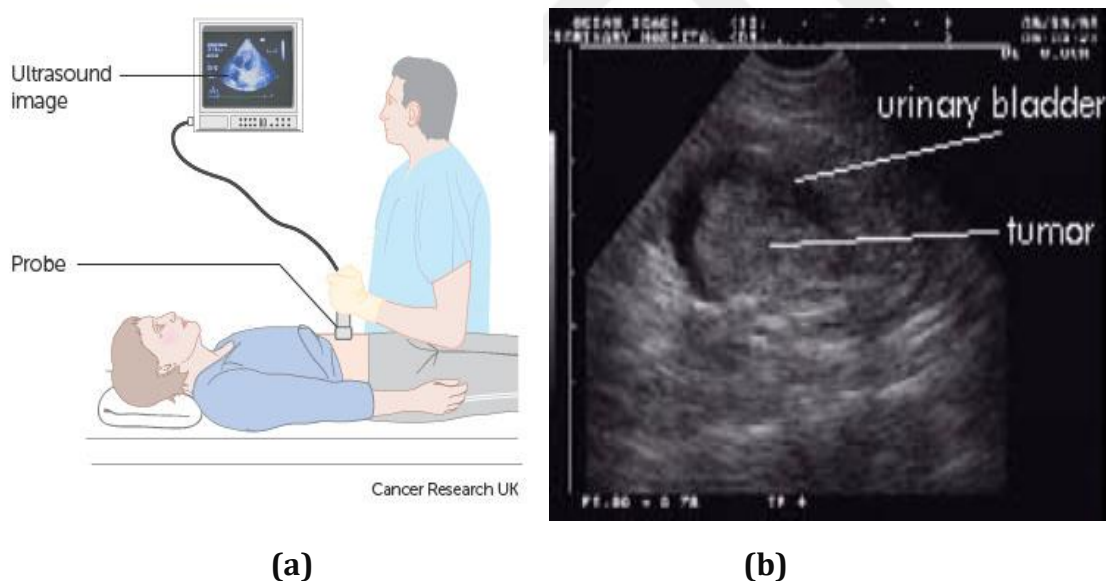


Figure 3.6 A cancer screening with ultrasound (a) The ultrasound probe is placed onto the desired field (b) a bladder cancer detected by ultrasound in the body of a dog [96], [97]

As technology evolves, smaller dimensions and the production of more specific integrated circuits have made ultrasound one of the most mobile imaging methods. Many ultrasound device manufacturers currently offer imaging systems in the size of laptop computers. Some of the new devices are only even smartphone size.

Although ultrasound does not offer as large volumetric imaging as other methods, image acquisition rates over small areas outperform MRI and other nuclear medicine imaging methods. Ultrasound can provide real-time images thanks to its high frame rate capability, which can be advantageous in many theranostic applications [48], [98]. Another advantage of ultrasound is that it visually visualizes soft tissues well. However, the difficulty in penetrating deep tissues greatly reduces the resolution of images [99], [100]. Although the safety of ultrasound contrast agents is still under investigation for many applications, it has been shown that side effects from contrast use are very rare [101]. Also, ultrasound contrast, MRI, and CT may play an important role in patients with contraindicated contrast agents (eg. renal failure) [102].

3.2.3 Magnetic Resonance Imaging (MRI)

MRI is a pain-free, non-invasive medical imaging system and it is used to contribute 2D or 3D images of the structures with a strong magnetic field and radio waves [103], [104]. The basis of the magnetic resonance signal is based on the principle of the motion of water hydrogen nuclei through an applied magnetic field as represent in Figure 3.7. After radiofrequency vibrations have been sent, images can be obtained during the ground state in which the nuclei return to their original positions. This method is used to obtain high-resolution anatomical images of the body while providing a perfect contrast image with millimetre resolution and MRI offers good depth penetration (10 cm) [51], [105].

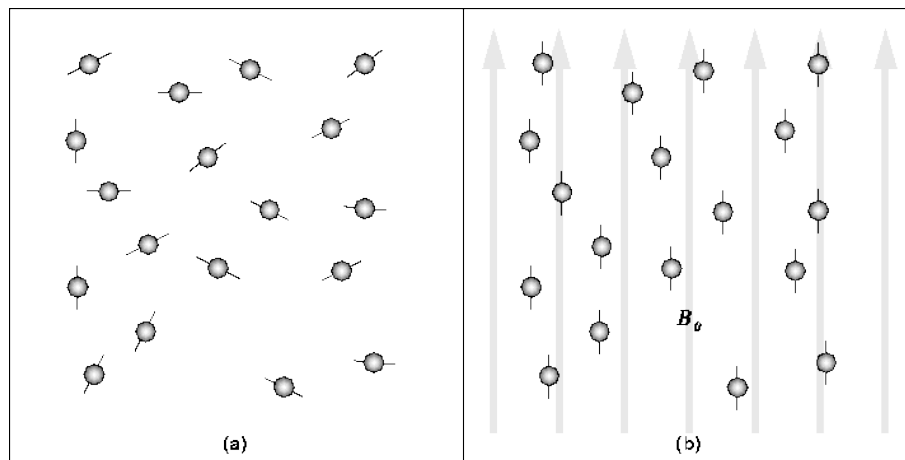


Figure 3.7 In the absence of a strong magnetic field, hydrogen nuclei are randomly aligned as in (a). When the strong magnetic field, is applied, the hydrogen nuclei precess about the direction of the field as in (b) [106]

MRI provides excellent information about soft tissue and function such as our organs, tissues, and skeleton by utilized from proton density, biochemical contrasts, perfusion, and diffusion to contribute diagnose and monitor a broad diversity of diseases [104], [107]. The MRI can be used in brain and spinal cord conditions such as multiple sclerosis (MS), stroke, heart and blood vessel structure issues, inflammatory bowel diseases, liver diseases like cirrhosis, joint and bone irregularities and tumours or abnormalities in organs [108]. MRI evaluates tumour microenvironment with many parameters. Assessment of tumour cell proliferation and metabolism can be done through MRI. MRI provides anatomical imaging and allows full-body imaging (Figure 3.8)[49].

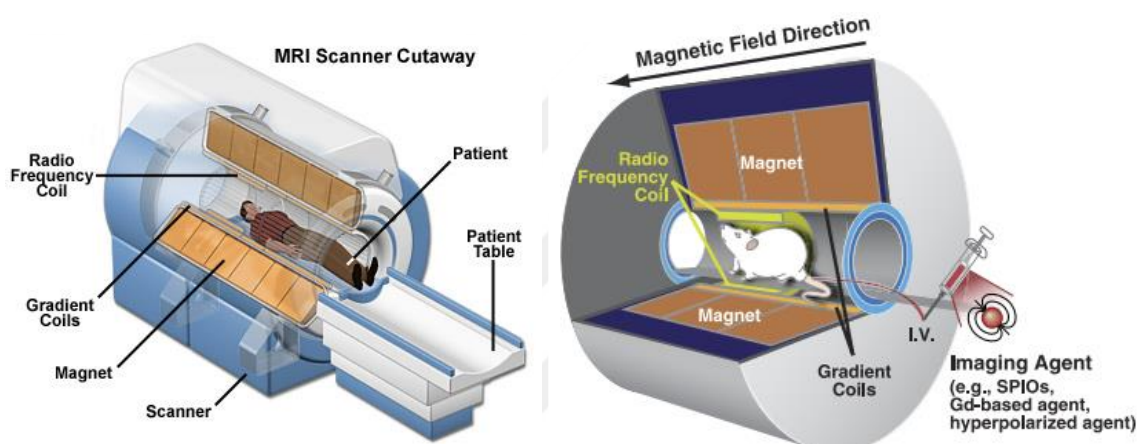


Figure 3.8 A schemes of typical MRI scanner [50], [109]

A lot of paramagnetic- and superparamagnetic-based molecular imaging agents have been tested for preclinical and clinical molecular imaging applications and some of them have been approved by the Food and Drug Administration (FDA) [51]. Paramagnetic molecules such as gadolinium (Gd) and manganese (Mn) used in MRI can be labelled into smaller molecules, macromolecules or nanoparticles. On the other hand, magnetic iron-oxide nanoparticles are spontaneously superparamagnetic and can be labelled with their charge on their surface. Compared to radionuclide or optical imaging, MRI has a good spatial resolution, but its sensitivity is low compared with other molecular imaging modes. To avoid this and to increase signal generation, a relatively high amount of contrast agent is required. High doses of these contrast agents raise concerns about tissue deposition and toxicity. Disadvantages of MRI include the need for lanthanide-based contrast

agents, expensive installation and maintenance fees, and the inability to use in patients using medical devices such as old implants [48].

Wang *et al.* developed chitosan magnetic nanoparticles filled with doxorubicin and plasmid DeoxyriboNucleic Acid (DNA) that were designed to affect lung and prostate cancer cells and could be used as a T2 contrast agent. With this system, tumours will be imaged with contrast-enhanced MR and a strong contrast will be created in T2-weighted series. Thus, therapeutics were efficiently delivered, and the effects of therapeutics were monitored in real-time [110]. Kim *et al.* synthesized a pH-sensitive micelle for use as an MRI contrast agent, and the amphiphilic block copolymers were methoxy poly [(ethylene glycol)-b-poly(L-histidine) (PEG-p(L-His)) and methoxypoly (ethylene glycol)-b-poly(L-lactic acid)-diethylenetriaminopentaacetic acid dianhydritadolinium chelate (PEG-p(L-LA)-DTPA-Gd) substance has established an interesting theranostic platform. These substances have a spherical shape of 40 nm at physiological pH but are separated from each other in the acidic environment of the tumour, contrasting tumour tissue in T1-weighted series [110].

3.2.4 Computed Tomography (CT)

X-rays are used in various devices in medical imaging. Computed tomography (CT), sometimes called "computerized tomography" or "computed axial tomography" (CAT), is the most comprehensive use in today's clinical practice [111], [112]. CT is a non-invasive medical imaging system that uses X-ray wavelength to perform the cross-sectional (tomographic) image of all body (Figure 3.9). Each cross-sectional view shows a slice of the displayed person, such as a loaf of bread slice [112]. The object to be examined with CT can be seen inside without any cuts. With CT, many two-dimensional X-ray images of an object are taken from different angles to obtain a three-dimensional image of the internal structure of that object. The basis of the X-ray machine is based on the above-mentioned events [113]. Allan McLeod Cormack and Godfrey Hounsfield shared the 1979 Nobel Prize in Physiology or Medicine for their work on X-ray-based CT in 1963 [114]. The use of CT has increased rapidly since its entry into the clinic in the 1970s. In the last two decades, CT use has increased 12-fold in the UK and 20-fold in the United States [115]. In fact, the number of examinations in the US reached 72 million annually in 2007 and it

was observed that there was a threefold increase compared to the 1990s [116]. In 2018, 271 people per thousand have been done a CT scan in the US, while in Turkey, 207 people per thousand have been done a CT scan [117].

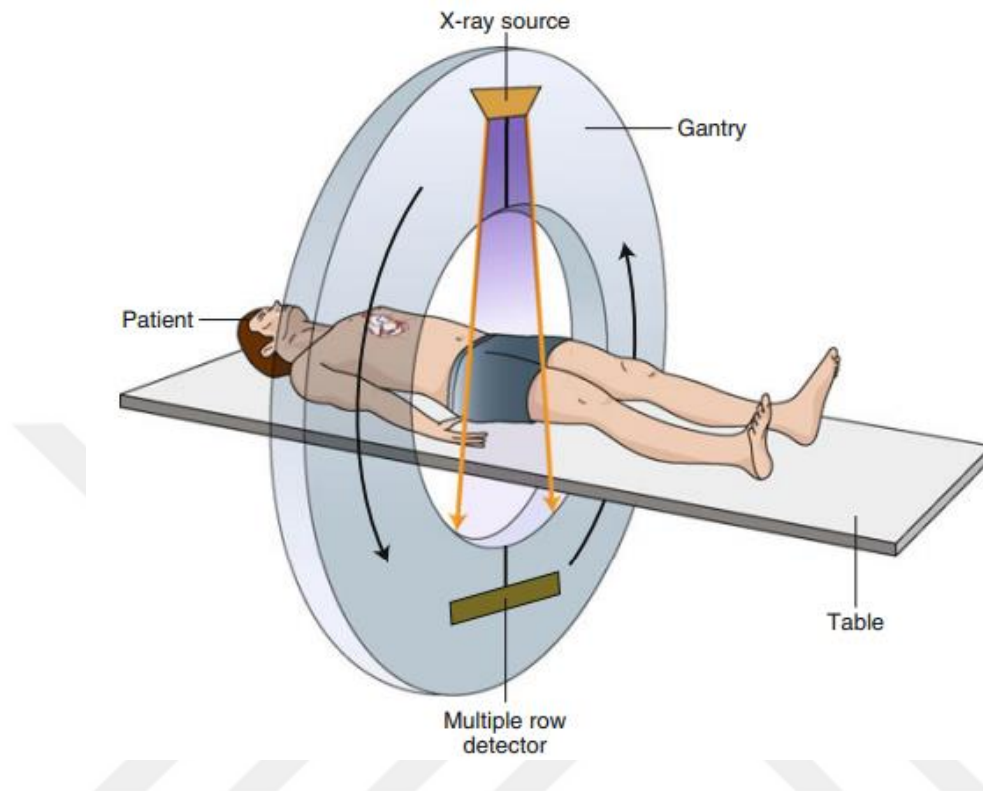


Figure 3.9 A scheme of a typical CT scanner [118]

The main function of CT is to collect anatomical information through a system based on the reduction of X-rays as they pass through different biological components such as bone, muscle, fat, water and air. While radionuclide-based imaging (such as PET/SPECT) is successful in providing information about physiological functions, CT collects anatomical information [48], [119], [120]. CT contrast agents are typical of low molecular weight and are identified by rapid extravasation and excretion. Despite the high spatial resolution of CT, the tissue contrast is low. It is difficult to represent subcutaneous and orthotopic tumours. It requires the use of some contrast agents. Functional vascular parameters in the tumour can be evaluated by dynamic contrast CT. However, contrast agents may not circulate sufficiently to accumulate in the tumour cells. Therefore, it requires applying a high volume of contrast agents, but this may not be tolerated by the body. Therefore, CT is especially used to evaluate tumours in lung and bone [49].

CT allows evaluating the structural features of the tumour and the presence of metastasis. Besides, advances in CT technology have led to the use of low-dose CT in lung cancer screening. It is possible to evaluate the inner surface of the tracheobronchial system with three-dimensional CT technology. One of the main difficulties in staging cancer by CT is the inability to detect the presence of metastases in normal-sized lymph nodes. MRI, another imaging modality, is not preferred for thoracic examination because of its tendency to artefacts caused by movement [121]. Numerous studies currently in progress or the planning phase in cancer screening studies with CT. In two large separate studies published to date, CT has been found to have superior features in terms of sensitivity than conventional radiography. The initial CT scan detected bronchogenic carcinoma in 1.1-2.7% of the patients screened. 62-93% of the tumours were stage I [122]–[124]. Sensitivity rates in bladder cancer diagnosis using this technique have been reported to be between 60% and 90% and specificity as 94%. During the examinations performed using CT virtual cystoscopy technique, it is possible to detect possible pelvic-paraaortic lymphadenopathies and upper urinary tract pathologies by keeping the examination range-wide. However, this technique involves catheterization if air-carbon dioxide is used as a contrast agent. If an intravenous contrast agent is used, it involves handicaps such as nephrotoxic effect and radiation exposure [125]–[128].

The macromolecules and nanoparticle substances are compounds that are suitable for vascular CT as they remain in the blood pool longer. High-atomic number of iodine, bismuth or gold-containing nanoparticles have been proposed as CT contrast agents [129]–[131]. Although the high load is required for the transmission of contrast agents in CT, many researchers use solid nanoparticles, some suggest liposomes in which water-based iodine molecules are trapped. Gold nanoparticles have become a popular choice for CT [132]. However, their optimal use is currently uncertain [133].

3.2.5 Radionuclide Imaging (PET/SPECT)

Radionuclide-based Imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) for molecular imaging are used effectively recently in imaging [48]. They are the most sensitive

molecular imaging techniques [134]. γ -ray emissions form the basis of the work of SPECT and PET and radiopharmaceuticals can be detected by a camera for both methods. In contrast to MRI, SPECT and PET images are performed with a lower background signal, and since the energy of the gamma rays is in the megavolt range, little signal amplification is required for images. SPECT and PET are quantitative techniques and have advantages over methods such as MRI and optical imaging [135], [136]. According to Coherent Market Insights report, A PET/CT, combining PET and CT device, scanner device is used mostly in oncology, cardiology, and neurology applications, respectively and they have US\$ 1.9 billion market value in 2017 [137].



Figure 3.10 A PET/CT device [138]

The PET device differs from the SPECT device in proportion to the technology to which it belongs. The SPECT device has one or two detectors which, in turn, detect X-rays from a large part of the body; Creates traceable images by taking Gamma Rays (γ). PET device (Figure 3.10); It has a frame that chassis the detector sets arranged in a circle. It detects the rays coming from the body (positron radiation β) employing detectors arranged in a series and forms a contrast image of the tissues. [139], [140]. PET is a tomographic scintigraphy technique based on the physical properties of certain radioactive isotopes, also known as positron emitters. Positron decay is a type of beta decay, which is a positively charged particle (shown as β^+) and occurs in proton-rich nuclei to become more stable. However, PET scanners do not display

positron emitted from the nuclei. The displayed are the gamma ray photons that are released to the PET detectors by positron annihilation as shown in Figure 3.11 [141]. Positrons, which are positively charged particles, can travel only a very short distance of a few millimetres without encountering a negatively charged electron. When a positron collides with an electron, the conversion of mass into energy takes place. As the masses of the colliding particles disappear, two annihilation photons of 511 keV (kilo electron volts) energy are released at an angle of 180 degrees to each other in the opposite direction. This event is called annihilation (disappearance), the resulting photons are called annihilation photons [142], [143].

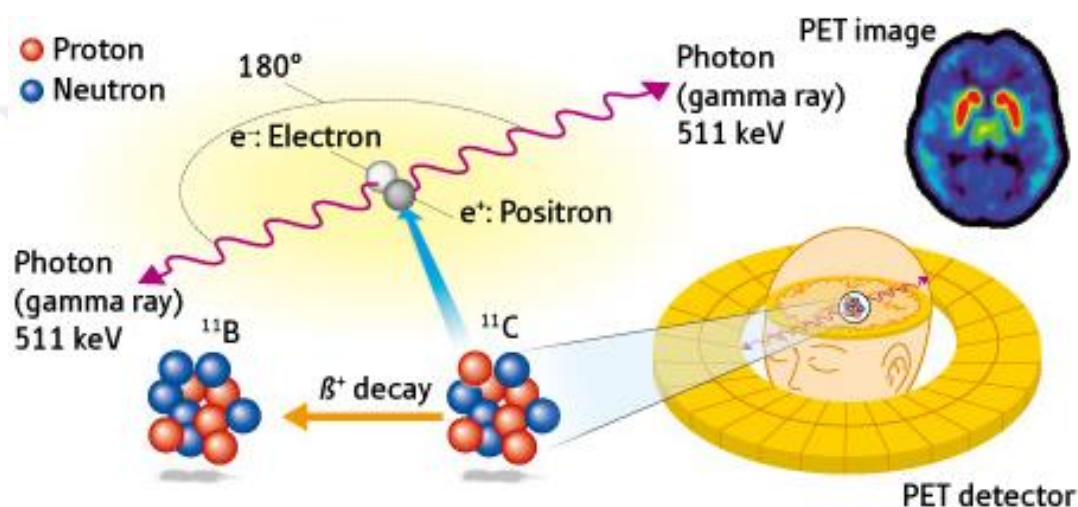


Figure 3.11 How the PET device works [144]

PET has been used to image the biochemical and biological process *in vivo* by using positron-emitting radioisotope bound to molecular probe which has a specific affinity to cancer cells [145], [146]. Organic radionuclides such as Carbon-11 (^{11}C), Nitrogen-13 (^{13}N), Oxygen-15 (^{15}O) used in the preparation of PET radiopharmaceuticals are isotopes of elements that are part of many biochemicals and drugs. Radiopharmaceuticals prepared with these radionuclides are indistinguishable from their natural versions in terms of their biochemical properties. But their short half-life is the biggest obstacle in the development of commercial radiopharmaceuticals for use in routine applications [147], [148]. Even though Chlorine (Cl^-) and Iodine (I^-) ions of Fluorine (F_2), Cl_2 , Bromine (Br_2) and I_2 elements in the halogen group are common in the human body, F and Br do not exist in the structure of natural molecules. Although the chemistry of radioiodine and radiobromide are very similar, the markings made with these radionuclides show

significant differences in the biological properties of the molecule in about their half-life. Gallium-68 (^{68}Ga), which is another group used in the preparation of PET radiopharmaceuticals, has a value of +3. Coordination chemistry is less complicated than Copper (Cu), Scandium (Sc) and Zirconium (Zr) radiometries. It is ideal for the development of PET radiopharmaceuticals such as $^{99\text{m}}\text{Tc}$ (Technetium-99m) radiopharmaceuticals routinely prepared in SPECT applications. Among other metallic radionuclides, zirconium-89 (^{89}Zr) is a more ideal radiometal for radiolabeled antibody fragments prepared for immuno-PET imaging studies compared to Copper-64 (^{64}Cu) [149], [150].

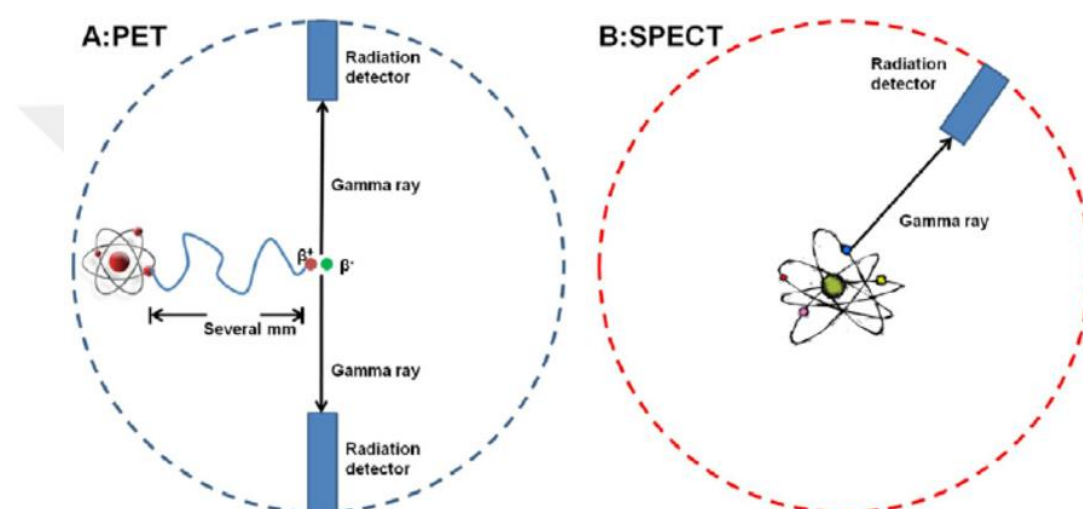


Figure 3.12 (a) Positron annihilation in PET: the emitted positron from the isotope travels in tissue or cell for a short range, during which time it loses kinetic energy until it decelerates to a point where it can interact with an electron. When an electron and positron collide, they produce two gamma photons to be emitted in opposite directions. Multi-headed gamma cameras detect these emissions 'coincident' in time. (b) The radioisotope emits the gamma photon that is detected by a simple gamma camera [157]

In parallel with the widespread use of 2-deoxy-2- (^{18}F) fluoro-D-glucose (^{18}F -FDG) and PET imaging in oncologic indications in the last 10-15 years, there is an increasing interest in this method in physicians and medicine. Only fifteen years ago, the number of PET application centres in the world, which is only mentioned in decimal numbers, is now expressed in thousands [151]. As of the end of 2004, the number of PET imaging centres in Turkey, which was first introduced in the last quarter of 2000, exceeded 10. Only three of these were in public hospitals (Istanbul Cerrahpaşa Faculty of Medicine, Ankara Gazi Faculty of Medicine and Ankara GATA Hospital), and others were held in private health institutions in Istanbul and Izmir.

As Turkey in January 2008, this number increased to 37 PET/CT device. Only 5 of the existing devices are PET, others are hybrid PET/CT. The most devices are in Istanbul with 16 units, 8 in Ankara, 4 in Izmir, 3 in Adana, 2 in Bursa and 2 PET/CTs or PETs [152]. Today, the number of PET imaging centres in our country has reached 120 in total as public and private hospitals. This is very positive and in line with the recommendations of the European Union [153]. “Cyclotron” systems, which are essential for radiopharmaceutical production, which is one of the basic elements of PET imaging, are not yet available to the public. At present, all centres supply their radiopharmaceutical needs from private sector commercial cyclotron companies in Istanbul and Izmir [143], [151].

To perform PET imaging, radioactive elements, namely unstable atom, are needed. The method for producing artificially radioactive elements is to unstabilize the stable atom. In other words, this process is called splitting the atom. Because the atom that becomes radioactive necessarily turns into another atom by radiating. These studies proceeded by Ernest O. Lawrence and Rutherford [153]. In the early studies of accelerated particles and radionuclide production, linear accelerator and high voltage were used to accelerate the particles that will bomb the target. Cyclotron, which produces high-energy particles such as protons for atomic research, was developed by Lawrence. The advantages of the cyclotron are that, unlike the linear accelerator, it takes up less space and requires less voltage [154].

The first cyclotron was discovered in 1929 by Ernest O. Lawrence [155]. The development of cyclotron technology has continued to the point where it can be located in a smaller area and operated with relatively little information. The use of cyclotron in the hospital was financed by the Medical Research Council and the Hammersmith Hospital Cyclotron Unit was opened in 1955 in London. This system was used both for the production of radionuclides and for treatment with neutrons and other particles [153], [154]. Second-generation cyclotrons with more complex design and improved engineering were produced by Scanditronix in Sweden, Thompson CSF in France and Sumitomo and Japan Steel Works in Japan. In 1988, Yves Jongen from the University of Louvain-laNeuve in Belgium developed Cyclone30, an industrial cyclotron designed for the production of medical radioisotopes. This led to the establishment of IBA SA, a new accelerator

manufacturer in Belgium. Later, some other companies producing industrial accelerators were established in Canada including EBCO. The desire to produce small amounts of PET radionuclide, such as single disease dose, did not materialize until 2009. In 2009, Ron Nutt's ABT Molecular Imaging Inc. developed a small cyclotron that accelerates protons at 7.5 MeV energy and uses an internal target for the production of single disease ^{18}F [153], [154], [156].

PET imaging was first developed by Phelps and Hoffman in the 1970s [158]; clinical use became widespread with the hybrid PET/CT device developed by Townsend in 2000 [159]. In the early 2000s, hybrid imaging techniques, such as PET/CT and SPECT/CT, combined molecular sensitivity and anatomic specificity in tumour imaging [160].

PET is one of the few FDA-approved molecular imaging technologies since 1997 and has been reimbursed by many insurance US companies, firstly Medicare, because of its FDA approval. PET has become a clinical vital element in for many malignancies after FDA approving [153], [161]. In our country, ^{18}F -FDG PET/CT reimbursement indications have been determined by the Social Security Institution (SGK) for cancer diagnosis, initial staging, re-staging and treatment response applications [162]. The most frequently used PET radiopharmaceutical imaging agent is ^{18}F -FDG. The first ^{18}F -FDG was performed in 1979 by Phelps *et al.*, the University of Pennsylvania using PET screening using ^{18}F -FDG synthesized at the Brookhaven National Laboratory in Long Island [163], [164]. ^{18}F -FDG is a glucose analogue and is very similar to glucose in structural and biochemical behaviour. It is selectively taken up by specific cells which has a high rate of glucose metabolism. Because of increased hexokinase enzyme activity in tumour cells increased glucose use [165]. This uptake difference provides to distinguish of malignant cells [56], [166]. ^{18}F -FDG-based PET has been approved for the staging of NSCLC, breast cancer, oesophageal cancer, head and neck cancer, colorectal cancer, melanoma, and lymphoma [56].

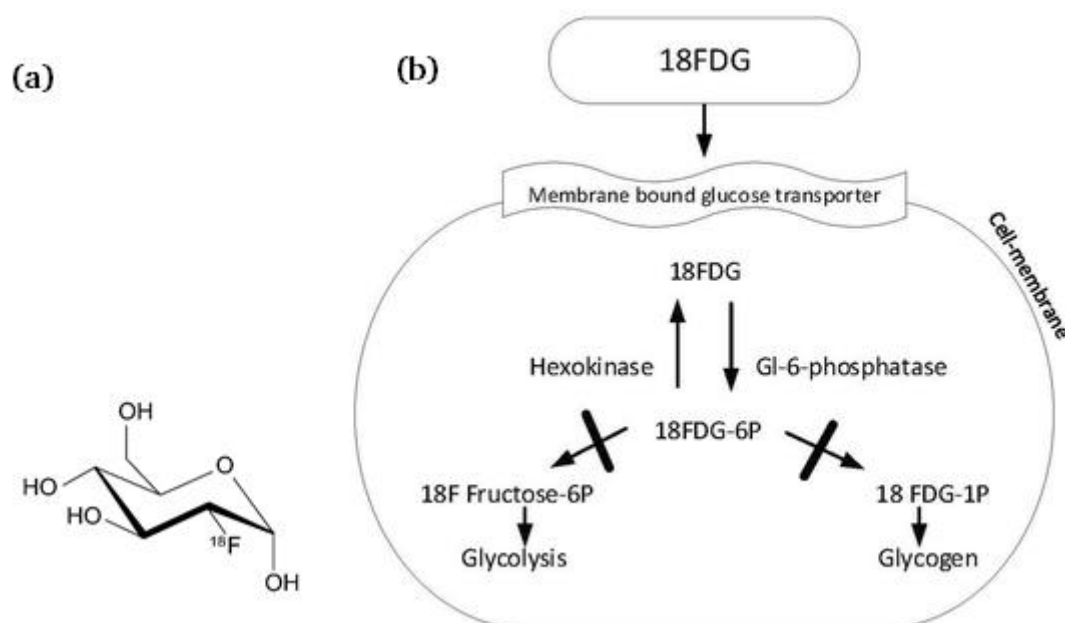


Figure 3.13 (a) Structure of the ^{18}F -FDG, ^{18}F is replaced -OH group of glucose (b) Cellular uptake of ^{18}F -FDG by membrane-bound glucose-transporter (GLUT). ^{18}F -FDG is phosphorylated to ^{18}F -FDG-6P in the cell. After, ^{18}F -FDG-6P trapped in the cell since it cannot enter the glycolytic pathways [170]

Five glucose transporter proteins (Glut 1-5), which are dominant in tumour tissue, have been identified. Significant biochemical characteristics of malignant cells include the increase of glucose transporter proteins (particularly Glut-1) on the cell surface [167]. Glucose is transported into tumour cells by facilitated transport by these carrier proteins and undergoes glycolysis. Pyruvate forms from glucose in aerobic conditions. However, in anaerobic conditions, as in a hypoxic necrotic tumour, tumour lactate levels increase. In ^{18}F -FDG, metabolically active tumours are transported by facilitated transport in the same way as glucose. Transport of ^{18}F -FDG by tumour cells is proportional to its metabolic activity. ^{18}F -FDG is phosphorylated in the same manner as glucose to form ^{18}F -FDG-6-phosphate. Unlike glucose, however, it does not continue to be metabolized and is increasingly trapped within the cell as shown in Figure 3.13 [168]. Thus, the increase in intracellular enzymes (hexokinase and phosphofructokinase) that provide glycolysis in tumour cells; on the other hand, the consumption of more glucose than normal cells as a result of low dephosphorylation rate due to decrease in glucose-6-phosphatase enzyme activity forms the basis of PET imaging using ^{18}F -FDG [168], [169].

Biochemists have shown that cancers are more metabolically active than normal tissues since 1920. Glucose uptake and use in cancer cells have increased due to this increased metabolism [171]. Standardized Uptake Value (SUV) is a semi-quantitative measure of relative ^{18}F -FDG metabolism in the lesion of interest. SUV is the ratio of ^{18}F -FDG concentration in the lesion to radiopharmaceutical concentration distributed throughout the body and has no specific unit [172]. It is expressed mathematically as Equation 3.1;

$$SUV = \frac{\text{Tumor Activity Concentration (MBq/ml)}}{\text{Injected Dose (MBq)}} \times \text{Body Weight (g)} \quad (3.1)$$

SUV is often used to evaluate cancer. Although it has certain significant limitations, it is generally an indicator for SUV malignancy higher than 2.5. [173]–[175]. If the SUV is smaller than 2.5, it supports benign, often inflammatory conditions. Besides, visually the metabolic activity of the lesion is higher than the mediastinal blood pool activity [143]. The degree of ^{18}F -FDG accumulation in the primary lesion was found to be prognostic value. Studies have shown that patients with SUV 10 or greater have a poor prognosis, even if there is no metastatic disease. Another important area of SUV use is post-treatment follow-up [143].

PET/CT is a hybrid imaging device created by integrating the PET scanner and the CT scanner. The CT component in PET/CT devices is used for two purposes. The first is to map the transmission to be used for attenuation correction of PET images, and the second is to perform detailed anatomical imaging to determine the exact localization of the lesions. PET/CT fusion images are obtained by overlapping the images obtained from the CT scanner with the PET scanner. Thus, both metabolic functional information and anatomical information can be evaluated simultaneously. Information from PET/CT is known or suspected malignancies; clinically more useful than information obtained from PET or CT alone or evaluated separately (Figure 3.14) [176].

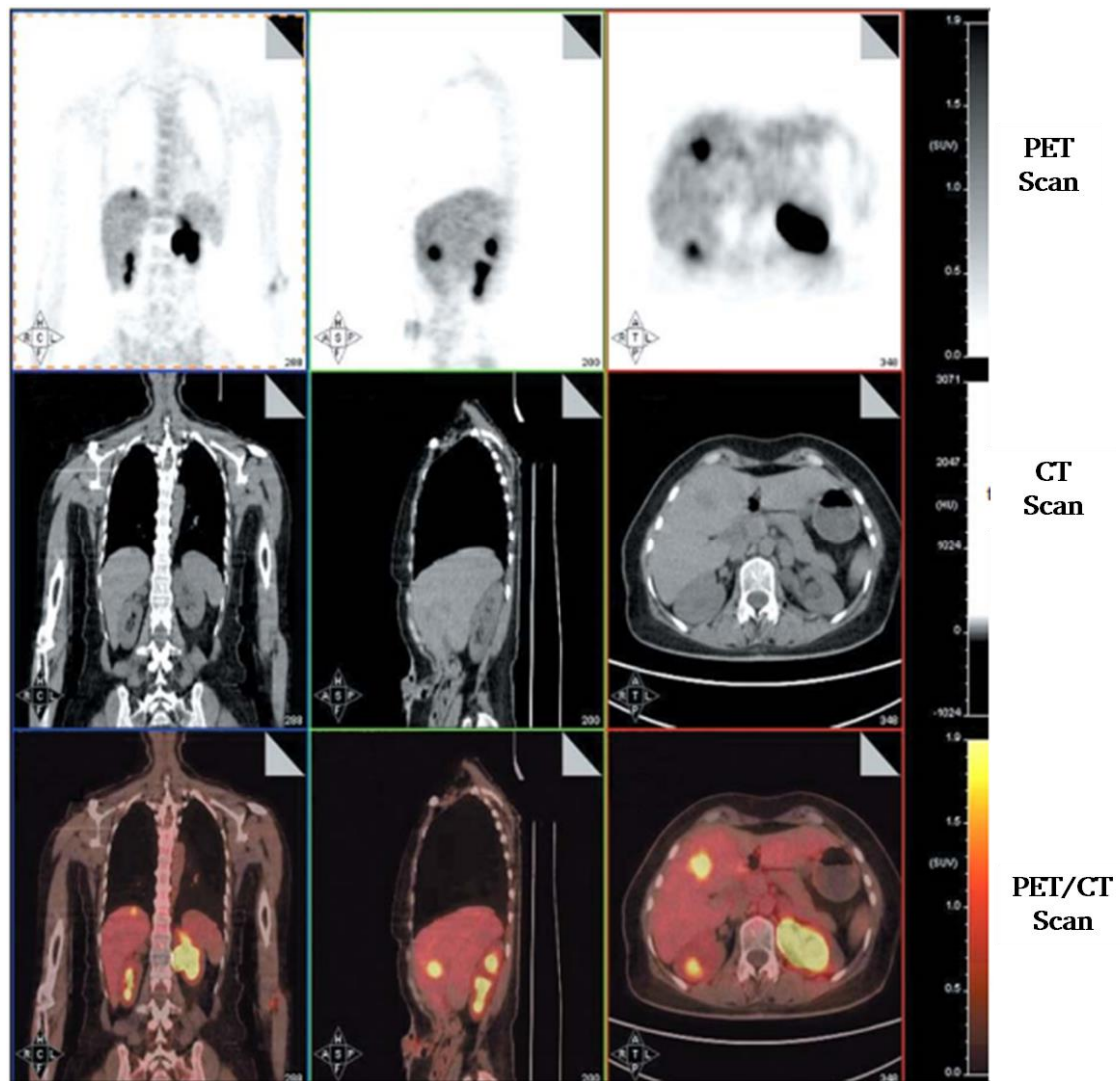


Figure 3.14 In integrated PET/CT systems, transmission images are generated by X-ray source (CT) and high-resolution morphological images are obtained. These images are used to correct the attenuation of PET images, and they are used with PET images to help localize the lesion [151]

Gupta *et al.* found that ^{18}F -FDG PET is highly accurate in the differentiation of benign-malignant Solitary Pulmonary Nodules (SPNs) of 6-30 mm in size with uncertain radiographic findings [177]. In 2001, a large meta-analysis of the accuracy of ^{18}F -FDG PET in pulmonary nodules and mass lesions was published in The Journal of the American Medical Association (JAMA). The researchers reviewed 40 previous 4-year studies. The sensitivity of ^{18}F -FDG PET scanning was found 96.8% and specificity was 77.8%, based on the analysis of 1,500 focal pulmonary lesions [173].

There are some limitations in the diagnosis made with ^{18}F -FDG, which is the most widely used radiopharmaceutical in PET imaging [178]. In cases of increased glucose consumption, such as inflammation and infection and glucose-flowed

organs such as the brain, ^{18}F -FDG's selectivity and sensitivity are lost. The main reasons that limit the imaging sensitivity and selectivity are the inability to clearly distinguish between tumour and tumour-neighbouring normal tissue, primary nodal staging can be performed with limited sensitivity, poorly labelled metabolically silent lesions, different results depending on the tumour's access to food and oxygen are obtained [178]–[180]. These effects have led to an increasing number of studies on the development of new radiopharmaceuticals [9], [181], [182].

Radiopharmaceuticals that can bind to specific receptors in tumour cells or cells within tumour mass are quite limited. The new trend in PET/SPECT imaging is peptides. Cyclic peptide (^{18}F -Galacto-RGD), which binds to active endothelial cells in the tumour, and peptide ligand (^{68}Ga -DOTATOC), which binds to the somatostatin receptor, which enables the imaging of meningiomas, are preferred as PET imaging agents [183], [184]. The use of ^{68}Ga -DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid)-peptides has been increasing for years. The reason behind this increase is that it does not require a cyclotron synthesis method and easy labelling. Also, unlike ^{18}F -DOPA and ^{18}F -FDG, ^{68}Ga -DOTA-peptides are not dependent on cell metabolism. Besides, it is an imaging agent used to locate, staging, and staging an unknown primary tumour in patients with proven Neuroendocrine tumours (NET) secondary lesions. ^{68}Ga -DOTA-peptides are used to determine whether the patient is suitable for administration for peptide receptor radionuclide therapy [185].

4.1 Biomarkers

Arising from the expression of biological markers, "biomarker" means medical marker (sign) constitute a subgroup and as a marker of a medical condition that can be measured accurately and reproducibly [186], [187]. Biomarkers can be defined as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids" [187]. Biomarkers are like molecular signs of the physiological state of a cell at a given time. Active genes, genetic material, related proteins and other organic chemicals produced by the cell to create these signs serve as biomarkers (Figure 4.1) [188]. The main benefit of biomarkers is the ability to provide a measurable factor to allow early diagnosis of disease, to ensure initial diagnosis and follow-up of the disease [189]–[191]. It has been shown that there is a significant change in the number of certain proteins, carbohydrates, and DNA during the development of various cancers and that these molecules can be potential biological markers for prediction, cause, diagnosis, progression, and even outcome of treatment of a disease [192], [193].

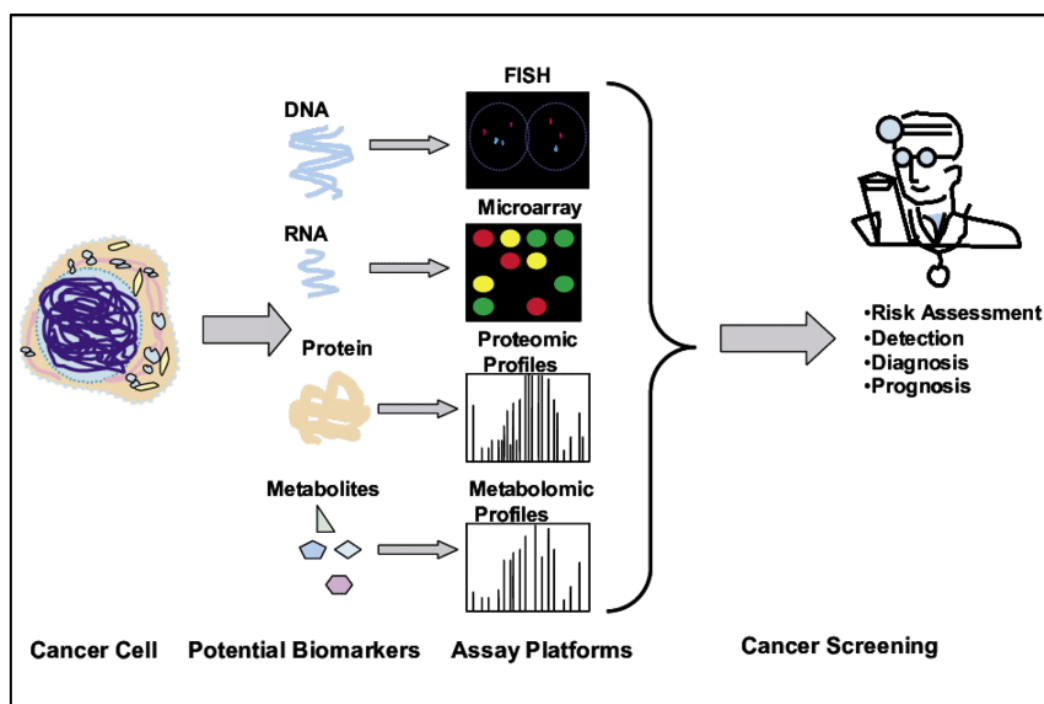


Figure 4.1 Biomarker clinical application for cancer [194]

4.1.1 Cancer Biomarkers

Cancer is among the most heterogeneous diseases due to both differences between individuals and genotypic and phenotypic differences in tumour cells (intratumoral). Tumour tissue contains transformed cells as well as various cells, including immune system cells, that support cancer development [195]. In particular, the new features gained by immune cells and stromal cells located in the tumour microenvironment can be identified by similar biomarkers (cell-type-specific proteins) in almost all solid tumour types. These markers are absent or expressed at very low levels in immune and stromal cells in normal tissues [195], [196]. The characteristics of immune and stromal cells change due to tissue physiology deteriorated during tumour development, factors secreted by tumour cells and developing inflammation. These cells are called tumour-related because they support the development of cancer. The number of tumour-associated cells that are embedded in the mass formed by the growth and/or progression of the tumour also increased [195], [196]. Even if they are located in tumours originating from different tissues or cell types, they are recognized by specific biomarkers indicating that they are present (tumour-related) in tumour tissue [197].

Cancer biomarkers are described by NCI “A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of cancer-related disease” [198]. Cancer biomarkers are biomolecules which provided from the tumour cells or by other body cells that respond to the tumour activity [199]. The cancer biomarkers can be used for cancer prediction, diagnostic, detection, and pharmacodynamics. Genomic signatures were used as a for the classification biomarker for cancers by Lander’s group [200]. Cancer biomarkers have not established classified, but it can be classified using different approaches as shown in Figure 4.2 [201], [202];

- Disease State
- Biomolecules
- Other Criteria

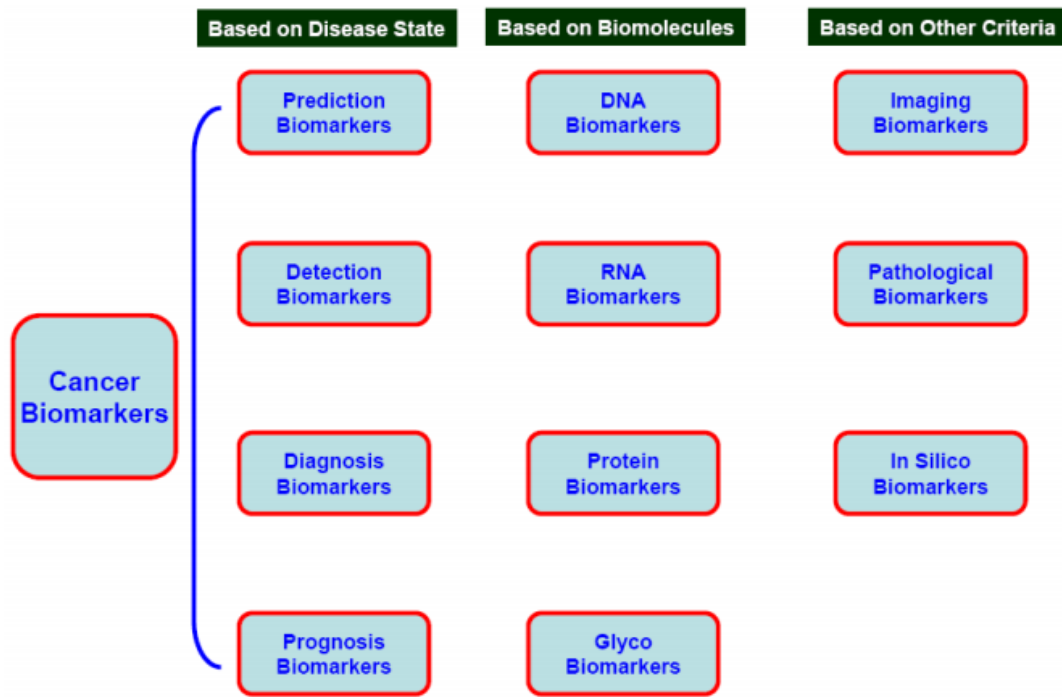


Figure 4.2 Classification of cancer biomarkers according to contemporary findings [202]

The first cancer biomarker was first seen in 1965 as the Bence Jones protein and carcinoembryonic antigen (CEA), which was found in colon cancer [203]. For all that, prostate-specific antigen (PSA) is known as the most important cancer biomarker and is still used in clinical practice [202], [204].

The presence of specific biomarkers of immune and stromal components in tumour tissue is supported by both literature and bioinformatics data. Also, immune and stromal cells can carry these biomarkers at high levels independent of the tissue from which the tumour originates [196], [205], [206].

4.1.1.1 Cancer Biomarkers for Cancer Diagnosis

Monitoring of the dynamics in the tumour microenvironment enables the follow-up of the disease status and treatment responses [207]. In these studies, cells isolated from experimental animals were labelled and traced back to the animals in *ex vivo* with a tracer or a special enzyme [208]–[211]. Various imaging methods are used to determine the location of *ex vivo* labelled cells in tumour foci. As a result of these experimental studies, it has been shown that imaging of tumour microenvironment can successfully monitor tumour growth, metastasis, angiogenesis, chemotherapy,

radiotherapy and immunotherapy responses [207]. The tracer used is generally selected from the luciferase gene, fluorescent protein genes, infrared-near fluorescent dyes and magnetic nanoparticles [207], [212], [213]. In particular, markings based on the fluorescent and luminescent principle can be visualized thanks to the advantage of small body volume of experimental animals but the not human body [212], [213].

The characteristics of immune and stromal cells change due to tissue physiology deteriorated during tumour development, factors secreted by tumour cells and developing inflammation [195]. The cancer cells can be isolated from other cells with high expression of Tumour-infiltrating lymphocytes, programmed death-1 (PD-1), glucocorticoid-induced TNFR-related protein (GITR), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), and similar molecules [214]–[218]. Endothelial and myeloid cells involved in tumour neo-angiogenesis can be detected by the presence of TEK receptor tyrosine kinase (TIE2) and Integrin beta 3 (CD61) receptors [219]–[224]. As a result of tumour-induced effects, PD-1 ligand 1 (PD-L1), macrophage mannose receptor (CD206), folate receptor 1 (FOLR1) levels in these cells are highly expressed [225]–[229].

Another approach which can provide information about the disease by imaging of stromal components in the tumour is the monitoring of proteolytic enzyme activity that breaks down the Extracellular Matrix (ECM) [207], [230], [231]. Substrates of enzymes such as Matrix metalloproteinases (MMP), hyaluronidase and transglutaminase are labelled with quenched fluorochromes. The increase in fluorescence signal as a result of enzymatic degradation of these substrates has coincided with the localization and size of the tumour. The activity of the corresponding enzymes such as matrix metalloproteinases and hyaluronidase must be present in the tumour for signal formation [207], [232].

4.1.1.2 Folate Receptor 1 (FOLR1) for Cancer Diagnosis

Folate Receptor alpha or Folate Receptor 1 (FOLR1, FRalpha, FR α FR1), a 38–40 kDa molecule, is a membrane-bound protein which is member of the Folate Receptor (FOLR) family [233], [234]. FOLR1 is encoded by the *FOLR1* gene, at band 11q13.4

[233]. FOLR family and FOLR1 have a high affinity to bind and transport active form of folate (5-methyltetrahydrofolate (5-MTHF)) molecule which is an essential component of some important cell metabolism activity such as DNA synthesis, replication and repair, protein synthesis and methylation process [234]–[236]. Besides, the FOLR1 receptor preserves and pursues a high level of tumour-specific apoptosis which prevents cell death of cancer cells [237].

FOLR1 expression is restricted in normal tissue such as apical surfaces of a few epithelia, predominantly in the lung, kidney, and choroid plexus while it is highly expressed in various nonmucinous solid tumours containing ovarian, NSCLC, breast, brain, kidney, colorectal cancer and in high-grade osteosarcoma [233], [239]. The high expression of FOLR1 in cancer cells may be due to the high level of DNA synthesis and rapid growth that cancer cells need [240]. FOLR1 is found common in cancer cells, it is overexpressed approximately 55% of human cancer (Table 4.1) and there is an effectively available to folate-linked drugs nowadays as shown Figure 4.3 [241]–[245]. Besides, the expression of FOLR1 surface protein in the ovarian cancers is 10-100 fold higher than its normal ovarian cell expression [235], [243].

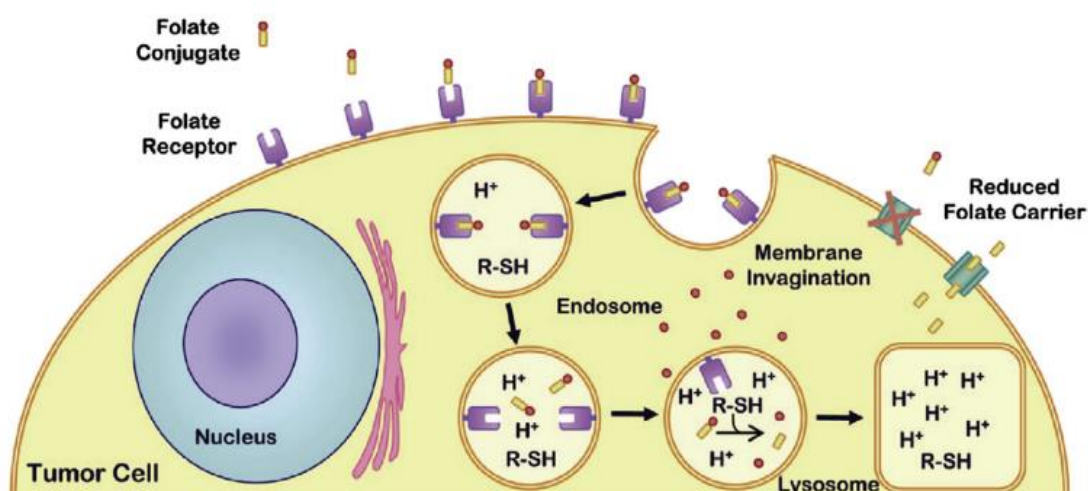


Figure 4.3 FOLR-mediated endocytosis of folic acid–drug conjugate. Folate conjugates bind FOLR with high affinity and are subsequently internalized into endosomes that can reduce disulfide bonds. A similar pathway could be exploited for the delivery of drug-loaded nanocarriers [238]

Table 4.1 FOLR expression number and ratio in chosen solid cancer [245]

Cancer Types	New cases per year	FOLR-expressing cancers per year	% FOLR
Breast	176.300	84.624	48
Lung	171.600	133.848	78
Colon-rectum	129.400	41.408	32
Head/neck	39.750	20.670	52
Uterus	37.400	33.660	90
Kidney	30.000	22.500	75
Pancreatic	28.600	3.718	13
Gastric	26.700	10.146	38
Ovarian	25.200	22.680	90
Endocrine	19.800	2.772	14
Brain and CNS	16.800	15.120	90
Testicular	7.400	1258	17
Total	708.950	392.404	~55

The cancer specificity of FOLR1 and FOLR1 overexpression on cancer cells make it a candidate for cancer that can be productive for both diagnostic and therapeutic strategies. For this purpose, new folate receptor-targeted therapies have been developed for the treatment of ovarian and lung cancer in clinical [246]. In this sense, the first produced folate-targeted imaging agent and the drug were ^{111}In -DTPA-folate, with binding affinity to FOLR1 at 1 nM [247]. Farletuzumab (MORAb-003) and Vintafolide (EC145) are among the examples for monoclonal anti-FOLR1 antibody and folate-conjugated small molecule, to target the folate receptor,

respectively [234]. Furthermore, FOLR-targeted imaging agents are started to use for diagnosis of cancer cells (Figure 4.4) [245]. In the study of Maurer *et al.*, ^{99m}Tc -Etarfolatide showed that cancer cells provide important information about the imaging, staging and prognosis because of etarfolatide affinity value for FOLR is high and have 0.92 and a 3.2 nM dissociation constant [248].

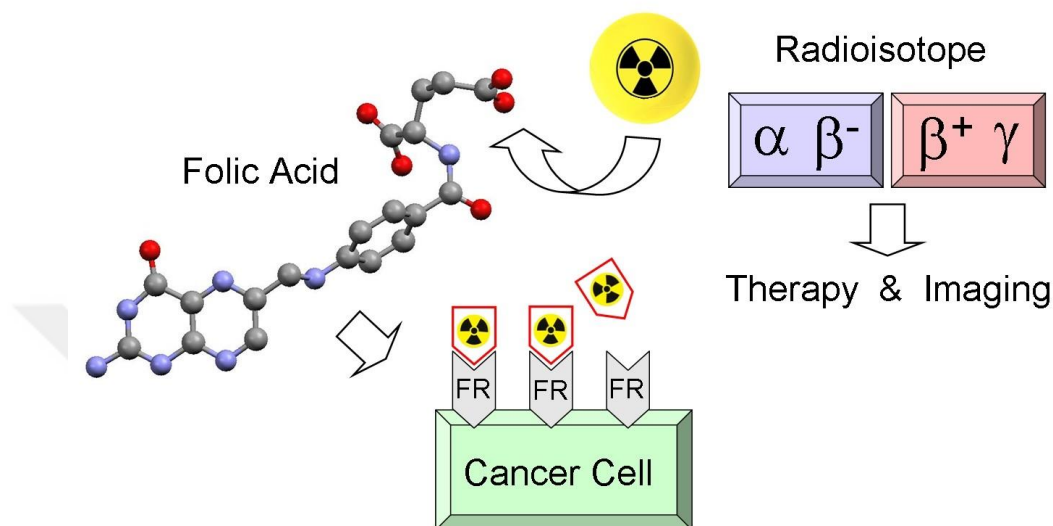


Figure 4.4 The folic acid linked-radioactive probe emitting gamma-rays or positrons are useful for FOLR-targeted nuclear imaging on SPECT and PET imaging [249]

FOLR1 as tumour markers in ovarian cancer has been guiding research [250], [251]. In a study conducted by Basal *et al.*, in June 2009, compared with ovarian cancer and control group found that FOLR1 levels are distinguishable for early and advanced stage patients [233]. Kimberly *et al.* demonstrated that FOLR1 expression is maintained on metastatic focus and recurrent tumours, as it is common in recurrent, high-grade serous tumours. Besides, it has been suggested that new folate-targeted therapies may be used in new diagnosis or recurrent ovarian cancer [252].

4.2 Imaging Agents

Personal information about physiology and biochemistry, imaging specific molecular pathways *in vivo*, the diagnosis of the disease following treatment, testing novel tools, and the process of imaging process are very important to overcome the disease. MI is the main element associated with drug discovery and development [63], [81]. In successful imaging agent; (a) the target binding molecule and the signal

portion (or known as “label”) should not be considered separately (b) should be separated from the non-target part of the body, such as blood [253]. Not only enzyme substrates and receptor ligands, but also higher molecular weight molecules such as antibodies and proteins can be utilized as MI probes for cancer imaging (Figure 4.5) [145]. It is undeniable that acquiring molecular and physiological information with imaging technologies increases our ability to fight cancer [56].

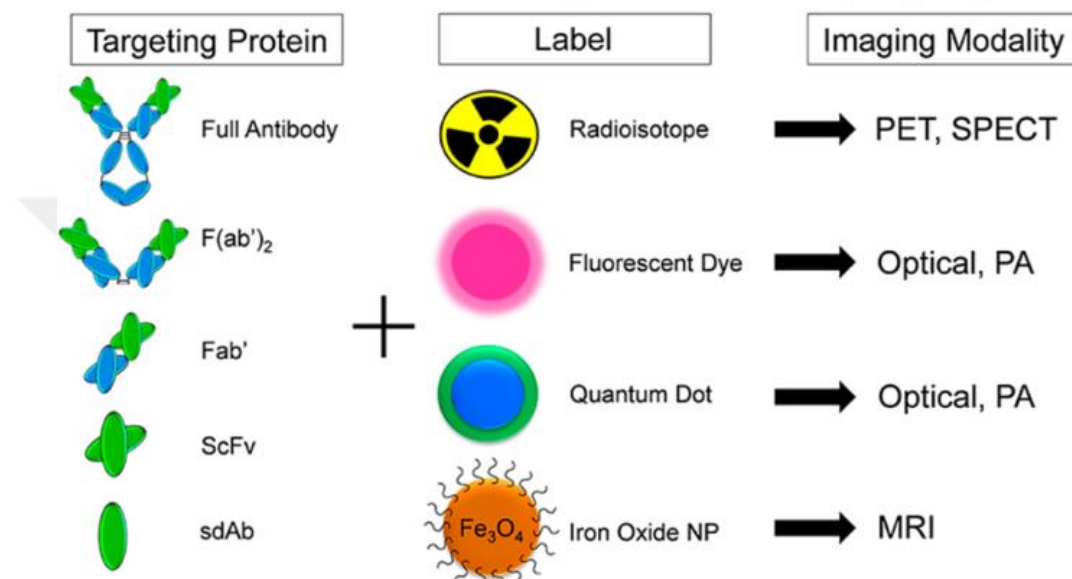


Figure 4.5 The target binding molecules and the different labels can change imaging modality. PA: photoacoustic, NP: Nanoparticles, F(ab')₂, Fab, ScFv and sdAb are some examples of antibody fragments [254]

Although molecular probes can be classified in many different ways, the most useful way is to reduce them into two basic groups, the basic radionuclide probes, and the activatable probes. The activatable probe connects (mostly physical) with its particular targets to create a measurable signal, whereas for the radionuclide probe it is not necessary to react with its particular targets for making a quantifiable signal. The radionuclide probe creates a notable signal before and after the interaction with its target while the activatable probe creates signal only in its interaction time [145]. The target of MI probes, biomarkers, can be classified according to their use purpose [255].

- Type 0 biomarkers: for imaging of the progression of the disease
- Type I biomarkers: for drug mechanism
- Type II biomarkers: for surrogation of endpoints

Presence, quantity, function, and location of diseased cells, especially cancer can be detected with target interacted probes. However, probes for MI do not have to be functional for the treatment of diseases. Nonfunctional probes like membrane lipids, extracellular peptides, and proteins can be used as a marker for disease progression. Chemical probes in MI technology given to the patient is very low concentrations for identifying ligand-target interaction without inducing any pharmacological (pharmacodynamic, pharmacokinetics, etc.) effect [255].

The imaging agent performances are based on its pharmacokinetic behaviours, signal production, and high signal-to-noise ratio. The chemical synthesis, genetic engineering production, and preclinical studies of imaging agent probes are repetitive for MI diagnostics applications [255]. The imaging probes have some crucial pharmacokinetic behaviours which can reach its target at a satisfactory amount, to overcome delivery barriers, and to remain there for enough time to detection [145].

Protein-protein interactions (PPIs) are a highly specific physical connection between more than one protein molecule. These interactions result from biochemical occurrence driven by electrostatic forces, such as the hydrophobic effect [256]. The designing of MI agents is based on PPIs. PPIs can be also defined for; particular cellular proteins connected for adjusting particular cellular functions with cellular signalling [257]. The particular cellular functions are the preeminent mechanism to figure out disease pathways and modelling tracers for these peculiar diseases. PPIs are altered dramatically by metabolic diseases such as cancer. Probe-biomarker interaction process can resemble PPIs pathways for developing this tracer [63].

The tumour microenvironment has many cell-surface receptors that may be possible biomarkers for tumour cells. Some cell-surface receptors may increase while some cell-surface receptors may emerge as completely new proteins such as PSA [258]–[260]. The development and radiolabeling of a new chemical probe against this cancer-related cell surface receptor are among the needs of medical fields.

4.2.1 Peptides for Cancer Imaging Agent

Peptides are organic substances composed of amino acids linked by covalent bonds, also known as amide or peptide bonds, and may exhibit hormone or drug-like activities. Peptides play an important role in the metabolic functions of living organisms and therefore, human health [261].

Peptides are small molecular structures that cause various effects at the cellular level through receptors in humans. Physiologically, the peptides bind to specific receptors in the cell membrane and act through the G-protein [262]. Peptides can be natural ligands to surface receptors. Small peptides have high selectivity and high affinity for cell-surface proteins, thus they supply effective internalization and selective binding to interested cells. Due to these characteristics, peptides can be used for cell-targeting or cell-penetrating molecules [63]. The high expression of specific peptide receptors in various tumour cells compared to normal tissues is the basis for the use of radiolabeled peptides as tumour imaging agents in nuclear medicine. Compared to large molecular structures such as monoclonal antibodies and other proteins, peptides are easy to migrate to all tissues except the brain because of their small size [262].

Since endothelial cell surface receptors are significantly increased in diseases, both imaging and treatment are achieved by targeting carriers to receptors [263]. In malignant tumours, angiogenesis is essential for tumour viability and growth. Because of angiogenesis, $\alpha v \beta 3$ integrin is a very common expression in tumour vascular endothelium. Specifically, peptide coated carriers such as $\alpha v \beta 3$ integrin can be reached to the vascular endothelium of the tumour. Experimental studies have shown that the systemically injected nanoparticle- $\alpha v \beta 3$ integrin complex is successfully targeted to the tumour and the tumour shrinks as a result of vascular endothelin apoptosis [264].

Somatostatin and vasoactive intestinal peptide analogues have been used successfully for imaging various tumours. In nuclear oncology, radiolabeled analogues of many peptides such as substance P, bombesin, cholecystokinin-B/gastrin and neurotensin have been synthesized for use in imaging [265].

^{111}In (Indium-111)-octreotide was the first developed labelled peptide. Octreotide, lantreotide and depreotide, which are somatostatin receptor (SSTR) analogues, are

labelled with different radionuclides (^{111}In , $^{99\text{m}}\text{Tc}$ (Technetium-99m)) and used for scintigraphic imaging. SSTR levels (especially SSTR2) increased significantly in breast cancer, lymphoma and neuroendocrine tumours [266]. Accordingly, primary or metastatic tumours showing intense SSTR expression can be imaged using radiolabeled somatostatin analogues. Intensive clinical studies show that the major indication of SSTR scintigraphy is the diagnosis and staging of gastroenteropancreatic neuroendocrine tumours. However, in lung cancer, ^{111}In octreotide with SCLC [267]; both SCLC and NSCLC [268] can be visualized with ^{111}In lantreotide. Another area using ^{111}In -octreotide scintigraphy is the demonstration of SSTR2 receptor expression in patients scheduled for octreotide treatment. The presence of octreotide uptake in the tumour in the scintigraphic examination is an indication that treatment will be effective [269].

$^{99\text{m}}\text{Tc}$ -depreotide is used for noninvasive staging of patients suspected of lung cancer preoperatively. The $^{99\text{m}}\text{Tc}$ -depreotide has been approved by the FDA for use as an adjunct diagnostic method in the assessment of SPN. As a SPECT agent, it may be preferred for ^{18}F -FDG-PET imaging in this clinical indication because SPECT cameras are more common and cost-effective [270]. In a multi-centre phase III study on the use of $^{99\text{m}}\text{Tc}$ -depreotide in the evaluation of SPN; histological examination revealed pathological depreotide involvement in 85 of 88 patients with malignant lesions. However, scintigraphy in 7 patients, 6 of which were granulomas and one hamartoma, gave false-positive results and sensitivity and specificity values for $^{99\text{m}}\text{Tc}$ -depreotide were 97% and 73%, respectively [271]. In a similar study, the same values in a smaller patient group were 93% and 88%, respectively [272].

Cholecystokinin-B (CCK-B) receptor is expressed in Small Cell Lung Cancer (SCLC) cells. ^{111}In labelled gastrin-derived peptides ([D-Asp26,Nle28,31]CCK26-33 (D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂)) have been successful for imaging tumours showing CCK-B receptor expression both *in vivo* and *in vitro* trials [273], [274].

Today, PET/CT, which is made with somatostatin analogues labelled with ^{68}Ga isotope in NET imaging, has started to replace ^{111}In -DOTA-octreotide. The ^{68}Ga isotope has a half-life of 68 minutes and is a $^{68}\text{Ge}/^{68}\text{Ga}$ generator product. Three different somatostatin analogues with ^{68}Ga labelling are used in clinical applications. ^{68}Ga -labeled somatostatin analogues commonly used in routine; DOTAD-Phe-Tyr3-

octreotide (DOTA-TOC), DOTA-1-Nal(3)-octreotide (DOTA-NOC) and DOTA-D-Phe-Tyr3-octreotate (DOTATATE). Studies have shown that the highest affinity for the SSTR2 which is the most commonly expressed in NETs, is DOTATATE. Reubi *et al.* reported that the SSTR2 affinity of DOTATATE was ten times higher than that of DOTATOC and DOTANOC. For these reasons, ^{68}Ga -DOTATATE is widely used in both routine clinical practice and research and is licensed in the United States [275].

According to preliminary results in the literature, PET/CT with ^{68}Ga -labeled somatostatin analogues can detect NETs with 87% accuracy. In current reviews, the sensitivity of PET/CT with ^{68}Ga -DOTA somatostatin analogues was shown to be 88-93% and specificity was 88-95% for NET diagnosis. In pancreatic NETs, this rate was found to be between 92% and 83%, whereas sensitivity for detecting bone metastases was 97-100% and specificity was 92-100% [276]–[279].

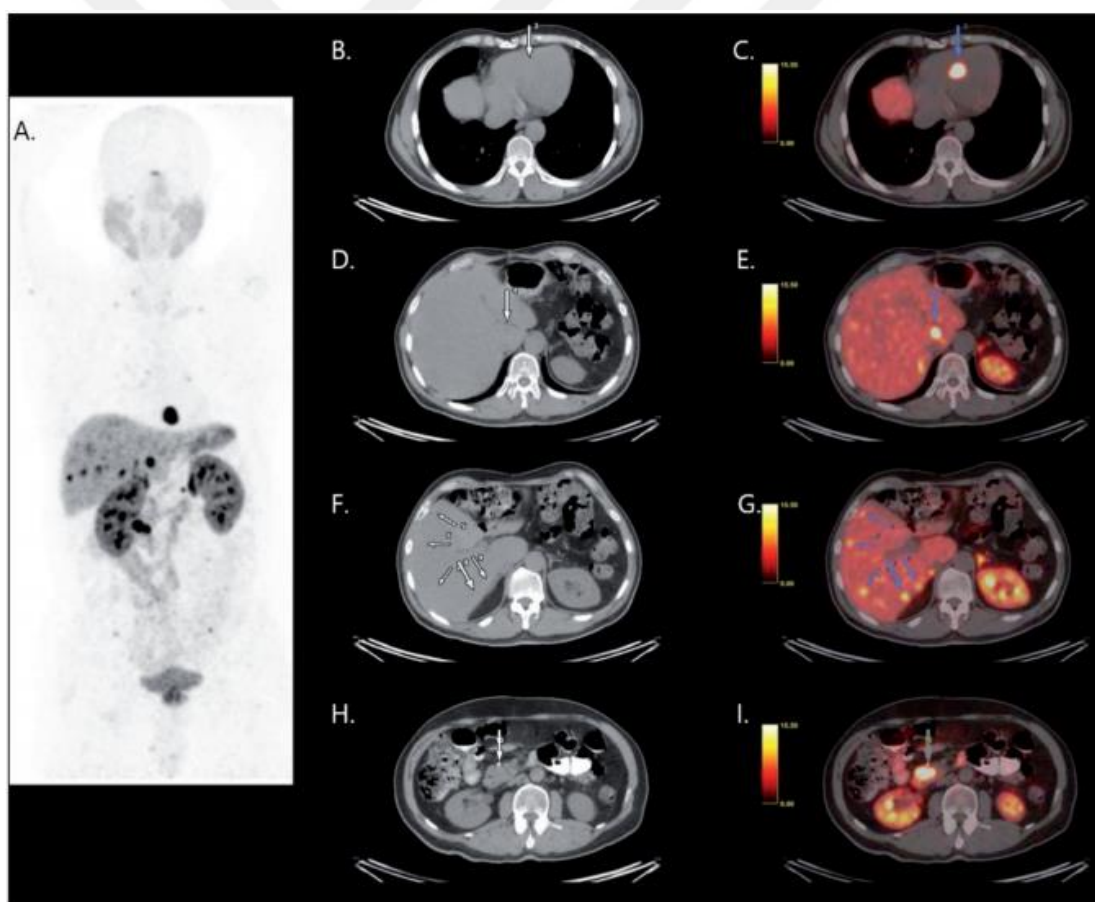


Figure 4.6 Intensive ^{68}Ga -DOTATATE involvement due to the expression of somatostatin in the primary pancreatic lesion (e, f), interventricular septum in the heart (b, c), both lobes of the liver (a, d, g) in a 58-year-old WHO Grade 3 NET patient is monitored. a, maximum intensity projection; b, d, f, h, Axial CT sections; c, e, g, i, Axial PET/CT fusion sections [275]

In a study performed with bifunctional micro PET/MR, ^{64}Cu labelled polyaspartic acid (PASP)-iron oxide (IO) nanoparticles were bound with DOTA and cyclic arginine-glycine-aspartic (RGD) peptides to monitor the distribution of integrin. It has been shown that tumour cells in stage 2 can be detected accurately [280].



5.1 Peptides and Aminoacids

Peptides (Greek *πεπτιδα*, "digested") are short polymers formed by the binding of α -amino acids in a defined order. The bond between one amino acid residue and another amino acid residue is known as an "amide bond" or "peptide bond". The simplest peptides are dipeptides (consists of two amino acids), followed by tripeptides (consists of three amino acids), tetrapeptides, oligopeptides, and polypeptides [281]. Structures consisting of more than 100 amino acids are called proteins. Peptides may exhibit different properties according to the functional groups and the sequence of amino acids present in their structures [282].

The term "amino acid" generally refers to an α -amino carboxylic acid [283]. 20 amino acids are the building blocks of all peptides and proteins in nature. Although almost all 20 amino acids have a common backbone, their side chains (R) differ. Thanks to these side chains, each protein gains a different structure and function [284], [285]. 20 amino acids (in fact, 19 α -amino acids and 1 α -imino acid) are used in living cells for protein synthesis in the control of genes. These 20 amino acids have a very special place because they are the most basic structural elements of peptides and proteins in all life forms. More than 700 amino acids have been identified in nature, most of which are α -amino acids. Almost all of these are obtained from bacteria, fungi and algae, either free or bound to large molecules (as a component of peptides or proteins or as part of other structures) [282].

The first carbon atom closest to the carboxyl group is called the α -carbon atom. Because the amino group in all amino acids is bound to α -carbon, it is called α -amino acid. The α -carbon of all amino acids except glycine is asymmetric carbon [286], [287]. Figure 5.1 represents the α -amino acid structure.

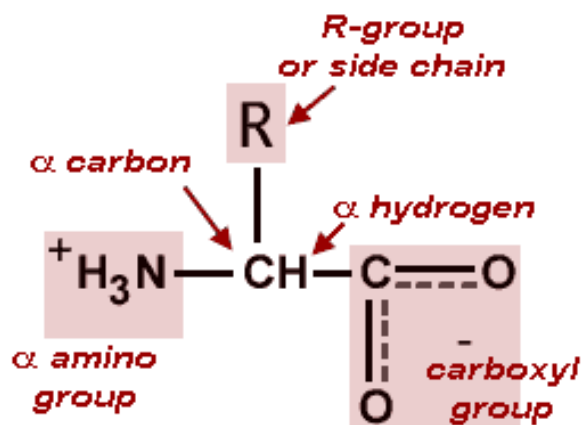


Figure 5.1 The general formula of an amino acids

Peptides and proteins are involved in many biological, metabolic and physiological processes within organisms. They play an important role in the transport of various substances through biological membranes, intercellular communication, the formation of hormones and neurotransmitters, and the formation of antibodies in the immune system [13]. Peptides have many health benefits, such as regulation of immune systems [288], lowering blood pressure [289], [290], cholesterol lowering effect [291], antithrombic [292], antioxidant therapy [293], positive effect in the diabetes [294], [295], opioid activity (morphine-like properties, showing positive effects on the nervous system) [296], increasing mineral absorption [297] and/or increasing its bioavailability [298]. Some peptides are multifunctional and have multiple positive effects on human health [299]. Besides, the enormous role of the peptides in physiological and biochemical processes has led to an increase in their potential drug nomination [300].

Peptides can be synthesised by chemically, whereas proteins can be synthesised by recombinant technology or purification from many organisms (Figure 5.2). There has been an increase in peptide research over the last 10 years. According to PubMed results between 2008 and 2018, an average of 120,000 articles is published annually in the field of peptide research [301].

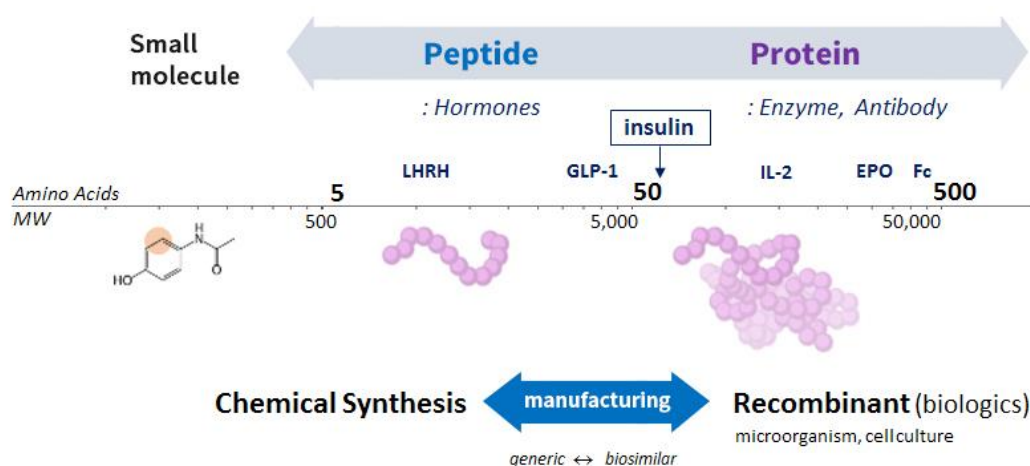


Figure 5.2 Scheme of amino acid, peptide and protein structures [302]

Especially since 1980, it is possible to list the reasons that encourage research on biomedical research into peptides and proteins [302];

1. With the development of analytical methods, it was possible to discover new hormones and peptides.
2. As a result of the development of solid-phase and conventional solution-phase techniques, synthetic oligopeptides could be obtained.
3. Molecular biology and genetic engineering have enabled the production of many polypeptides that have not been achieved by synthesis or isolation to date.

Peptides can generally be obtained by 3 methods [303].

1. Extraction methods from natural sources,
2. Genetic engineering methods,
3. Chemical synthesis methods

Each of these methods has its advantages and disadvantages. Only chemical peptide synthesis allows the incorporation of unnatural amino acids and the production of large amounts of pure peptides [287], [303]. The majority of synthetic peptides are of commercial importance and are used in pharmaceutical products. More than 400 synthetic peptides have been used in clinical trials. Chemical synthesis of peptides is of interest because it is faster, more efficient and reliable than other methods [304].

5.1.1 History of Peptide Synthesis

The history of peptide synthesis begins in 1881. Theodor Curtius with H. Kolbe obtained the first N-protected dipeptide, benzoylglycylglycine, by reacting the silver salt of glycine with benzoyl chloride by azide coupling during his doctoral dissertation [305]. Emil Fischer studied on peptide synthesis in 1901 [306]. The first synthetic dipeptide, glycoglycine, was synthesized by Emil Fischer by hydrolysis of diketopiperazine of glycine and is considered as the beginning of peptide chemistry [303], [307]. In the following years, chemists' increased interest in peptide synthesis has continued. In 1932, the $N\alpha$ protecting group Carboxybenzyl (Z, Cbz) was developed [308]. In 1953 du Vigneaud synthesized the Oxytocin hormone, a cyclic peptide [308]. With this work, du Vigneaud was awarded the Nobel Prize in Chemistry in 1955 [309].

Peptide synthesis is divided into two categories. One of them is the classical solution method and another is the solid-phase method. Classical methods, 20th century, is a method that has been used since the beginning. In recent years, even though the synthesis of short peptide sequences by solution method remains important in the pharmaceutical sector, peptide synthesis by Solid Phase Peptide Synthesis (SPPS) method (especially in long peptide sequences) has become widespread [310]. Peptide synthesis in the first years was carried out in the solution phase. In 1962, Bruce Merrifield developed a new groundbreaking SPPS method [311]. He was awarded the Nobel Prize in Chemistry in 1984 [312]. Merrifield also developed the first semi-automatic peptide synthesizer [282].

The SPPS method consists of three basic steps as shown in Figure 5.3. According to this; Deprotection, activation of the carboxyl group (Activation), and peptide bond formation (Coupling). Following these procedures, the final deprotection of the last added amino acid is removed and the N-terminal is released. In the last step, the cleavage of the protecting groups (cleavage), and the separation of the resin-bound peptide from the solid support (deprotection), and the completion of the peptide synthesis ends [313]–[315].

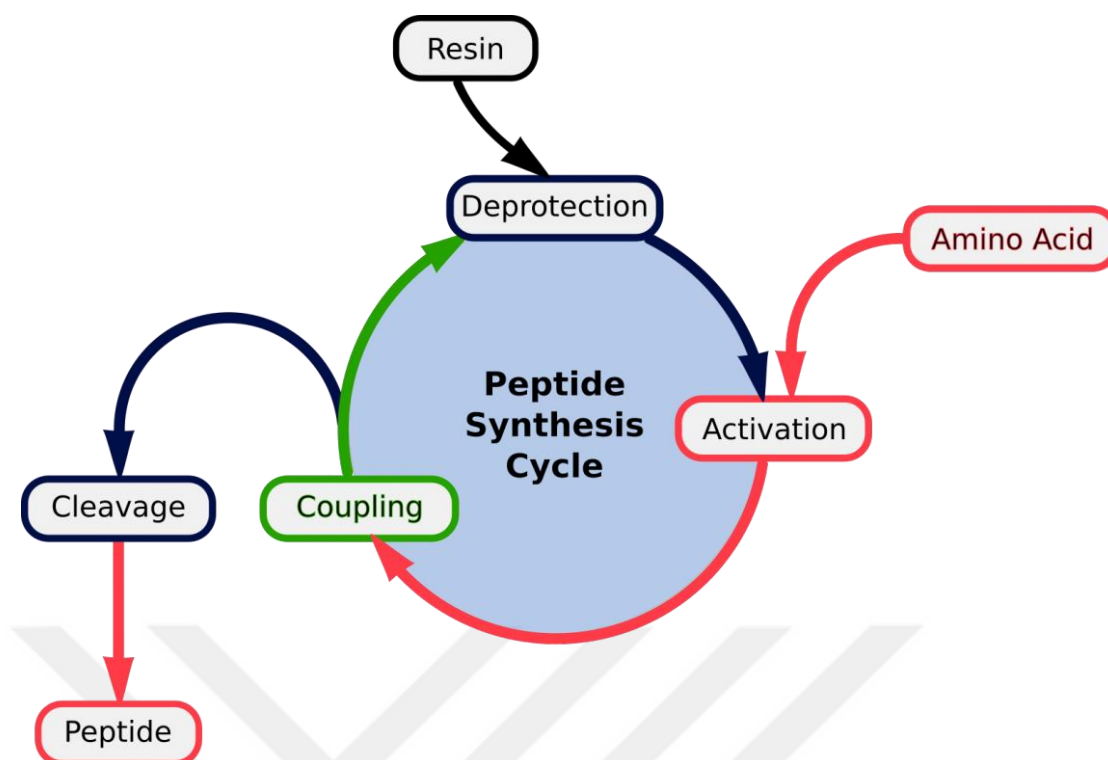


Figure 5.3 A short view of peptide synthesis. Repeated cycles of (1) Deprotection; (2) Activation of the aa; (3) Coupling [316]

In the method developed by Merrifield, it is necessary to use (Trifluoroacetic acid) TFA to remove *t*-Boc (*tert*-butoxycarbonyl), the protecting group at the N-terminus ($N\alpha$) at each amino acid addition [317]. TFA is a very powerful chemical and its vapours in the air form hydrogen fluoride (HF), a hazardous chemical for health [285]. These difficulties have been remedied to some extent by the use of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group, a more easily removable $N\alpha$ protecting group, in peptide synthesis [318]. Since the Fmoc protecting group is sensitive to bases, it can be easily removed with bases such as piperidine and piperazine (Figure 5.4 and 5.5) [282], [319]. The synthesis of the Fmoc protecting group under more mild conditions compared to the *t*-Boc method has made the Fmoc protecting group synthesis advantageous in routine synthesis [320].

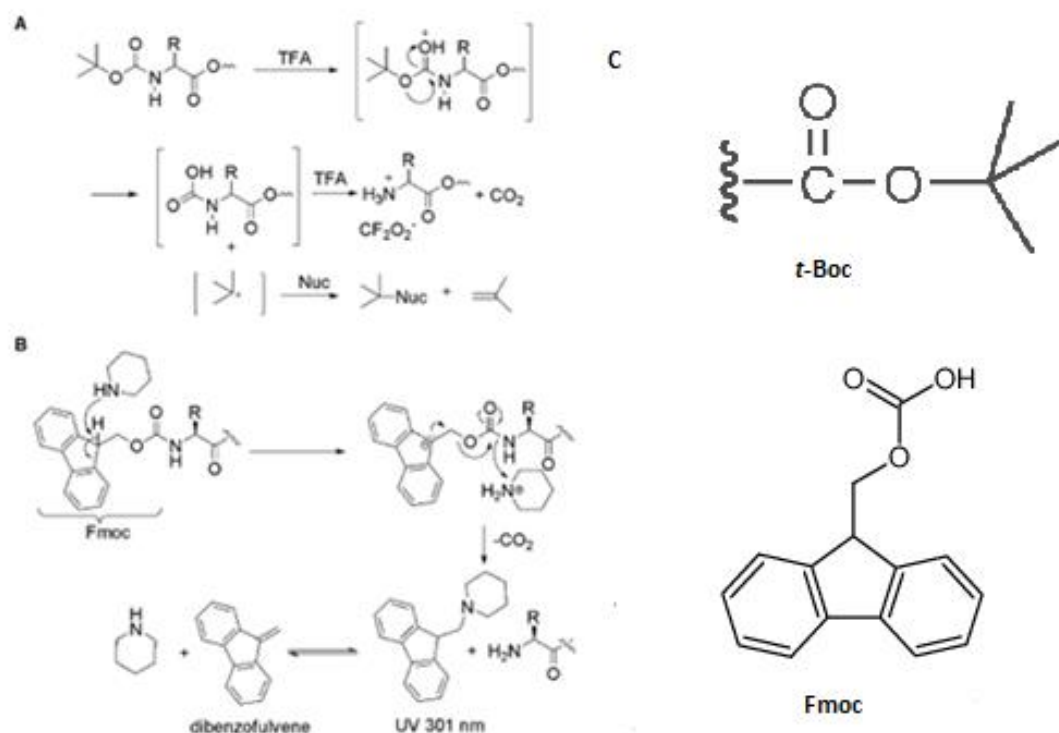


Figure 5.4 Deprotection scheme of (a) *t*-Boc deprotection strategy (b) Fmoc deprotection strategy. (c) Chemical structures of *t*-Boc and Fmoc N α protection groups [282], [320]

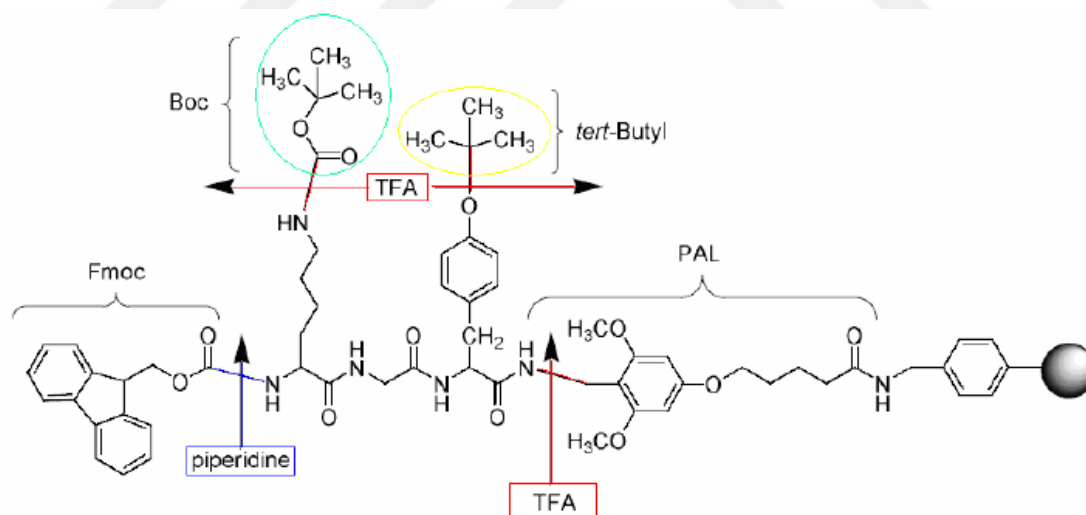


Figure 5.5 Fmoc/*t*-Boc protecting groups strategy in SPPS. Piperidine cleaves Fmoc group while TFA cleaves *t*-Boc, *tert*-Butyl and resin [287]

5.2 Mechanism of Peptide Synthesis

Peptide synthesis is categorized in two ways: the solution phase synthesis (SPS) and the solid-phase peptide synthesis (SPPS) method [321].

5.2.1 Solution Phase Synthesis (SPS) Method

In the “classical” SPS method, an α -amino protected and α -carboxyl group activated amino acid and an amino acid having a free amino group and protected α -carboxyl group react to form a dipeptide as shown in Figure 5.6. The α -amino protecting group is then selectively removed from the dipeptide and the next α -amino-protected amino acid is an activated α -carboxyl group is then coupled to form a tripeptide. This process is continued until the desired peptide sequence is obtained. All protective groups are removed in the last step [322]. The advantage of this method is that the resulting intermediates (missing sequence peptides) can be isolated and purified after each synthesis step, and the deprotection groups can be removed. Besides, the desired sequence is recombined with the previously synthesized small peptides to obtain larger peptides. This technique is highly flexible concerning the chemistry of coupling and the combination of the peptidic blocks [321]. The pharmaceutical companies used the SPS method especially between the 1970s and 1980s [323]. By the SPS method, high purity peptides can be synthesized as mentioned above, however, the SPS method has some indestructible disadvantages. The SPS method is quite slow, laborious and time-consuming. To successfully achieve a high purity peptide, it is necessary to incorporate amino acids in a step by step manner starting from the peptide's C-terminus end. After each amino acid has been added, the intermediate peptides need to be separated, purified and characterized. As chain length increases, the yield of intermediate peptides decreases, and solubility decreases. Besides, the solubility may decrease as the peptide chain lengthens. This causes problems both in purification and in the next binding step. As a result, the elongation of the peptide chain may end [324], [325].

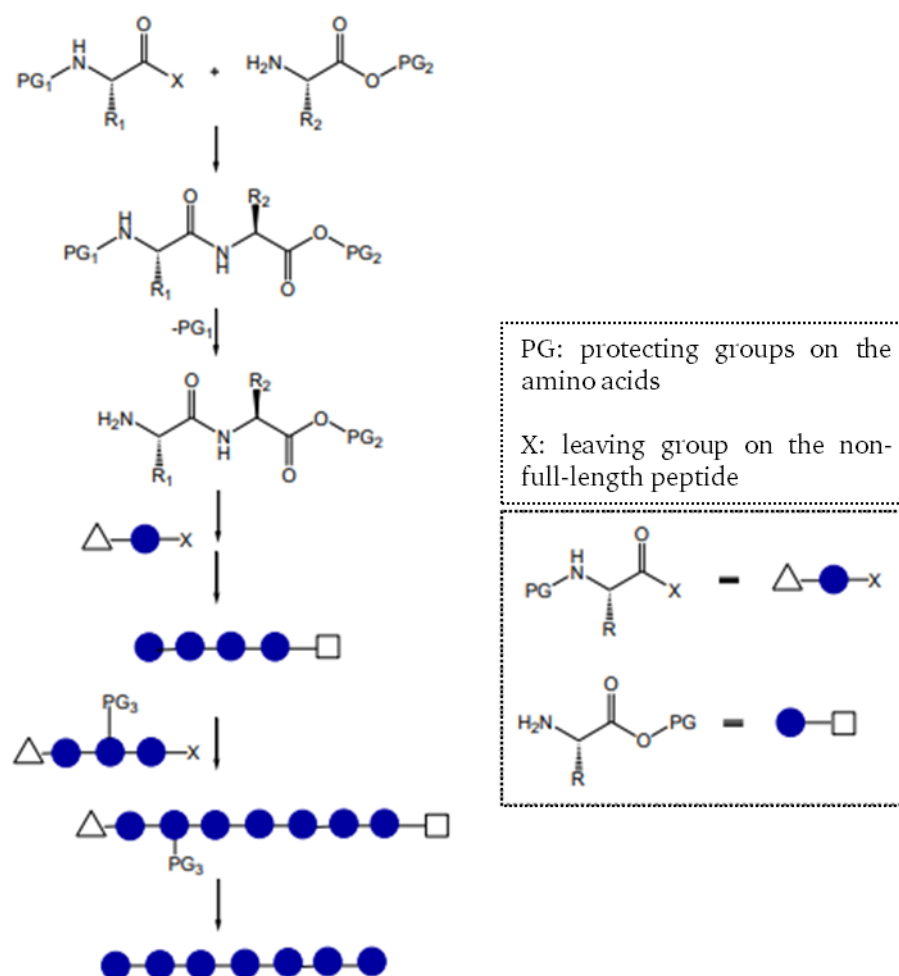


Figure 5.6 The classical solution phase peptide synthesis method will be produced with most protecting groups. All non-reacting functional groups in the peptide chain are reversibly protected. These groups are removed in the final step for synthesizing of the demanded and designed full-length peptide [322]

5.2.2 Solid-Phase Peptide Synthesis (SPPS) Method

Solid-phase peptide synthesis (SPPS), which is the most widely used method in peptide synthesis, was described by Merrifield in 1963, and has become a reliable method and was reported to be about fifty times less laborious than conventional solution peptide synthesis [321], [326]. The main difference between SPPS and SPS is that the peptide synthesis is performed on a special solid support, also called a resin on the SPPS method [15]. In this method, the reaction can be automated, the problem of solubilization of the peptide is eliminated and loss is prevented since the reaction is carried out in a single vessel [321], [326].

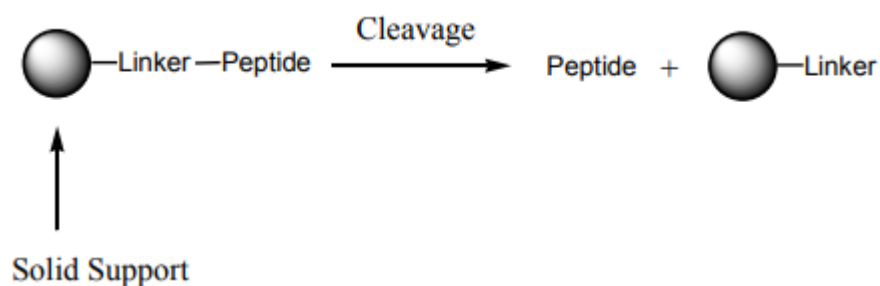


Figure 5.7 Schematic representation of an SPPS [323]

The principle of SPPS is quite simple. SPPS is formed by the elongation of a peptide chain bound to inert a solid support (resin, solid phase) by a linker and cleavage of the peptide from the solid support when the desired sequence is reached as shown in Figure 5.7 [321].

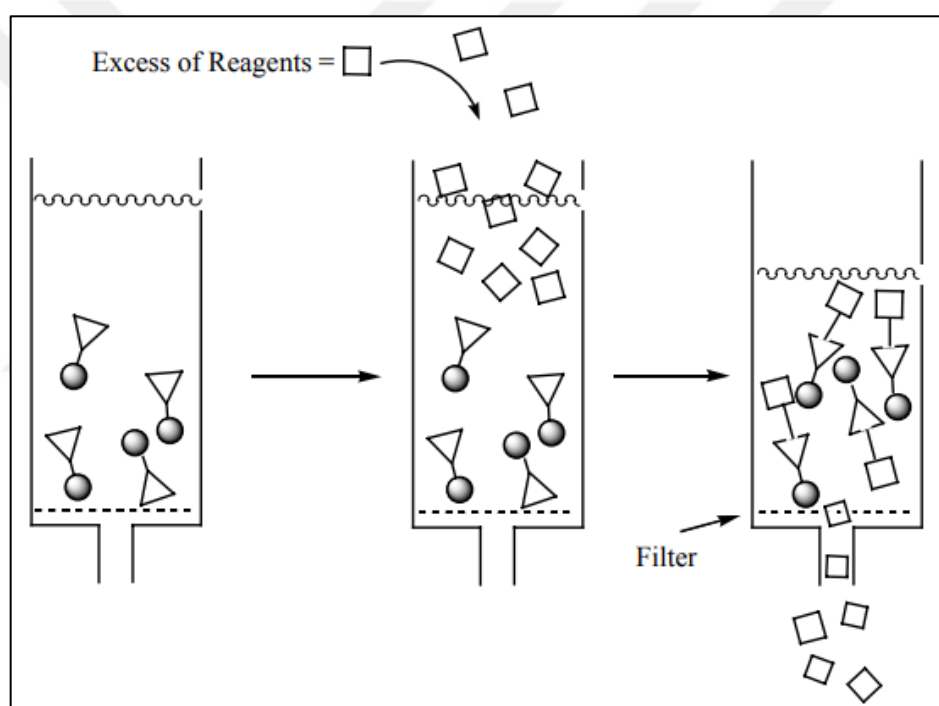


Figure 5.8 A representation of purification of compounds bound to the solid support (peptides with bound protection groups) from excess reagents (unbounded amino acids, activators etc.) in solution by simple filtration [323]

The growing chain (which may be a peptide or other type of oligomer) is attached to a stable and solid particle (solid phase, resin). During the synthesis period, the growing chain remains bound on the resin, and other soluble chemicals are removed by filtration and washing (Figure 5.8). In the last step, the desired product is separated from the solid phase (from resin). The purification and characterization are continued in the free solution of the desired product [319], [327]. All reactions

that occur during peptide synthesis take place in a single reaction vessel, which provides avoiding possible material losses that may occur during the transfer of the reaction vessels [328].

SPPS generally uses about 100-200 mesh (150-75 μm) polystyrene resin as the solid phase. The peptide chain is elongated by the addition of amino acids on this resin. In this method, the amino acid side chains (R) and the N-terminus of the amino acids are also protected by protecting groups. With protecting groups, both the side chains of the amino acid are protected from reaction with each other and the sequence is correct (ie, only 1 amino acid extension of the sequence is provided at each amino acid insertion) [282].

In the SPPS method, the direction of synthesis takes place from the C-terminus to the N-terminus of the peptides and consists of a total of 5 steps as shown in Figure 5.9 [327], [329], [330].

- **Step 1 - Attaching of an amino acid to the polymer.** The first step is to bind the first amino acid at the C-terminus to the solid support at the $-\text{COOH}$ terminal.
- **Step 2 – Activation.** The second step is an activation of the carboxyl group of the penultimate amino acid at the C-terminus of the sequence to be synthesized.
- **Step 3 – Coupling.** The third step is the coupling of the carboxyl end of the amino acid to be added to the sequence with the amine end of the amino acid attached to the solid support to form the amide peptide bond. After that, washing steps takes place for removing excess reagent and by-products. The second and third steps are repeated each time the amino acid is added.
- **Step 4 – Deprotection.** In the fourth step, the N-terminus can be released by removing the last deprotection of the last added amino acid. If this is not done, the N-terminus of the peptide sequence remains inactive until the deprotection is removed. As a fourth step, acetylation (Capping) can also be performed. By acetylation, the N-terminus of the peptide is acetylated and protected.

- **Step 5 - Polymer removal and achieve full-length peptide.** The final step can be summarized as the cleavage of the peptide from the resin and the deprotection of the protecting groups.

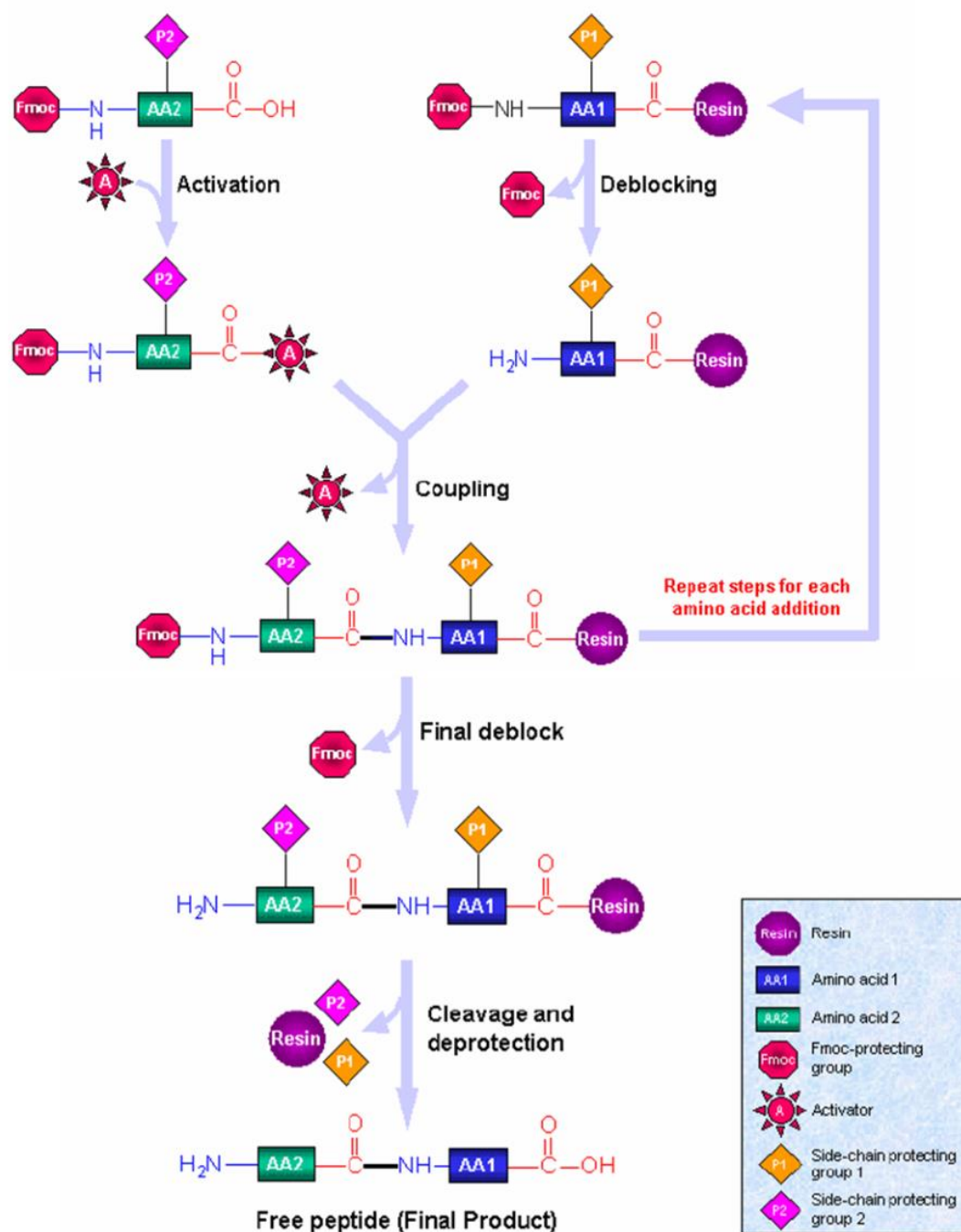


Figure 5.9 General reaction mechanism of SPPS by Fmoc chemistry [331]

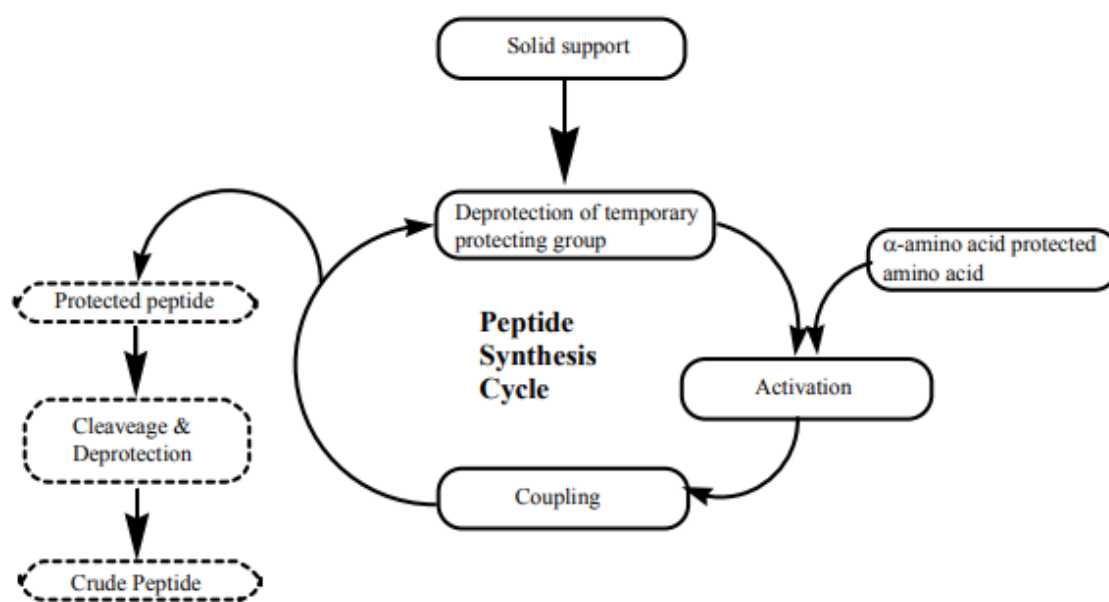


Figure 5.10 A general representation for the synthesis of peptides by SPPS method [332]

The resin is small and spherical shape polymer-based beads that carry some special functional groups to which the first amino acid of the desired sequence to be synthesized can be attached. Resins are commonly used in a size of about 100–200 mesh (75–150 μm) or 200–400 mesh (35–75 μm) in peptide synthesis [15]. The solid phase used in SPPS (resin) must be chemically inert, mechanically stable, have a narrow range of resin bead sizes, insoluble in any solvents used and easily filterable. Resins are like a sponge. Dry resins should swell up to 5–6 times their size in solution such as Dichloromethane (DCM), N,N-Dimethylformamide (DMF) before first coupling step [15], [282], [333]. The most common resins are polystyrene (PS), polyamines (poly-N,N-dimethyl acrylamide) (PDMA), poly(N-acryloyl pyrrolidine) [poly(APr)], poly(N-acryloyl morpholine) (PNAM), etc.), polyethylene glycols (PEG)-PS, pure cross-linked PEG resins and silica [15], [331], [334]–[336]. Most PS resins contain cross-linked with 1% of divinylbenzene (DVB) which provides an insoluble and swelling feature in all common solvents [15]. The resin swelling is an important matter in organic solvents, and it may change with different organic solutions (Table 5.1). Because swelling has a high impact on diffusion and accessibility of the reagents into the core of the polymer which affects reaction yield and it lets to expand and maximize the exposure of their functional groups to the incoming reactants. The most common solvents for Fmoc SPPS are DMF or N-methyl pyrrolidone (NMP) [333].

Table 5.1 The typical swelling factor for a 1% DVB cross-linked PS resin in different organic solvents and water [320], [335]

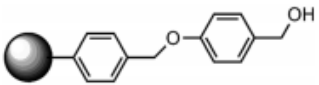
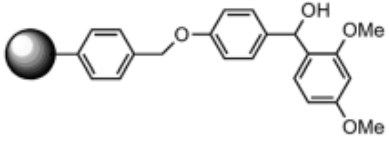
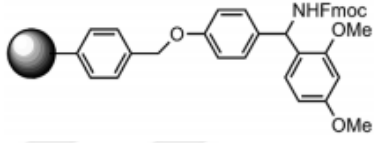
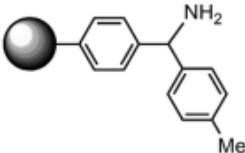
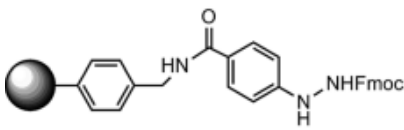
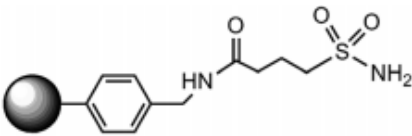
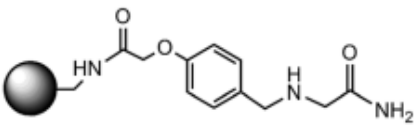
Organic Solvent	Swelling Times
Tetrahydrofuran (THF)	5-6
Toluene	5-6
DCM	5-6
1,4-Dioxane	5-6
DMF	4
Ethanol	2
Acetonitrile	2
Methanol	1,6
Distilled water	0

The resins used in the synthesis may be pre-loaded and unloaded. The term loading is used to bind the first amino acid to the resin. It may be advantageous to use loaded resin. Because in the loaded resins, the first amino acid of the sequence is present on the resin. Adding the first amino acid to the resin has a very difficult procedure. Loaded resins are of two types: lower loaded and higher loaded. This property due to loading is very important for synthesis. This is because, when a highly loaded resin is used, the aggregation of peptide sequences growing on the resin (particularly susceptible to long and hydrophobic interaction) will increase and this will lead to an undesired amino acid deletion. Therefore, less loaded resins are generally preferred [249], [282].

The amide bond is the focal point for the prevention of proteolytic degradation in peptides. The risk of exposure of an amide bond to enzymatic attack depends on the environment in which it is located and the amino acid side chains around it. Peptidases need a charge bearing group to recognize the peptide substrate. For example, aminopeptidases need a free amino group. N-Acylation, N-alkylation or C-

amidation is usually sufficient to block the effect of this enzyme because of reducing the overall charge of a peptide [337]. N-Acylation, N-alkylation or C-amidation produce a closer imitation of the native protein. These modifications in the terminal increase the stability of the peptides. Modifications at the C-terminus can easily be made with commercial resins [338].

Table 5.2 Different resins for SPPS method [320]

Resin Name	Resin Structure	Peptide product
Wang resin		Acid
Rink acid resin		Acid
Rink amide resin		Amide
MBHA resin		Amide
Hydrazine resin		Acid, ester, amide
Sulfonamide resin		Acid, ester, thioester, amide, etc.
PEGA-BAL resin		Acid

Since the resins have been used for the first time, they can be modified with structures called “linkers” to meet the requirements of the final product and, due to changes in the synthesis and cleavage stage. A lot of different linkers for PS core resin have been evolved in the last years. The linkers can be cleaved from the resin under certain conditions to form the final peptides product [320], [333]. Every linker modified resin has peculiar cleavage conditions (TFA, HF, THF, etc.) and after cleavage, final C-terminal functionalization is obtained. C-terminal can remain carboxylic acid, amide, ester or alcohol depending on the type of the linker on the resin beads (Table 5.2) [320].

The success of peptide synthesis depends on the activity of the activators selected for the extension of the peptide chain by the arrangement of temporary (Fmoc, *t*-Boc) and permanent (side chain protecting groups) protecting groups [282]. The α -carboxyl group of the amino acid must first be activated to bind to the amino group of the previous amino acid for a rapid and quantitative amide bond formation. The excess reactant is added to accelerate the coupling reactions. Activator efficiency increased with the development of new activators over time [282], [320], [333].

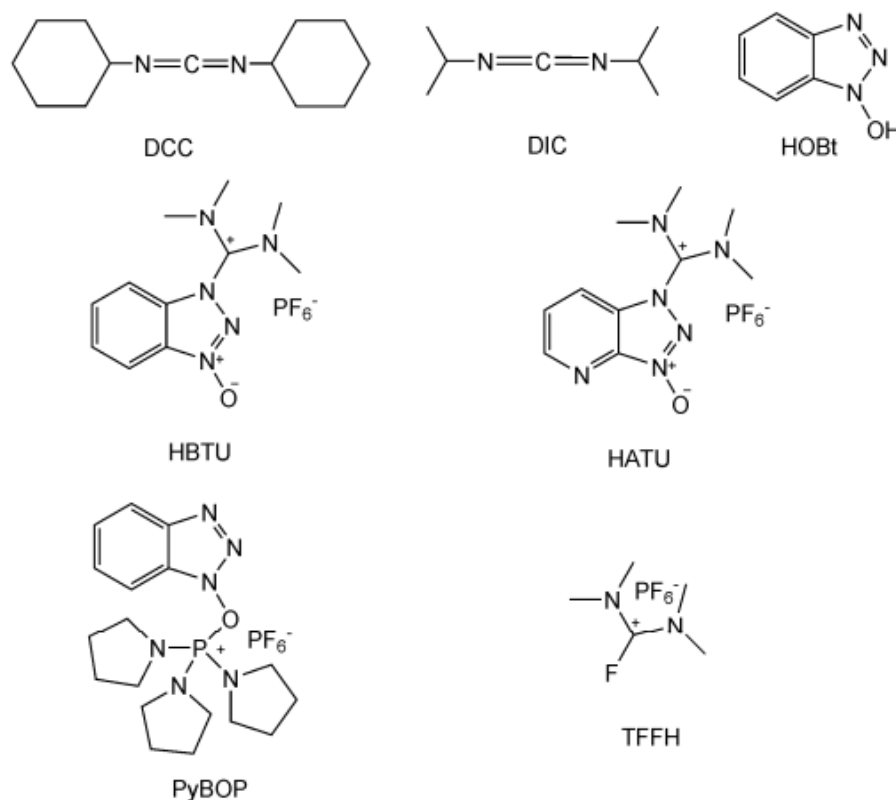


Figure 5.11 Conventional coupling reagents for carboxylic acid activators used in peptide synthesis [320]

Activation of the α -carboxyl group of the amino acid can be carried out with the carbodiimides (DCC (N,N'-Dicyclohexylcarbodiimide), DIC (N,N'-Diisopropylcarbodiimide)) as shown in Figure 5.11, as well as with newly developed activators of the Uronium (HBTU (O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate), HATU (N-((Dimethylamino)-1H-1,2,3-triazolo[4,5-b]-pyridino-1-yl)methylene)-N-methylmethan-aminium hexafluorophosphate)) and Phosphonium (PyBOP (Benzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate)) type. Nowadays, these new types of activators are preferred because they cause less racemization. DCC is a carboxyl activator used first by Merrifield [339]. Later, it is the most popular and simple used carboxyl activator used in SPPS. However, dicyclohexylurea, a byproduct of DCC, is insoluble in most organic solutions. This makes it a suitable activator for the SPS method, but not SPPS due to solubility of resin. Instead of DCC, DIC can be used in the SPPS method, because the urea by-product is dissolved in the most organic solution. However, a partial racemization process, loss of chiral integrity of the amino acid, was observed [320].

These problems during the binding of carbodiimides have led to the development of new activators. In this context, the so-called onium salts, such as aminium, uronium, and phosphonium salts have been developed as new carboxyl activators. The most commonly used salts are HBTU, PyBOP, and HATU. The mechanism of the α -carboxyl group of the amino acid activation with HBTU is presented in Figure 5.12 and 5.13 [320].

During the α -carboxyl group activation, proton removal from the carboxyl end of the amino acid is required to initiate the reaction. N,N-Diisopropylethylamine (DIPEA) is a base and used to deprotonate the acid group of the Fmoc-bounded amino acid. So, they are good acid or proton scavenger. Since DIPEA consists of central nitrogen attached to an ethyl group and two isopropyl groups, only small electrophiles, such as protons, can react with the nitrogen lone pair [340], [341].

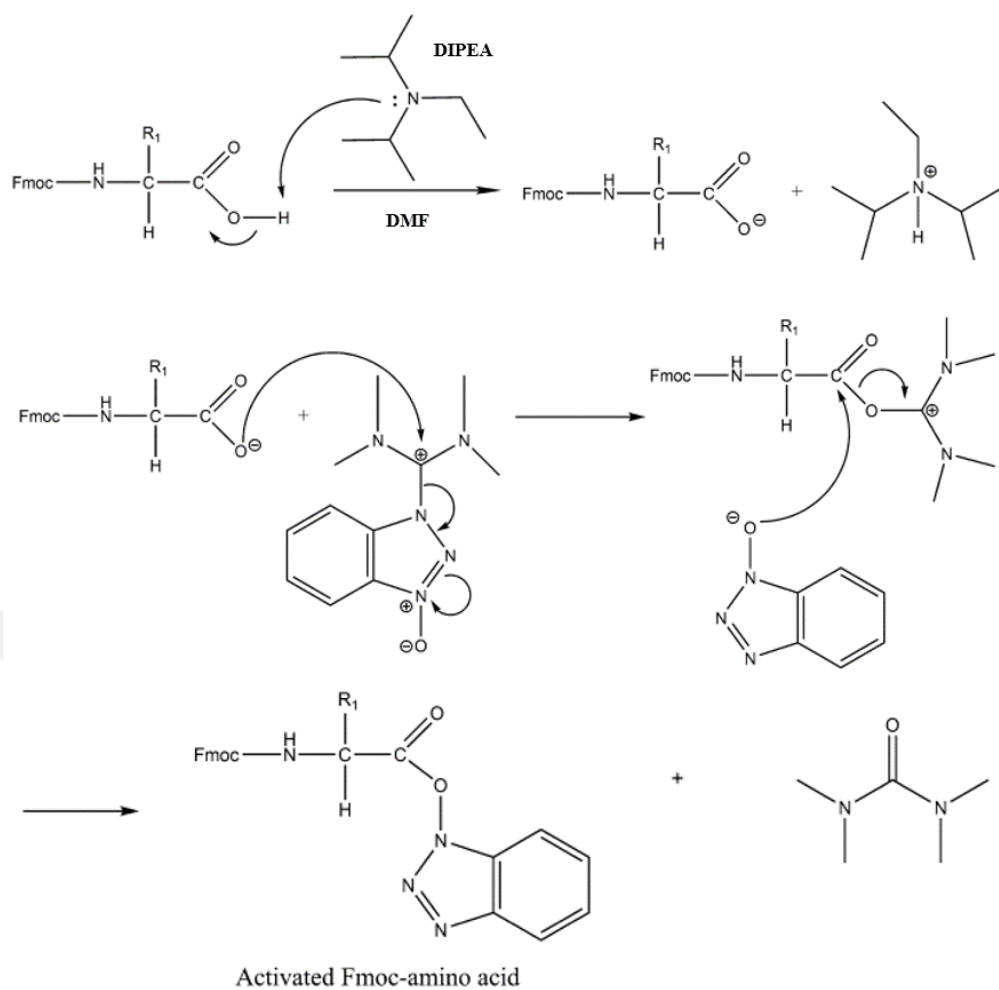


Figure 5.12 Mechanism for the activation reaction of the Fmoc-amino acid with HBTU and DIPEA [340]

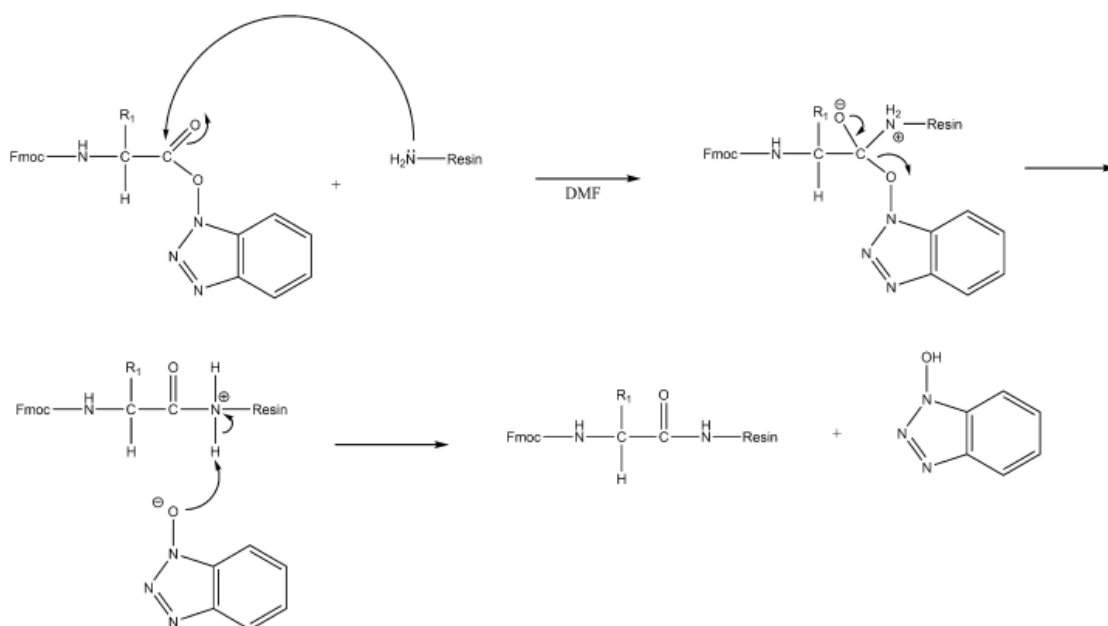


Figure 5.13 Mechanism for coupling the activated Fmoc-amino acid to the previous amino acid or resin [340]

6.1 Devices Used in Experimental Studies

- Thermo Fisher Scientific Q Exactive Orbitrap LC-MS/MS brand Liquid Chromatography-Mass Spectrometry (LC-MS) was used for characterization of synthesized peptides.
- Waters brand preparative Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) was used for characterization and purification of synthesized peptides.
- Shimadzu brand analytical RP-HPLC was used for characterization of synthesized peptides
- Lyophilizator was used for freeze-drying of samples.
- GFL brand pure water device was used for supplying pure water to be used in studies.
- Eppendorf Micropipettes (1-10 mL, 100-1000 μ L, 5-50 μ L) were used for measuring and taking liquid samples
- Precisa brand analytical balance was used for highly sensitive and accurately measuring solid samples mass.
- KNF brand Vacuum system was used for the separation of excess chemicals during peptide synthesis.
- Refrigerator was used for refrigeration of the samples to use further application
- Heidolph brand vortex and Protech brand sonicator were used for agitation particles in a sample

6.2 Chemical Substances Used in Experimental Studies

Table 6.1 The chemicals used in the experiment

Name of Chemical	Company	Catalog Number
4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl- (Rink amide MBHA resin) (with Norleucine) (0.3-0.8 meq/g, 200-400 mesh)	Chem-Impex International	03642 (Cas No: 431041-83-7)
N ^α -Fmoc-N ^ω -(2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl)-L-arginine (Fmoc-L-Arg(Pbf)-OH) ≥ 99.9%	Chem-Impex International	01964 (Cas No: 154445-77-9)
N ^α -Fmoc-N ^{im} -trityl-L-histidine (Fmoc-L-His(Trt)-OH) ≥ 99.7%	Chem-Impex International	00713 (Cas No: 109425-51-6)
Fmoc-L-alanine ≥ 99.7%	Chem-Impex International	02369 (Cas No: 35661-39-3)
N ^α -Fmoc-N ^ε -Boc-L-lysine ≥ 99.7%	Chem-Impex International	00493 (Cas No: 71989-26-9)
Fmoc-L-phenylalanine (Fmoc-L-Phe-OH) ≥ 99.7%	Chem-Impex International	02443 (Cas No: 35661-40-6)
Fmoc-L-Pro-OH (S)-Fmoc-pyrrolidine-2-carboxylic acid (Fmoc-L-proline) ≥ 99.7%	Chem-Impex International	02448 (Cas No: 71989-31-6)
O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU)	Alfa Aesar	B23597 (Cas No: 94790-37-1)
Acetonitrile (ACN)	Sigma-Aldrich	34851-2.5L (Cas no: 75-05-8)

Table 6.1 The chemicals used in the experiment (Continued)

Piperidine	Sigma-Aldrich	8222992500 (Cas no: 110-89-4)
N,N-Diisopropylethylamine (DIPEA)	Alfa Aesar	A10280 (Cas no: 108-18-9)
Triisopropylsilane (TIPS)	Sigma-Aldrich	233781-10G (Cas no: 6485-79-6)
Trifluoroacetic acid (TFA)	Sigma-Aldrich	302031-100ML (Cas no: 76-05-1)
Dichloromethane (DCM)	Sigma-Aldrich	24233-2.5L-R (Cas no: 75-09-2)
N,N-Dimethylformamide (DMF)	Merck	1030342500 (Cas no: 68-12-2)

6.3 Synthesis of Peptides Determined by CADD Method by SPPS

The binding affinity research of the target molecule (FOLR1 receptor) by Computer-Aided Drug Design (CADD) method was carried out in Bezmialem University, Faculty of Pharmacy by GOLD Suite software [342]. As a result of the preliminary study, the synthesis of linear peptides shown below has been proposed for cancer-specific imaging on PET/CT and its structure has shown in Figure 6.1, 6.2, 6.3, and Figure 6.4. All amino acids which were used in peptide chains below have the L conformation and these peptides were synthesized by SPPS method with Fmoc protection group.

- NH₂-RPAAK-CONH₂
- NH₂-RFAAK-CONH₂
- NH₂-HRAFAK-CONH₂
- NH₂-RKAFK-CONH₂

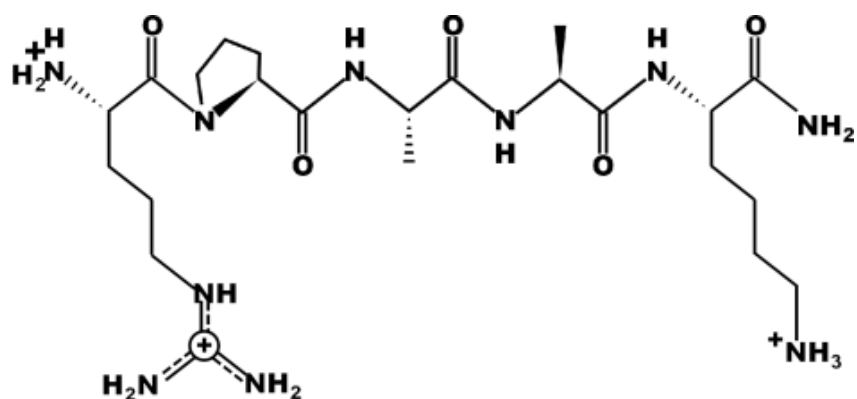


Figure 6.1 Structure of NH₂-RPAAK-CONH₂ sequence. R and K give hydrophilic features to peptide while P gives hydrophobic feature

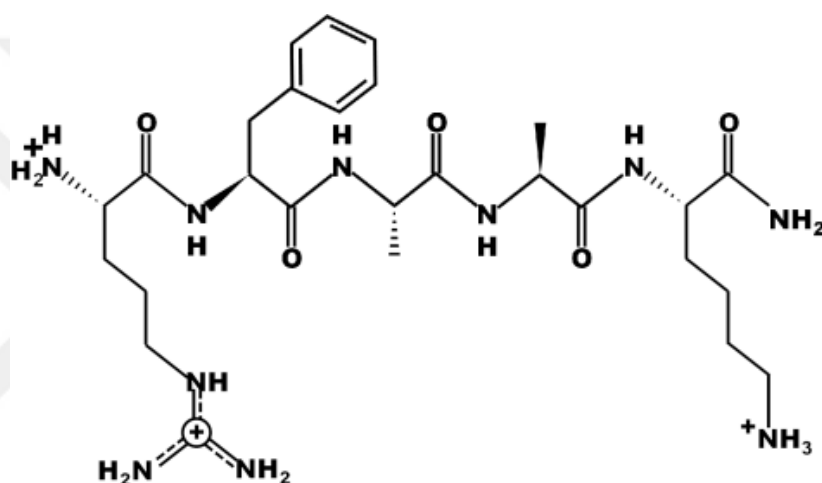


Figure 6.2 Structure of NH₂-RFAAK-CONH₂ sequence. R and K give hydrophilic feature to peptide while F gives hydrophobic feature

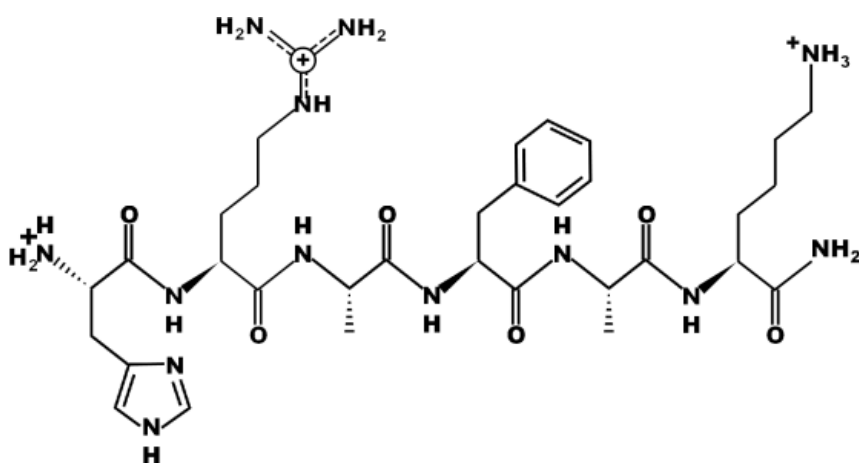


Figure 6.3 Structure of NH₂-HRAFAK-CONH₂ sequence. H, R, and K give hydrophilic feature to peptide while F gives hydrophobic feature

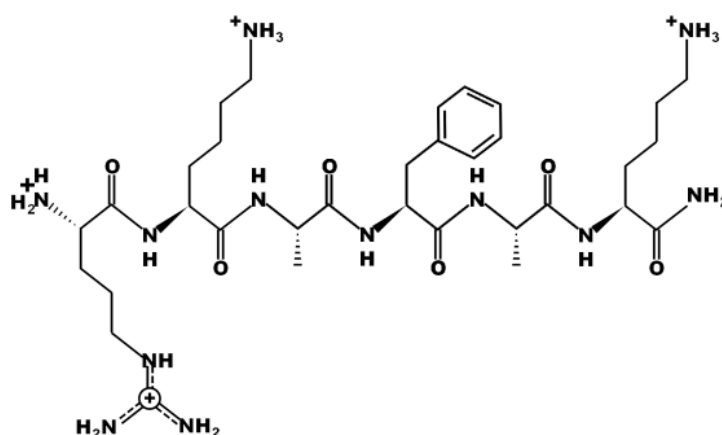


Figure 6.4 Structure of NH₂-RKAFAK-CONH₂ sequence. R, and K give hydrophilic feature to peptide while F gives hydrophobic feature

6.3.1 Synthesis of RPAAK Sequence with Fmoc-Chemistry

RPAAK peptide sequence contains 5 amino acid residues. According to CADD results, K at the C-terminal adds activity to these sequences for FLOR1 protein. The schematic representation of the hydrophilic and hydrophobic amino acid residues of the RPAAK peptide, the pH-dependent titration graph and the net charge at each pH were shown in Figure 6.5. The schematic view of peptide synthesis was shown in Figure 6.6. The synthesis of RPAAK will be given in detail in next sections.

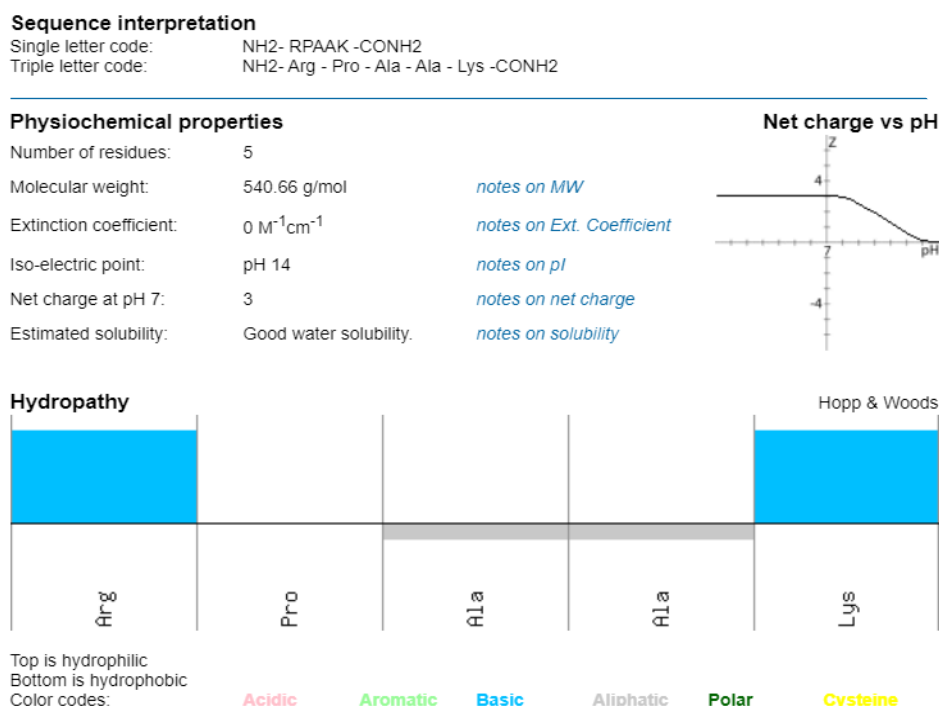


Figure 6.5 Some properties of the RPAAK peptide [343]

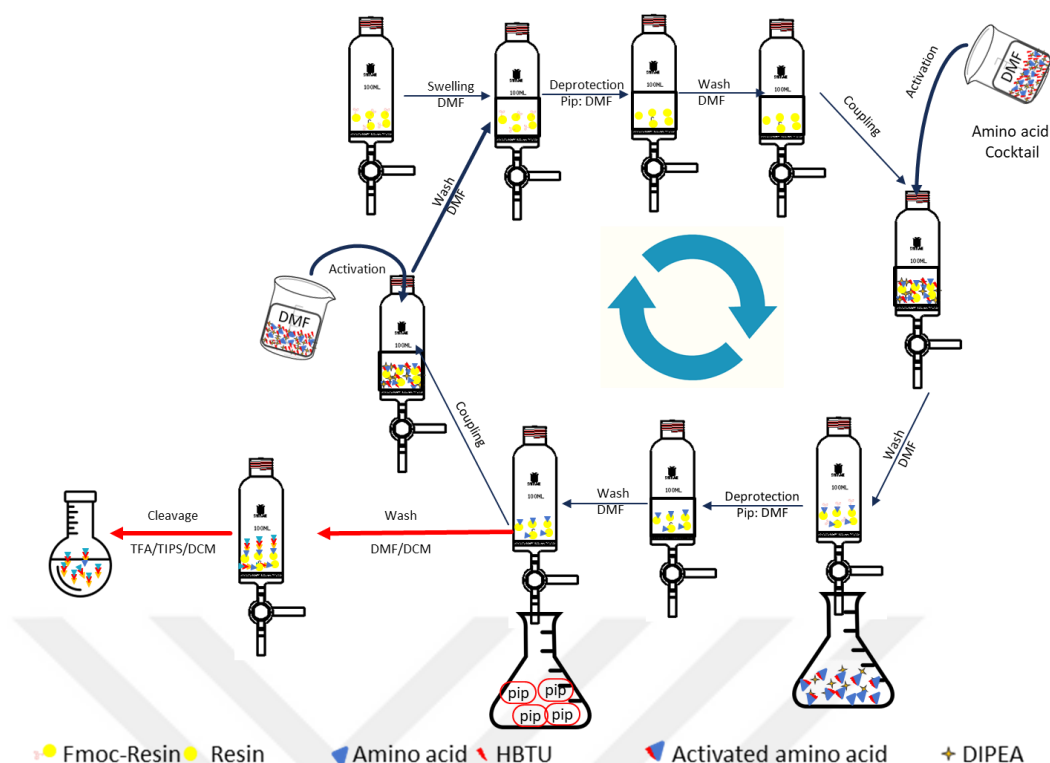


Figure 6.6 The short view of peptide synthesis

6.3.1.1 Swelling of the Rink Amide MBHA Resin

50 mg (0.022 mmol, 0.44 meq/g) rink amide MBHA resin (RAM) was weighed and poured into a reaction vessel. 10 mL DMF was used for swelling of RAM for 30 minutes. Then, all DMF was drained with vacuum applying.

6.3.1.2 Removal of Fmoc Group from MBHA Resin

Pre-swollen MBHA resin treated with two times 20% piperidine solution (855 μ L) in DMF for 10 minutes for 2 times. After the solution was vacuumed and resin was several times washed with DMF (approximately 5 mL for each washing step, the washing step was repeated ten times).

6.3.1.3 Coupling of Lysine (K)

For preparing lysine cocktail, 56.7 mg K (5.5 eq) Fmoc-protected amino acid K dissolved with 42 mg (5 eq) HBTU in 1.5 mL DMF solution. After, 50 μ L (10 eq.) DIPEA was added onto the solution. After DIPEA addition the solution was stirred by a vortex device and was added to the reaction column within 1-2 min (on some amino acid cocktail, there will be a colour change after addition of DIPEA. The

additional time can be determined from these colour change). After cocktail addition, the solution was kept in reaction vessel within 1 hour for binding first amino acid to the resin. Then, the solution was filtered off with vacuum device and the resin was washed with 50 mL DMF for removing possible residue amino acid cocktail chemicals.

6.3.1.4 Removal of Fmoc Group Amino Acid

The same procedure was applied for cleaving of Fmoc group like in 6.3.1.2 section. Briefly, 855 μ L 20% piperidine in DMF solution was poured into reaction column for 10 minutes for 2 times. After, the solution was vacuumed, and resin was several times washed with DMF for removing excess piperidine, possible residue amino acid cocktail chemicals and other by-products.

6.3.1.5 Coupling of Alanine (A), Prolin (P), and Arginine (R)

For every amino acid addition step, 0.121 mmol (5.5 eq) amino acid was added to amino acid cocktail with a certain amount of HBTU, DMF and DIPEA. After, the reaction column was washed with DMF before and after every cleavage step of Fmoc from last added amino acid. At below, addition A, P, and R represents;

For preparing an amino acid cocktail,

- 37 mg K (5.5 eq) Fmoc-protected amino acid A,
- 40.6 mg K (5.5 eq) Fmoc-protected amino acid P,
- 78 mg K (5.5 eq) Fmoc-protected amino acid R

was dissolved separately, in 42 mg (5 eq) HBTU and 1.5 mL DMF solution. After, 50 μ L (10 eq) DIPEA was added onto the solution. After DIPEA was added, the solution was stirred by a vortex device and added to the reaction column within 1-2 min. After cocktail addition, the solution was kept during 1 hour for binding amino acid to the previous amino acid. Then, the solution was vacuumed, and the resin was washed with 50 mL DMF. The Fmoc group was removed from last binding amino acid with 855 μ L 20% piperidine in DMF solution for 10 minutes for 2 times.

6.3.1.6 Removal of the Last Fmoc Group

After 20% piperidine treating and DMF washing for eliminating excess piperidine, and other by-products, the resin was washed with DCM several times until eliminating DMF from the reaction column. If DMF remains in the milieu, it will completely interfere to final cleavage step. Then, the resin was dried.

6.3.1.7 Cleavage of the Peptide

Cleavage cocktail was prepared with 98% TFA, 1% DCM and 1% TIPS in total 1.1 mL (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS). The cocktail was added to (550 μ L) the reaction column and waited for 1 hour for 2 times. Then, the solution was collected. TFA and DCM were removed with an evaporator device under reduced pressure at 40 °C, 200 rpm in 15 minutes with the addition of DCM at least 3 times. After the evaporation process, in which the cutting cocktail was substantially removed, it was left in the lyophilizer for 48 hours to ensure complete drying. The peptide was submitted to HPLC and LC-MS for purification and characterization.

6.3.2 Synthesis of RFAAK Sequence

This peptide sequence is based on the RFAAK peptide synthesis previously shown step by step, and only the amino acids to be used differently. The amounts of amino acids and other reagents used to obtain a single peptide sequence were indicated in the Table 6.2.

Table 6.2 Reagents of the RFAAK sequence

Reagent	Amount	Reagent	Amount
Resin	50 mg	Phenylalanine (F)	46,7 mg
HBTU	5 x 42 mg	Alanine (A)	2 x 37 mg
DMF	5x1.5 mL	DIPEA	5 x 50 μ L
Arginine (R)	78 mg	DMF-piperidine	5 x 2 x 855 μ L
Lysine (K)	56,7 mg		

After coupling of amino acids and 20% piperidine treating, cleavage cocktail was prepared with 98% TFA, 1% DCM and 1% TIPS in total 1.1 mL (1078 μ L TFA, 11 μ L

DCM, 11 μL TIPS). The cocktail was added to (550 μL) the reaction column and waited for 1 hour for 2 times. Then, the solution was collected. TFA and DCM were removed with an evaporator device under reduced pressure at 40 $^{\circ}\text{C}$, 200 rpm in 15 minutes with the addition of DCM at least 3 times. After the evaporation process, in which the cutting cocktail was substantially removed, it was left in the lyophilizer for 48 hours to ensure complete drying. The peptide was submitted to HPLC and LC-MS for purification and characterization.

The schematic representation of the hydrophilic and hydrophobic amino acid residues of the RFAAK peptide, the pH-dependent titration graph and the net charge at each pH were shown in Figure 6.7.

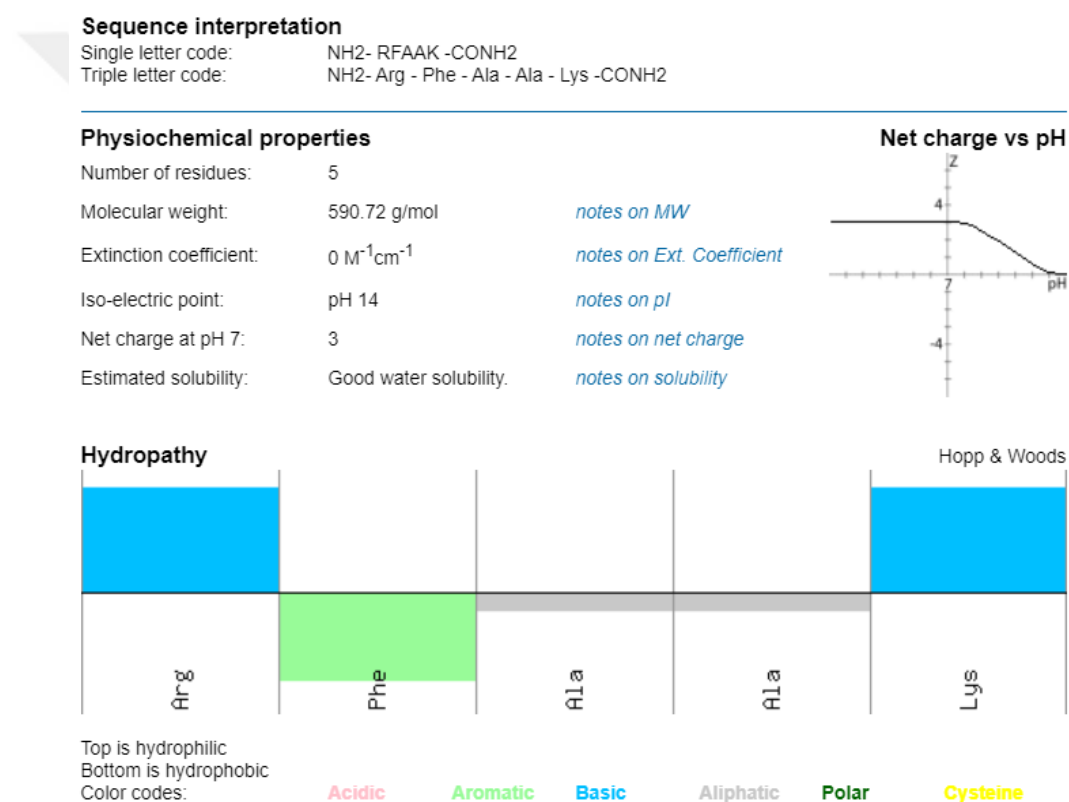


Figure 6.7 Some properties of the RFAAK peptide [343]

6.3.3 Synthesis of HRAFAK Sequence

This peptide sequence is based on the HRAFAK peptide synthesis previously shown step by step, and only the amino acids to be used differently. The amounts of amino acids and other reagents used to obtain a single peptide sequence were indicated in the Table 6.3.

Table 6.3 Reagents of the HRAFAK sequence

Reagent	Amount	Reagent	Amount
Resin	50 mg	Phenylalanine (F)	46,7 mg
HBTU	6 x 42 mg	Alanine (A)	2 x 37 mg
DMF	6 x 1.5 mL	Histidine (H)	74 mg
Arginine (R)	78 mg	DIPEA	6 x 50 μ L
Lysine (K)	56,7 mg	20% Piperidine	6 x 2 x 855 μ L

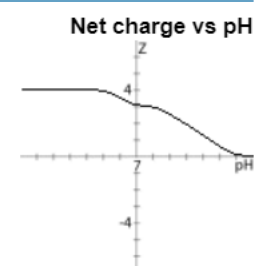
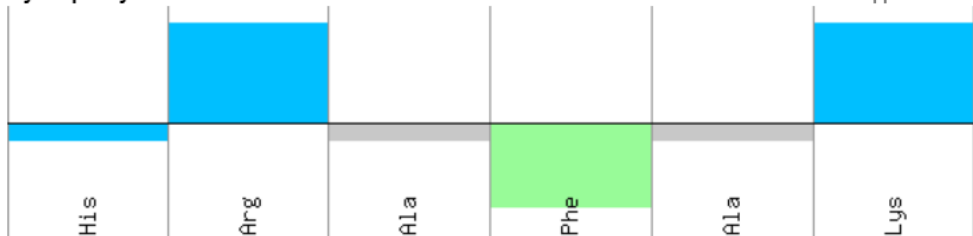
After coupling of amino acids and 20% piperidine treating, cleavage cocktail was prepared with 98% TFA, 1% DCM and 1% TIPS in total 1.1 mL (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS). The cocktail was added to (550 μ L) the reaction column and waited for 1 hour for 2 times. Then, the solution was collected. TFA and DCM were removed with an evaporator device under reduced pressure at 40 °C, 200 rpm in 15 minutes with the addition of DCM at least 3 times. After the evaporation process, in which the cutting cocktail was substantially removed, it was left in the lyophilizer for 48 hours to ensure complete drying. The peptide was submitted to HPLC and LC-MS for purification and characterization.

Sequence interpretation

Single letter code: NH₂- HRAFAK -CONH₂
 Triple letter code: NH₂- His - Arg - Ala - Phe - Ala - Lys -CONH₂

Physiochemical properties

Number of residues: 6
 Molecular weight: 727.86 g/mol *notes on MW*
 Extinction coefficient: 0 M⁻¹cm⁻¹ *notes on Ext. Coefficient*
 Iso-electric point: pH 14 *notes on pI*
 Net charge at pH 7: 3.1 *notes on net charge*
 Estimated solubility: Good water solubility. *notes on solubility*

**Hydropathy**

Top is hydrophilic
 Bottom is hydrophobic
 Color codes:

Acidic Aromatic Basic Aliphatic Polar Cysteine

Figure 6.8 Some properties of the HRAFAK peptide [343]

The schematic representation of the hydrophilic and hydrophobic amino acid residues of the HRAFAK peptide, the pH-dependent titration graph and the net charge at each pH were shown in Figure 6.8.

6.3.4 Synthesis of RKAFK Sequence

This peptide sequence is based on the RKAFK peptide synthesis previously shown step by step, and only the amino acids to be used differently. The amounts of amino acids and other reagents used to obtain a single peptide sequence were indicated in the Table 6.4.

Table 6.4 Reagents of the RKAFK sequence

Reagent	Amount	Reagent	Amount
Resin	50 mg	Phenylalanine (F)	46,7 mg
HBTU	6 x 42 mg	Alanine (A)	2 x 37 mg
DMF (for aminoacid cocktail)	6 x 1.5 mL	DIPEA	6 x 50 μ L
Arginine (R)	78 mg	20% Piperidine	6 x 2 x 855 μ L
Lysine (K)	2 x 56,7 mg		

After coupling of amino acids and 20% piperidine treating, cleavage cocktail was prepared with 98% TFA, 1% DCM and 1% TIPS in total 1.1 mL (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS). The cocktail was added to (550 μ L) the reaction column and waited for 1 hour for 2 times. Then, the solution was collected. TFA and DCM were removed with an evaporator device under reduced pressure at 40 °C, 200 rpm in 15 minutes with the addition of DCM at least 3 times. After the evaporation process, in which the cutting cocktail was substantially removed, it was left in the lyophilizer for 48 hours to ensure complete drying. The peptide was submitted to HPLC and LC-MS for purification and characterization.

The schematic representation of the hydrophilic and hydrophobic amino acid residues of the RKAFK peptide, the pH-dependent titration graph and the net charge at each pH were shown in Figure 6.9.

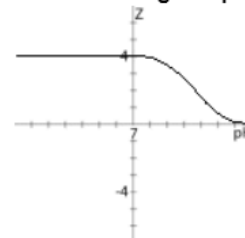
Sequence interpretation

Single letter code: NH₂- RKAFAK -CONH₂
Triple letter code: NH₂- Arg - Lys - Ala - Phe - Ala - Lys -CONH₂

Physiochemical properties

Number of residues:	6	
Molecular weight:	718.89 g/mol	notes on MW
Extinction coefficient:	0 M ⁻¹ cm ⁻¹	notes on Ext. Coefficient
Iso-electric point:	pH 14	notes on pI
Net charge at pH 7:	4	notes on net charge
Estimated solubility:	Good water solubility.	notes on solubility

Net charge vs pH



Hydropathy

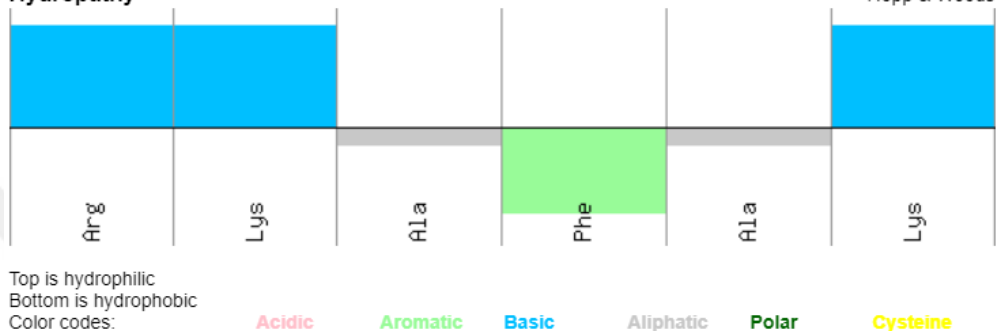


Figure 6.9 Some properties of the RKAFAK peptide [343]

6.4 Purification and Characterization of the Peptides

6.4.1 Purification of Peptides by Analytical RP-HPLC

The peptides from the lyophilizer were stored at -20 °C. They were then sequentially subjected to RP-HPLC studies. All four peptides were analysed on the analytic RP-HPLC instrument under the following conditions and at room temperature.

- Flow rate: 1.0 mL/min
- Mobile Phase A: Water (0.08 % TFA)
- Mobile Phase B: Acetonitrile (0.08 % TFA)
- Column: GL Science Inertsil ODS-3 C18 column (150 mm x 4.6 mm, 5 µm)
- UV Detector: 214 nm
- Injection Volume: 20 µL
- Column Temperature: 40 °C

The four peptide sequences listed above were assigned to analytical RP-HPLC. Analytical RP-HPLC was performed on the SHIMADZU Semi-Preparative Liquid Chromatography system. The peptides were prepared by dissolving 500 µL of

acetonitrile and 500 μ L of water at low concentration ranging from 0.3 mg/mL to 1.3 mg/mL. The prepared samples were passed through the filter (0.45 μ m) and transferred to a clean vial and placed in the device.

6.4.2 Purification of Peptides by Preparative RP-HPLC

All four peptides were analysed and purified on the preparative RP-HPLC instrument after analytical HPLC under the following conditions and at room temperature.

- Flow rate: 5.0 mL/min
- Mobile Phase A: Water (0.08 % TFA)
- Mobile Phase B: Acetonitrile (0.08 % TFA)
- Column: Thermo Scientific Hypersil GOLD C18 column (250mm x 10.0 mm, 12 μ m)
- UV Detector: 214 nm
- Injection Volume: 3500 μ L
- Column Temperature: Room temperature

After lyophilization, all peptides were prepared by dissolving 2 mL of acetonitrile and 2 ml of distilled water. The prepared samples were passed through the filter (0.45 μ m) and transferred to a clean vial and placed in the device. Two different methods were applied. The first method (a) at a flow rate of 5 ml/min for 70 min with acetonitrile concentration increasing from 5% to 100% and the second method (b) 5 ml/min for 30 min with acetonitrile concentration increasing from 5% to 20% and 10 min with acetonitrile concentration increasing from 20% to 100% in total 40 min. During elution, peaks that are considered to be peptide peaks are collected, in glass test tubes in the fraction collection unit of the apparatus when the detector gives peaks. It was then placed in clean glass containers and placed in a lyophilizer. A 48-hour program allows the solvents to completely evaporate and dry. It was then stored at -20 °C.

6.4.3 Characterization of Peptides by LC-MS/MS

The molecular weight of all four peptides thought to be purified was performed on the Thermo Scientific Q Exactive Orbitrap LC-MS/MS instrument. 0.01 mg of the

peptide was prepared by dissolving 0.5 mL of acetonitrile and 0.5 mL of water and then passing through a "syringe filter" (0.45 μm) and transferred to a clean vial and placed in the apparatus.



Peptides have been started to use as imaging probes for cancer imaging in nowadays because of ability of high affinity and selectivity to cancer cells [344], [345]. Aim of the thesis is that synthesizing, developing, and testing functionally for new imaging probes formed by peptides for cancer imaging at the cellular and molecular level for PET device. The peptides were designed by GOLD Suite program methods to bind with high affinity, specificity and selectivity to FOLR1 protein which is overexpressed in most cancer types. So, on the thesis, the certain peptide sequences were designed, synthesized, purified, and characterized. After that, peptides will be performed Isothermal Titration Calorimetry (ITC) for the examination of binding affinity to FOLR1 protein for future studies. After peptides were selected, these peptides will be labelled with fluorescent dye and radionuclides for *in vitro* and *in vivo* tests, respectively.

7.1 Peptide Synthesis by SPPS method

These four peptides were synthesized on rink amide resin using SPPS method. Peptide synthesis basically has a cycle as illustrated in Figure 7.1.

In this cycle, the carboxylic acid of the amino acids is activated by HBTU and DIPEA to react with the free amino group of the resin or the subsequent amino acid to form the peptide bond. After coupling reaction, the Fmoc protecting group on the coupled amino acid is cleaved with piperidine to make it ready for coupling of the next amino acid. The synthesized peptide is cleaved with TFA/TIPS/DCM from resin, and any other protecting groups such a *t*-Boc.

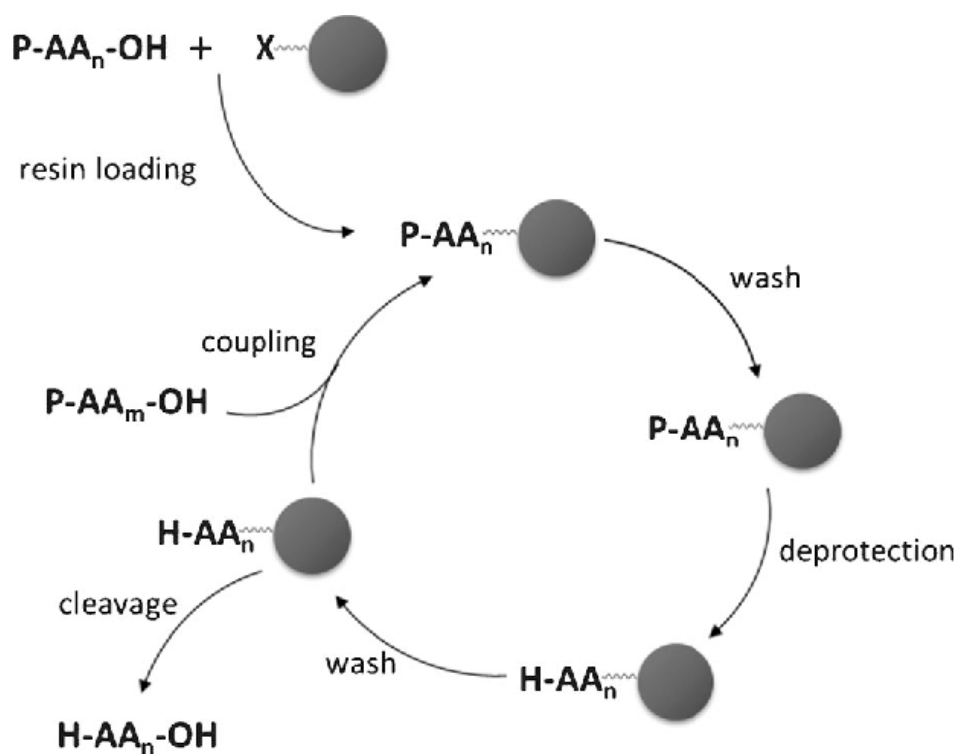


Figure 7.1 Scheme of the general SPPS in which P-AA-OH depicts an N-terminal and side-chain protected amino acid and H-AA-OH depicts an unprotected amino acid [346]

In general, the peptides should be prepared by orthogonal protection, which provides strict control over the reactivity of the amine residues of both the side chain and the N- terminal. There are two main protection groups: Fmoc and *t*-Boc. Fmoc is cleaved under milder reaction conditions (e.g., piperidine in DMF) relative to *t*-Boc, and therefore the Fmoc protecting group is more preferred in SPPS. The reason why piperidine is preferred to cleavage the Fmoc group is that using cyclic secondary amine is more effective than primary and tertiary amines because of their nucleophilicity features [347]. In addition to Fmoc and *t*-Boc, there are other protecting groups to protect other reacting groups to be attached to the amino acid and each is cleaved under different conditions such a pH (Table 7.1). Differences in these cleavage conditions minimize the possibility of by-products, allowing control of which functional groups can become reactive.

Table 7.1 Commonly used protecting groups for SPPS method. *t*-Bu: *tert*-Butyl, Trityl: Triphenylmethyl, Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl [348]

Protecting group	Resistant to	Labile to	Removed with
Fmoc	Acids	Bases	10–20% Piperidine
<i>t</i> -Bu	Bases	Acids	≥50% TFA in DCM
<i>t</i> -Boc	Bases	Acids	≥50% TFA in DCM
Trityl	Bases	Acids	≥2% TFA
Pbf	Bases	Acids	~95% TFA

7.2 Removing of the TFA and DCM

The evaporator is used to remove the cleaved peptide solution from TFA and DCM to achieve crude peptide. Another way to remove TFA and DCM is by precipitation with cold ether. However, precipitation with cold ether is not suitable for short peptides due to high solubility of peptides in ether solution. Nevertheless, removing the TFA in the evaporator may not be enough for the first time. It may be easy to follow, especially with visible colour changes. For example, in the HRAFAK sequence, a colour change occurs when the TFA containing cleavage solution is added to the peptide solution and the peptide solution colour turns orange from transparent (Figure 7.2). This orange color may not disappear during the first evaporation. So, the presence of TFA in the solution. To clear away TFA, add some DCM to the peptide solution at the end of each evaporation process and continue to evaporate. Thus, the colour will turn white colour completely and so TFA will be removed.

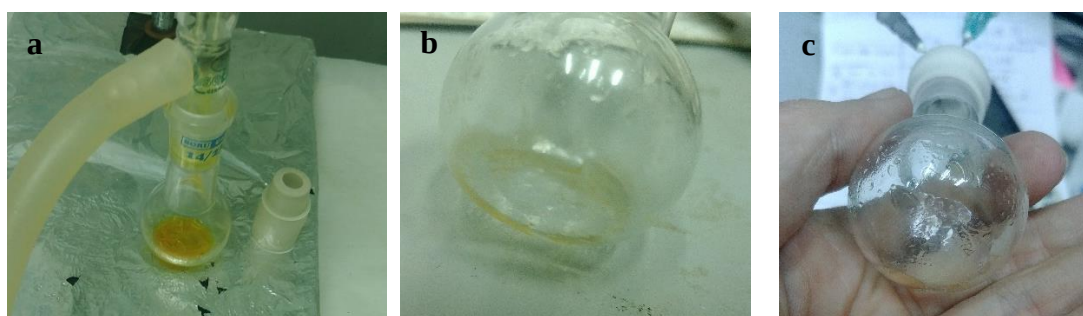


Figure 7.2 Colour change during evaporation process. (a) After cleavage of the peptide (b) after first evaporation and (c) after many evaporations

7.3 Rink Amid Resin for Peptide Synthesis

Resin selection in SPPS can dramatically affect the structure of the final peptide, and the synthesis conditions, as previously mentioned. In the first formed resins, the final peptides have the amine group at the N-terminus and the carboxyl group at the C-terminus. In the thesis, Rink Amide (RAM) resin is preferred because it forms an amide group at the C-terminus. The presence of the amide group at the C-terminus allows the peptides to remain stable *in vivo* conditions. This is because the amide group is resistant to serum protease enzymes. This resistance may result from the amides by masking the C-terminus used as a recognition site for proteases. It also helps peptide stabilization as it facilitates the formation of α -helix structure in the peptide chain [349], [350]. Additionally, amidation increases cell penetration. The amidated peptides can be very useful for intracellular experiments, *in vivo*, and *in vitro* studies [351]. The structural difference of the modified peptide which synthesized with RAM resin and natural peptide is shown in Figure 7.3.

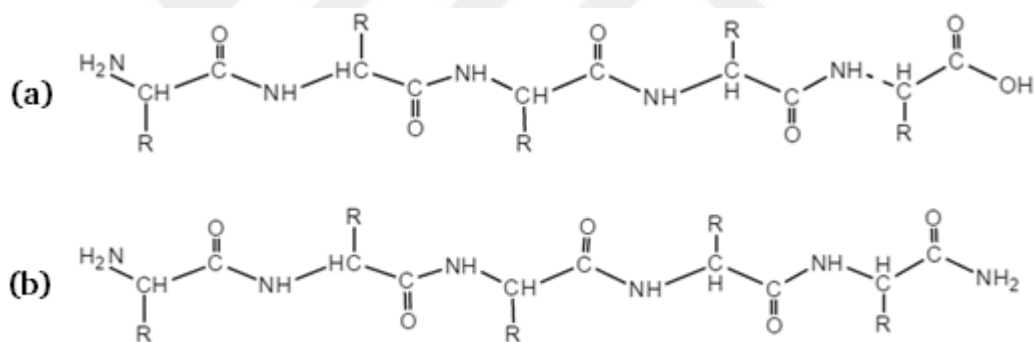


Figure 7.3 (a) The natural structure of the peptide chain and (b) the structure of *de novo* synthesized modified peptide chain with RAM resin

7.4 HPLC and LC-MS Analysis of the Four Peptides

Since the amino acid contents of each peptide were different from each other, their hydrophobic and hydrophilic properties may show differences. Therefore, the peak of each peptide can be emerged at different minutes. The HPLC and LC-MS results of RPAAK, RFAAK, HRAFAK and, RKFAFAK were shown in the below.

7.4.1 Analysis of RPAAK Sequence

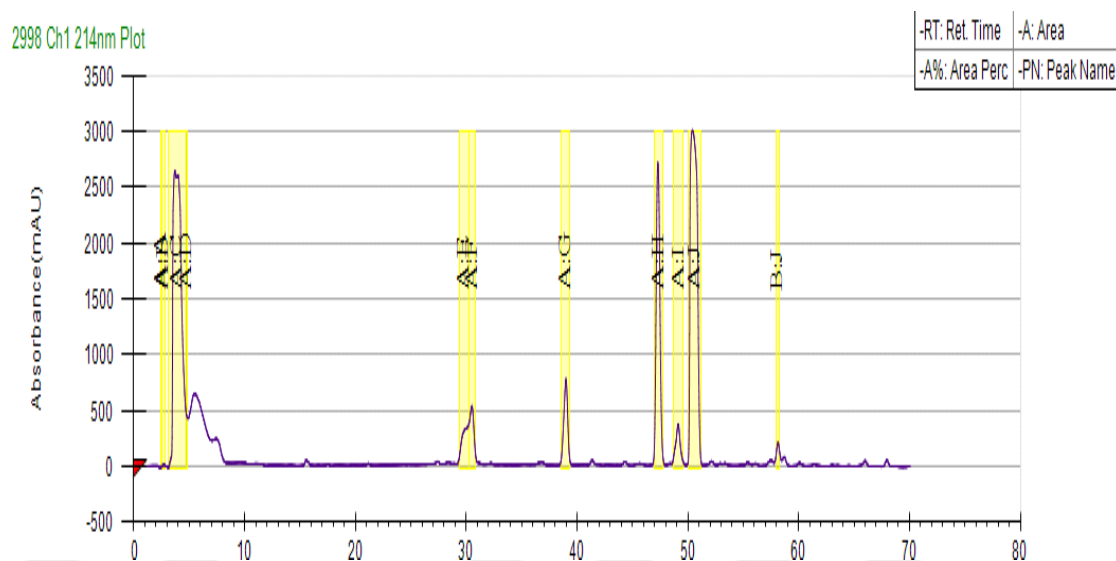


Figure 7.4 Preparative HPLC chromatogram of the synthesized RPAAK crude peptide

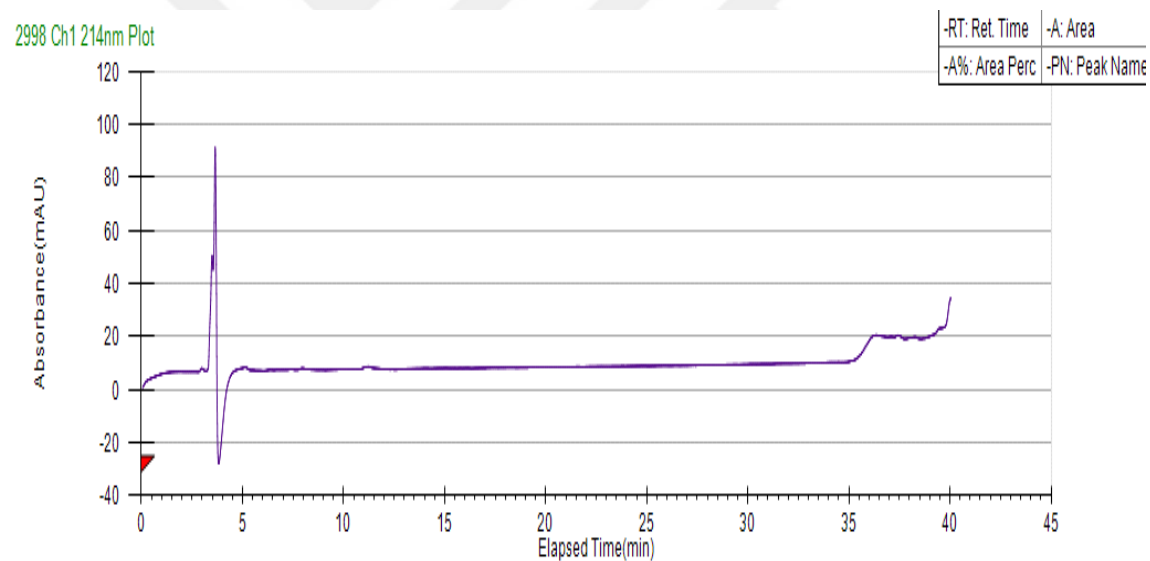


Figure 7.5 Preparative HPLC chromatogram of the synthesized RPAAK purified peptide

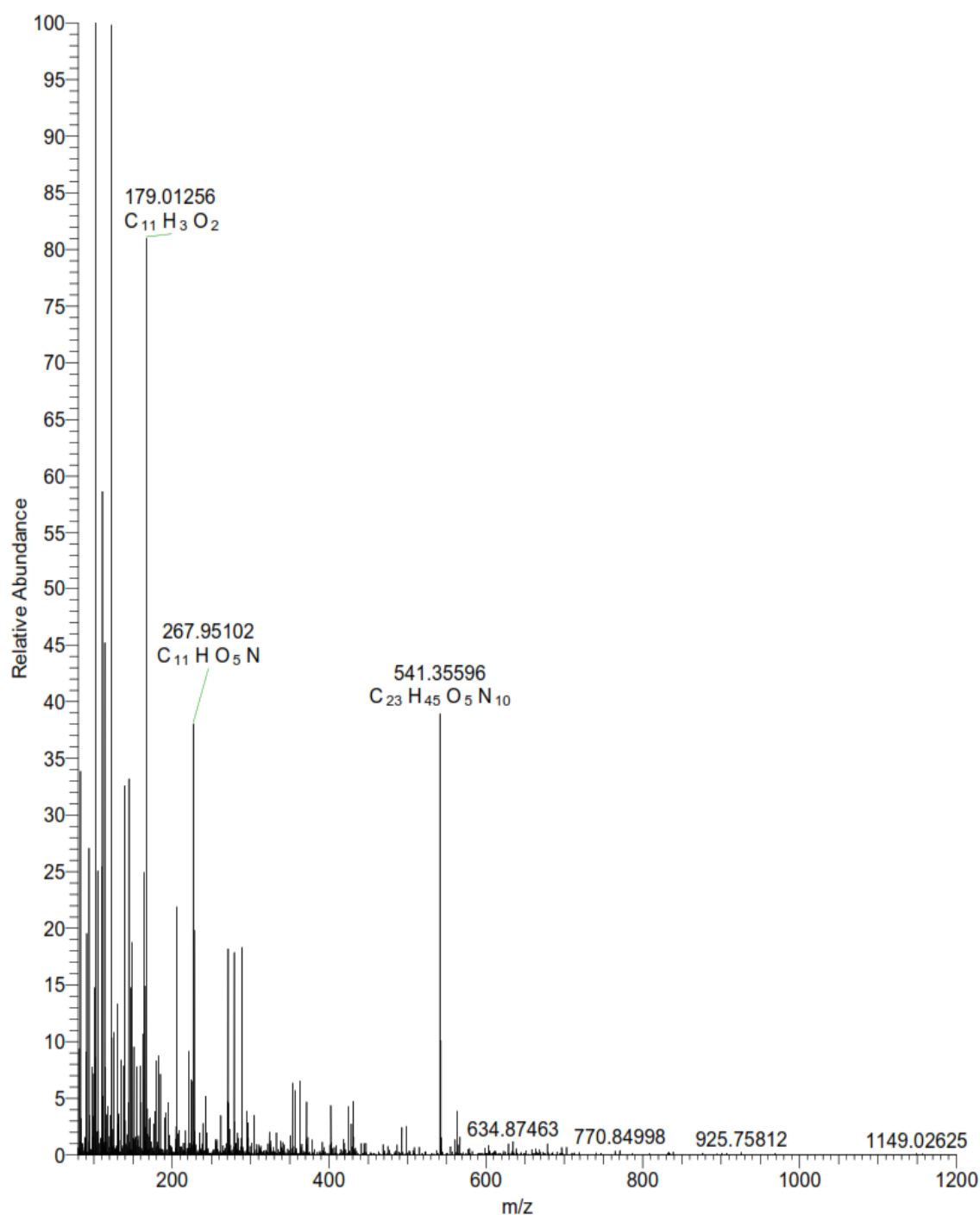


Figure 7.6 LC-MS chromatogram of the purified RPAAK peptide

HPLC chromatogram was obtained at 214 nm. The crude peptide and purified peptide chromatogram are seen in Figure 7.4 and Figure 7.5, respectively. As seen from chromatograms, the peptide is successfully purified. Since the RPAAK peptide is a very hydrophilic, the first peak was considered as peptide to be purified. Therefore, it comes with high gradient water. The first peak was taken and then analyzed by LC-MS. The 3., 4., 5., 6., and 7. peaks in the Figure 7.4 are probably "ghost

peak" which can result from the different effect such as pumping and mixing problems, inorganic impurities in water, systematic sample contamination, reagents and additives for mobile phases, etc. [352], [353]. Samples from all peaks are dried with lyophilizer after the purification process. No powder particles were observed in tubes with peaks 3., 4., 5., 6., and 7. whereas white powder was observed in the first two peaks. So, these peaks are probably the "ghost peak", they are not peptides.

According to the LC-MS chromatogram, it was observed that RPAAK peptide was synthesized successfully. The molecular weight of the peptide sequence was calculated as 540.66 g/mol with the help of 'PepCalc.com peptide calculator' program [343]. In the chromatogram of the purified peptide sequence (Figure 7.6), the experimental molecular weight was calculated using m/z ratio 267.95 and 541.35 in the mass spectrum.

$$[M + 2H^+]^{2+} = 267.95 \rightarrow M_W = 533.90 \text{ g/mol}$$

$$[M + H^+]^+ = 541.35 \rightarrow M_W = 540.35 \text{ g/mol}$$

$$M_{W\text{average}} = 537.13 \text{ g/mol}$$

$$M_{W\text{teoritical}} = 540,66 \text{ g/mol}$$

It is seen that the experimental molecular weight calculated using LC-MS is similar to the calculated molecular weight from 'PepCalc.com peptide calculator' program.

7.4.2 Analysis of RFAAK Sequence

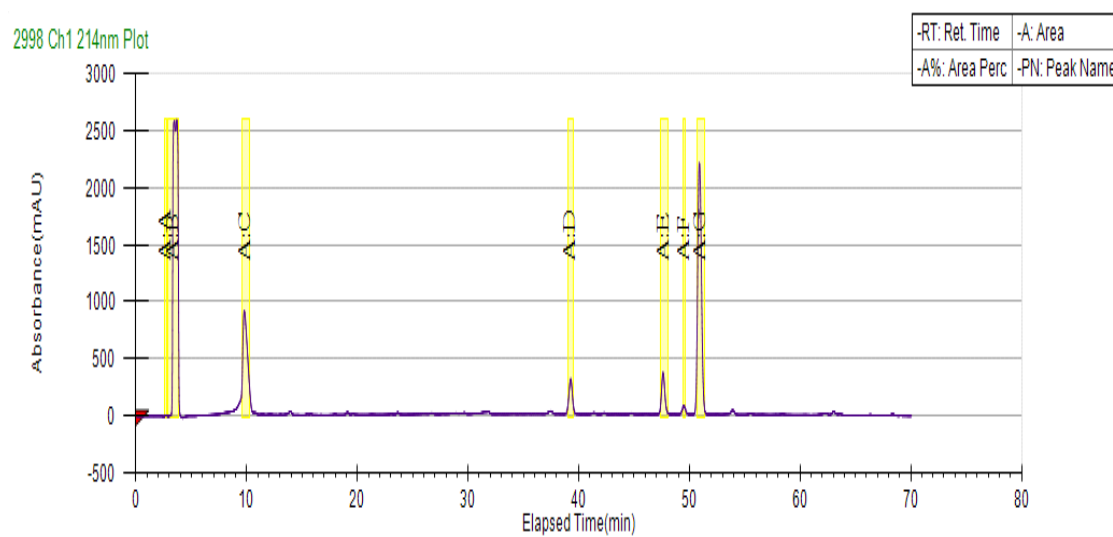


Figure 7.7 Preparative HPLC chromatogram of the synthesized RFAAK crude peptide

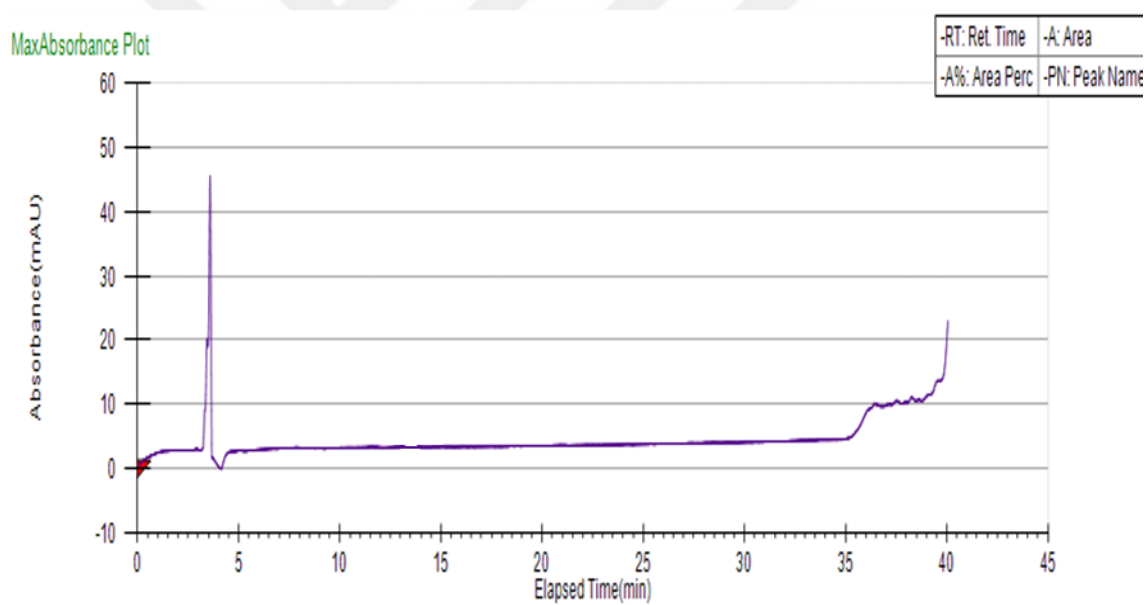


Figure 7.8 Preparative HPLC chromatogram of the synthesized RFAAK purified peptide

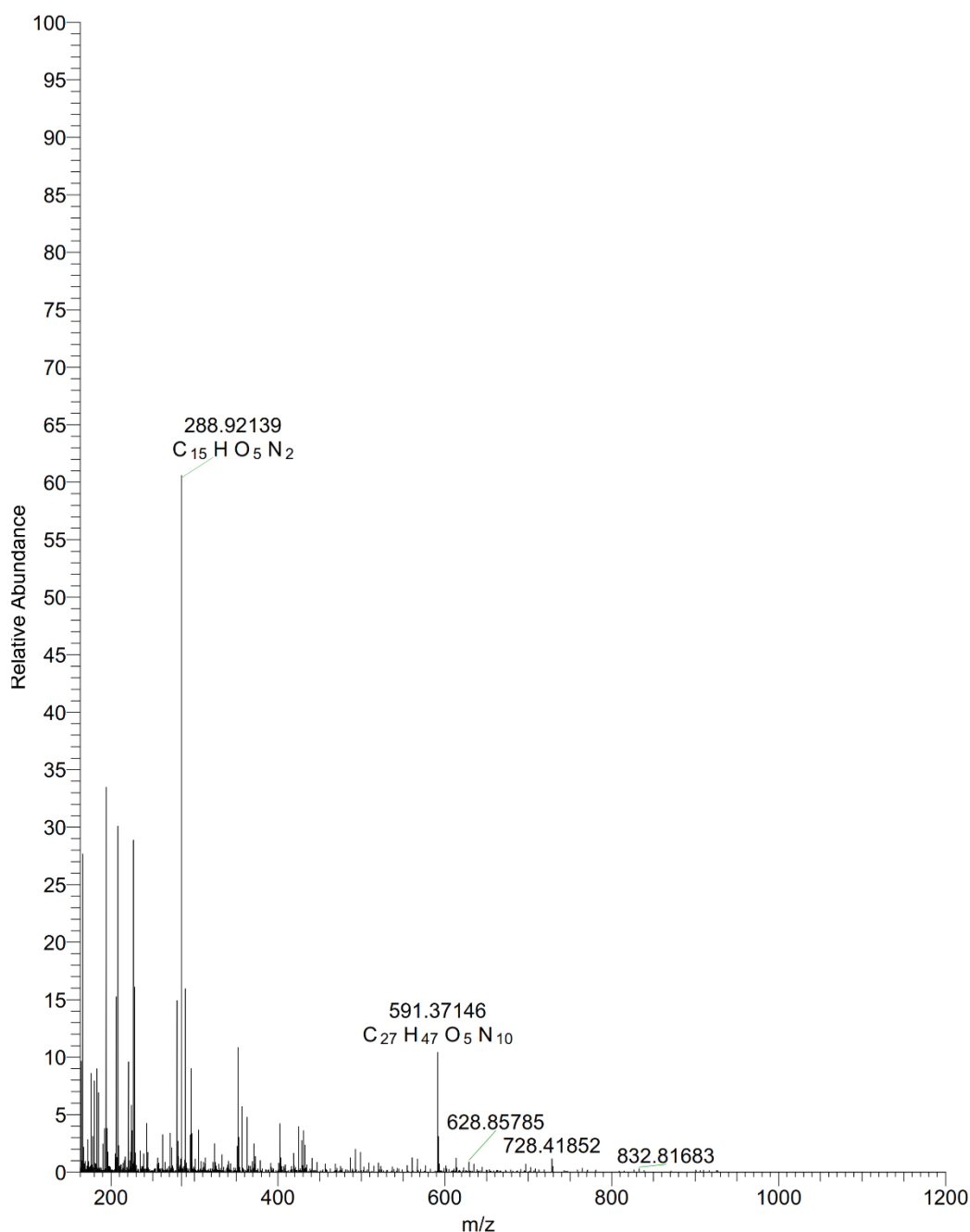


Figure 7.9 LC-MS chromatogram of the purified RFAAK peptide

HPLC chromatogram was obtained at 214 nm. The crude peptide and purified peptide chromatogram are seen in Figure 7.7 and Figure 7.8, respectively. As seen from chromatograms, the peptide is successfully purified like previous sequence. Since the RFAAK peptide is a very hydrophilic, the first peak was considered as the peptide to be purified like RFAAK peptide synthesis. Therefore, it comes with high gradient water. The first peak was taken and then analyzed by LC-MS. The same “ghost peak” phenomenon has also appeared at 3., 4., 5., and 6. on this sequence.

The molecular weight of the RFAAK sequence was calculated as 590,72 g/mol with the help of 'PepCalc.com peptide calculator' program [343]. In the chromatogram of the purified peptide sequence (Figure 7.9), the experimental molecular weight was calculated using m/z ratio 288.92, and 591.37 in the mass spectrum.

$$[M + 2H^+]^{2+} = 288.92 \rightarrow M_w = 575.84 \text{ g/mol}$$

$$[M + H^+]^+ = 591.37 \rightarrow M_w = 590.37 \text{ g/mol}$$

$$M_{W\text{average}} = 584.61 \text{ g/mol}$$

$$M_{W\text{theoretical}} = 590,72 \text{ g/mol}$$

It is seen that the experimental molecular weight calculated using LC-MS is similar to the theoretical molecular weight.

7.4.3 Analysis of HRAFAK Sequence

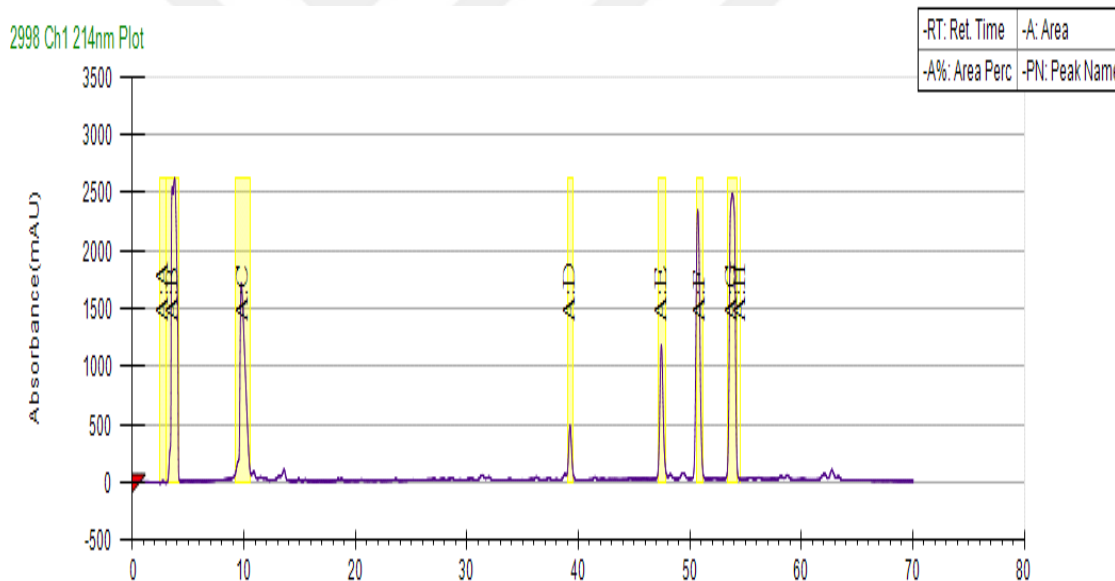


Figure 7.10 Preparative HPLC chromatogram of the synthesized HRAFAK crude peptide

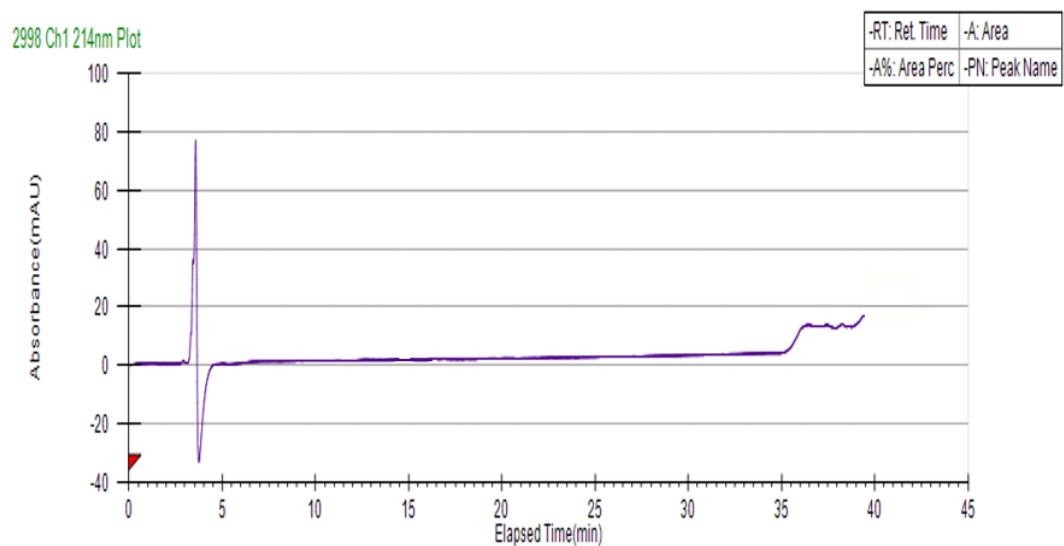


Figure 7.11 Preparative HPLC chromatogram of the synthesized HRAFAK purified peptide

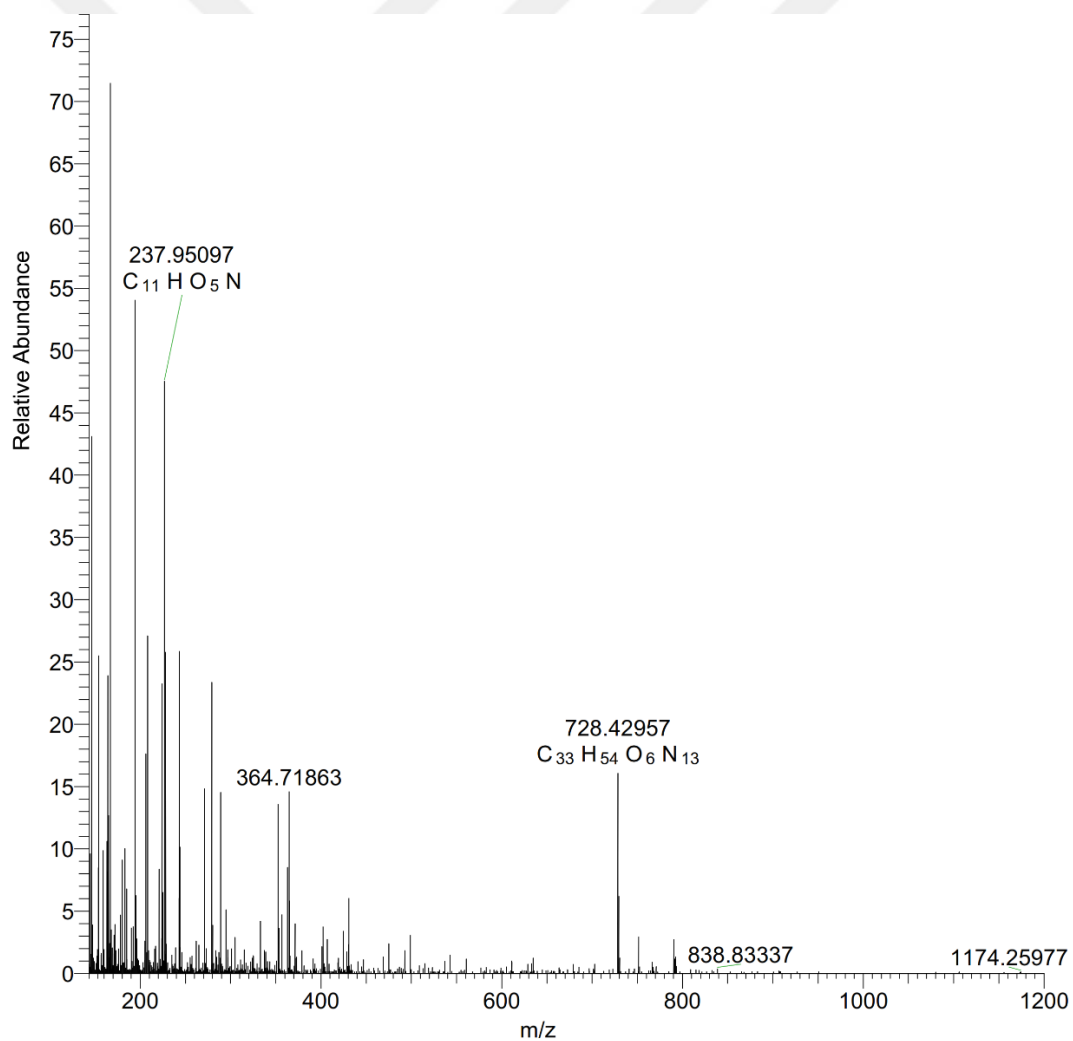


Figure 7.12 LC-MS chromatogram of the purified HRAFAK peptide

The crude peptide and purified peptide chromatogram are seen in Figure 7.10 and Figure 7.11, respectively. As seen from chromatograms, the peptide purification was obtained successfully. The first peak was considered as peptide to be purified due to hydrophilic feature of the peptide. Hydrophilic structures are expected to come with high water concentration. Therefore, the first peak shows the hydrophilic solution containing the peptide to be purified. The first peak was taken and then analyzed by LC-MS. The same “ghost peak” phenomenon has also appeared at 3., 4., 5., and 6. on this sequence.

According to the LC-MS chromatogram, it was observed that HRAFAK peptide was synthesized successfully. The molecular weight of the peptide sequence was calculated as 727.86 g/mol with the help of ‘PepCalc.com peptide calculator’ program [343]. In the chromatogram of the purified peptide sequence, the experimental molecular weight was calculated using m/z ratio 364.72 and 728.43 in the mass spectrum as shown Figure 7.12.

$$[M + 2H^+]^{2+} = 364.72 \rightarrow M_w = 727.44 \text{ g/mol}$$

$$[M + H^+]^+ = 728.43 \rightarrow M_w = 727.43 \text{ g/mol}$$

$$M_{W\text{average}} = 727.43 \text{ g/mol}$$

$$M_{W\text{theoretical}} = 727.86 \text{ g/mol}$$

The experimental molecular weight calculated using LC-MS was found very similar to the ‘PepCalc.com peptide calculator’ program calculated molecular weight.

7.4.4 Analysis of RKAFAK Sequence

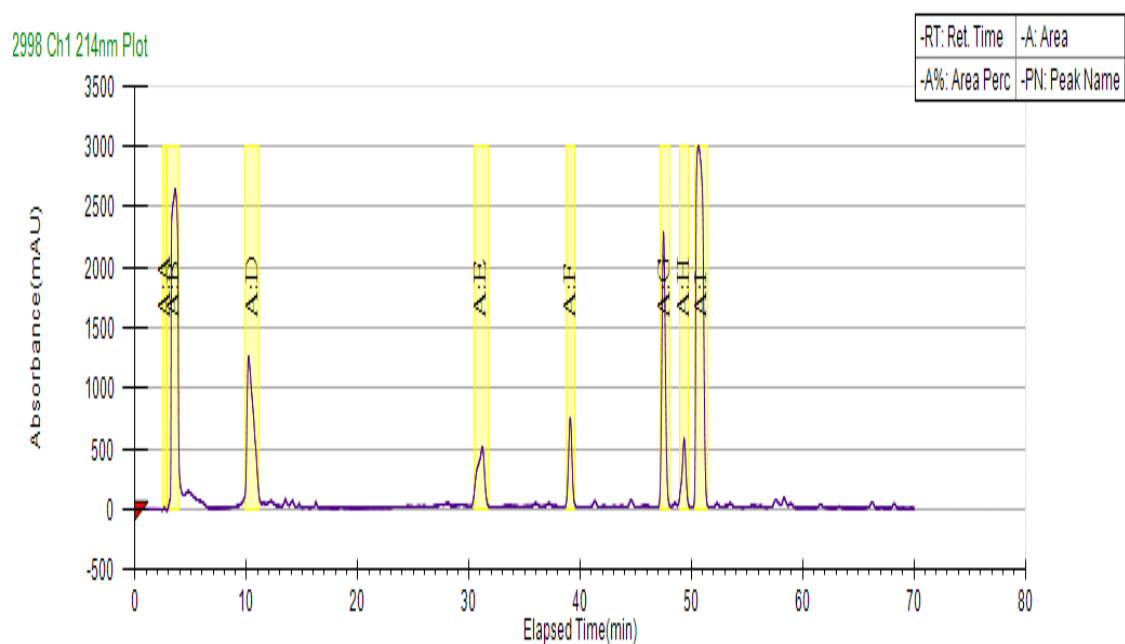


Figure 7.13 Preparative HPLC chromatogram of the synthesized RKAFAK crude peptide

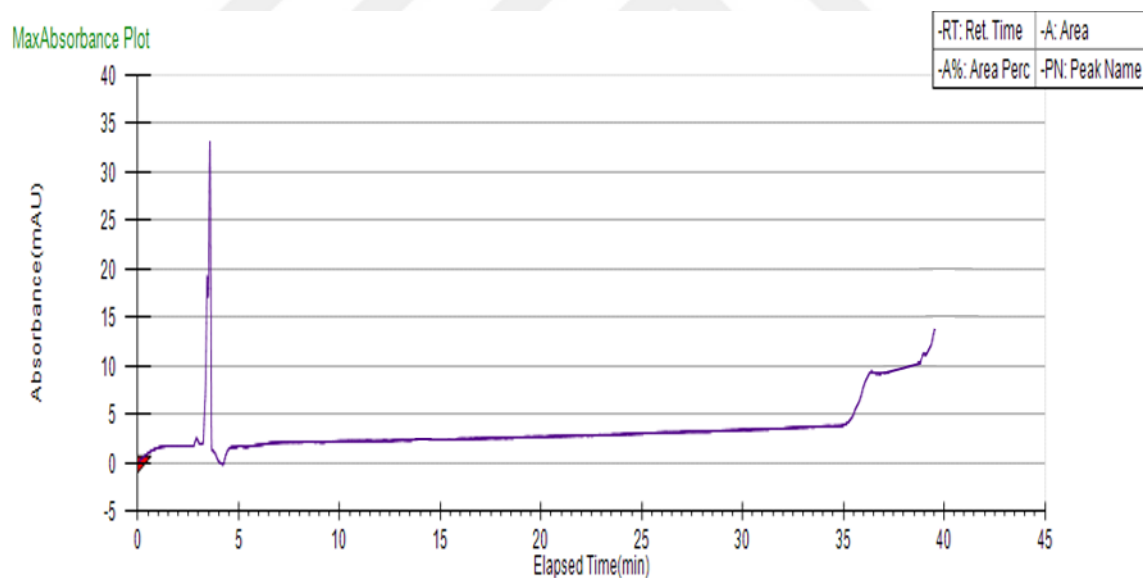


Figure 7.14 Preparative HPLC chromatogram of the synthesized RKAFAK purified peptide

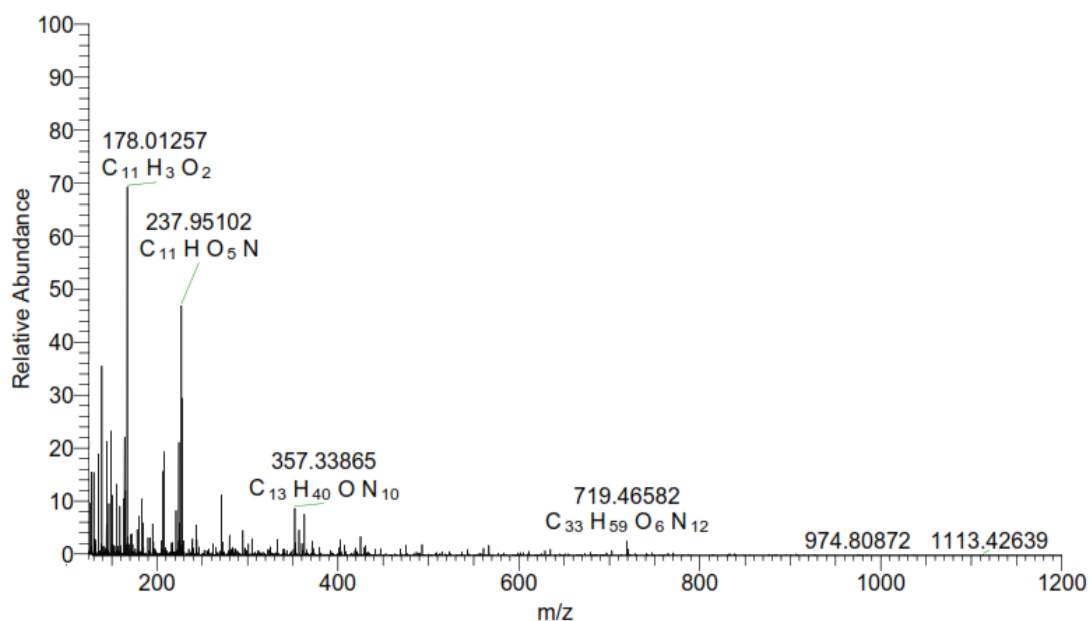


Figure 7.15 LC-MS chromatogram of the purified RKAFK peptide

HPLC chromatogram was performed at 214 nm. The crude peptide and purified peptide chromatogram are seen in Figure 7.13 and Figure 7.14, respectively. According to HPLC chromatograms, complete purification of the RKAFK peptide was performed. The peptide, which is thought to be pure as in other peptides, was seen at the first peak on the chromatogram. This was determined by the LC-MS. Similarly, ghost peaks were seen on the chromatogram at 3., 4., 5., 6., and 7. on this sequence.

According to the LC-MS chromatogram, RKAFK peptide was synthesized successfully. The peptide molecular weight was calculated as 718,89 g/mol with the help of 'PepCalc.com peptide calculator' program. In the chromatogram of the purified peptide sequence, the experimental molecular weight was calculated using m/z ratio 237.95, 357.34 and 719.46 in the mass spectrum as shown Figure 7.15.

$$[M + 3H^+]^{3+} = 237.95 \rightarrow M_w = 710.85 \text{ g/mol}$$

$$[M + 2H^+]^{2+} = 357.34 \rightarrow M_w = 712.68 \text{ g/mol}$$

$$[M + H^+]^+ = 719.46 \rightarrow M_w = 718.46 \text{ g/mol}$$

$$M_{W\text{average}} = 714.00 \text{ g/mol}$$

$$M_{W\text{theoretical}} = 718,89 \text{ g/mol}$$

The above values indicate that the experimental molecular weight and the molecular weight calculated by program have very close levels.

7.5 Conclusion and Future Studies

Within the scope of the thesis, synthesis of four peptides which designed by CADD method was performed. These designed peptides have high binding potential against FOLR1, which is known to be highly expressed in cancerous cells. The proposed RPAAK, RFAAK, HRAFAK, and RKAFAC peptide sequences were synthesized by SPPS method. The peptides were successfully purified by HPLC. The peaks 3., 4., 5., 6., and 7. (if any) observed in all HPLC chromatograms were probably “ghost peaks”. The purified peptides were analyzed by LC-MS. It was observed that all peptides were synthesized successfully. The experimental molecular weight and theoretical molecular weight have very similar for all peptides.

In the next studies, the synthesized peptides will be examined for their binding affinity to the FOLR1 protein on the ITC device. The selected peptides will then be stained with fluorescence dye to proceed *in vitro* cell testing. The binding affinity of the peptides to the cancer cells will be determined by fluorescence dye in the cell test. Rhodamine-B will be used as a fluorescence dye. The structure of Rhodamine-B is shown in the Figure 7.16. This is because the carboxyl group of Rhodamine-B reacts with the amine group easily any without any additional reaction in the peptide sequence [354], [355]. Thus, Rhodamine-B pretended to be an amino acid and attached to the peptides.

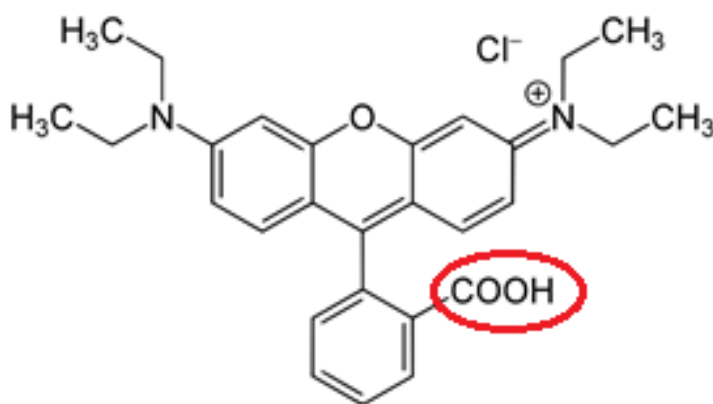


Figure 7.16 Chemical structure of Rhodamine-B dye

PET/CT is the most advanced technology used for oncological imaging, which not only provides anatomical information but also information on the metabolic and functional status of tissues and organs, and therefore compounds which are found to be biologically active will be identified after ITC and *in vitro* tests. The biologically active peptides obtained in this thesis will be evaluated as PET imaging agents. With this aim, selected peptides will then be radiologically labeled with ^{68}Ga and DOTA-chelates (Figure 7.17) for use in PET/CT devices. Amide bonding between the free carboxylic acid group on the DOTA and the amine group at the ϵ -ammonium group (NH_3^+) of the lysine will allow the peptide to bind to DOTA. Thus, the final molecule will allow the DOTA to be attached to the structure while preserving the main nucleus that constitutes the biological activity in the designed compounds. Binding of DOTA to the peptide will be bound by EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) / (N-hydroxysuccinimide) chemistry due to the amine group on DOTA [356]. After the DOTA group has been attached, all three *t*-Boc protecting groups on DOTA will be removed and the resulting molecules will be ready to label with ^{68}Ga .

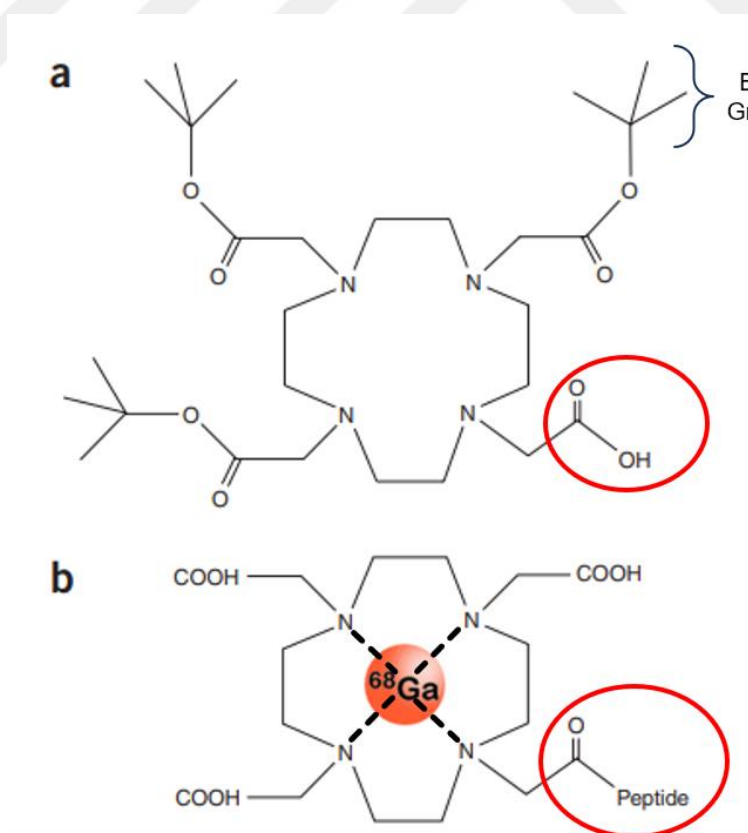


Figure 7.17 Chemical structure of (a) DOTA-tri-*t*-Butyl ester (tri BOC-DOTA) (b) the conjugated ^{68}Ga -DOTA-peptide [357]

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PUBLICATIONS FROM THE THESIS

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Conference Paper

1. C. Özel, Ö. Yılmaz, and S. Yücel, "Design and Evaluation of Peptide Usage for Cancer Imaging," in XIII International Scientific Conference of postgraduates, masters and young researchers on "ACTUAL PROBLEMS OF CHEMISTRY," 2019, pp. 14–15.

