

**T.C.
BURDUR MEHMET AKIF ERSOY UNIVERSITY
INSTITUTE OF SCIENCES
DEPARTMENT OF CHEMISTRY
DOCTORAL THESIS**

**DESIGN AND SYNTHESIS OF CISPLATIN-LOADED
MULTIFUNCTIONAL NANOCARRIERS AND
EVALUATION OF THEIR ANTICANCER ACTIVITY
AGAINST AFATINIB-RESISTANT TRIPLE-
NEGATIVE BREAST CANCER (TNBC) CELLS**

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BURDUR, 2025

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PREFACE

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LIST OF SYMBOLS AND ABBREVIATIONS

A	: Absorbance
UV	: Ultra Violet
BH	: Boltorn H
mL	: Mili Liter
mm	: Mili Meter
nm	: Nanometer
λ	: Wavelength
°C	: Centigrade
FA	: Folic Acid
HA	: Hyaluronic Acid
DMSO	: Dimethyle sulfooxide
BC	: Breast Cancer
TNBC	: Triple Negative Breast Cancer
-OH	: Hydroxyl group
MTD	: Maximum Tolerated Dose
%	: Percentage
IC50	: Inhibition Concentration
MDR	: Multidrug resistance
DLE	: Drug Loading Effeciency
NMR	: Nuclear Megnatic Resonance
FT-IR	: Fourier Transform Infrared Spectroscopy
ROS	: Reactive Oxygen Species
MS	: Mass Spectrometry
SEM	: Scanning Electron Microscopy
TEM	: Transmission Electron Microscopy
DSC	: Differential Scanning CALorimetry
μl	: Microliter
h	: Hour
min	:Minutes
MS	: Mass Spectrometry

ÖZET

Doktora Tezi

Sisplatin Yüklü Multifonksiyonel Nanotaşıyıcıların Tasarımı, Sentezi ve Afatinib Dirençli Üçlü Negatif Meme Kanseri (TNBC) Hücrelerine Karşı Antikanser Aktivitesinin Değerlendirilmesi

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Tez çalışmasında, ilaç taşıyıcı olarak kullanılabilecek çok işlevli hiperdallanmış polimerik nanopartiküllerin sentezi gerçekleştirilmiştir. Sentezlenen nanopartiküllerin affatinib dirençli triple negatif meme kanseri hücreleri için hedefli ilaç taşıyıcı olarak kullanılması amaçlanmıştır. Sentezlenen nanotaşıyıcılar infrared spektroskopisi (FT-IR), ultraviyole-görünür spektroskopisi (UV-Vis), nükleer manyetik rezonans (NMR), kütle spektroskopisi (MS), diferansiyel taramalı kalorimetri (DSC), termogravimetrik analiz (TGA), Zeta Sizer (DLS) ve floresans spektrometrisi kullanılarak karakterize edilmiştir. Yapılan analizler, nanotaşıyıcıların çekirdek molekülü olan BHX (40,30,20)'in HFBCE ve Flamma 774 izotiyosiyanat ile başarılı bir şekilde fonksiyonelleştirildiğini göstermiştir. Ayrıca kabuk kısmı, hyaluronik asit ile birlikte folik asit ve -R8-c(RGD) peptidi ile işlevsel hale getirilmiştir. Zeta Sizer analizleri, partiküllerin 200 nm'nin altında olduğunu göstermiştir ve bu sonuçlar SEM ve TEM analizleriyle doğrulanmıştır. Termogravimetrik analizler, hem BHX hem de HA'nın fonksiyonelleştirilmesini desteklemiş, bu durum NMR ve MALDI-TOF analizleri ile de teyit edilmiştir. Nanotaşıyıcıların sitotoksikite analizleri, hem normal CC14CO hücrelerine hem de kanser hücre hattı CAL 51'e karşı toksik etkisinin olmadığını göstermiştir. Cisplatin yüklü çok işlevli nanotaşıyıcıların floresans spektrometrik analizleri, kanser hücreleri tarafından başarıyla alındığını göstermiştir. Cisplatin yüklü nanotaşıyıcıların kanser hücrelerine karşı, normal hücre hattına göre daha fazla etki gösterdiği ve hedefleme özelliğinin kanser hücrelerinde nanotaşıyıcı alımını artırdığı gözlemlenmiştir. Benzer sonuçlar serbest cisplatin ile karşılaştırıldığında da elde edilmiştir. Cisplatin yüklü nanotaşıyıcıların antikanser aktivitesi, afatinib dirençli CAL51 hücrelerde serbest cisplatine göre daha etkili bulunmuştur.

Anahtar Kelimeler: boltorn, dendrimer, nanomalzeme, afatinib, hiyaluronik asit

Hazırlanan bu Doktora tezi TÜBİTAK tarafından (Proje no: **121S877**) proje numarası ile ve Burdur Mehmet Akif Ersoy Üniversitesi BAP Koordinatörlüğü tarafından (Proje no: **0825-DR-22**) proje numarası ile desteklenmiştir.

ABSTRACT

Ph.D. Thesis

Design and Synthesis of Cisplatin-Loaded Multifunctional Nanocarriers and Evaluation of Their Anticancer Activity Against Afatinib-Resistant Triple-Negative Breast Cancer (TNBC) Cells

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Department of Chemistry**

Supervisor: Prof. Dr. Mehmet Fatih EMEN

August, 2025

In this thesis the synthesis of multifunctional nanocarrier as a drug delivery material was carried out. The aim of the study was to apply these designed nanocarrier as targeted delivery carriers in triple negative breast cancer. These nanocarriers were synthesized and characterized by using infrared spectroscopy (IR), ultraviolet-visible (UV-Vis), Nuclear magnetic resonance (NMR) and mass spectroscopy (MS), Differential scanning, thermogravimetry, Zeta Sizer (DLS), and fluorescence spectrometry.

The results of analysis showed that core molecule BHX (40,30,20) of nanomaterial was successfully functionalized with HFBCE and Flamma 774 isothiocyanate. As well as shell of hyaluronic acid with folic acid and -R8-c(RGD) peptide. Zeta sizer analysis showed that particles are less than 200 nm which was further confirmed by SEM and TEM analysis. Thermogravimetric analysis supports the functionalization of both BHX and HA, which was further confirmed by NMR, and MALDI-TOF analysis. Cytotoxicity analysis of these nanomaterials showed no toxic effect to both normal CC14CO and cancerous cell lines CAL-51. Fluorescence spectrometric results of these nanosized multifunctional nanocarrier loaded with cisplatin, had shown successful uptake by cancerous cells. It was observed that cisplatin loaded nanocarriers significantly affect the cancerous cells compare to normal cell line which shows targeting enhance the uptake of nanocarriers in cancer cells compare to normal cell line. Similar results were observed in free cisplatin and loaded onto nanocarriers. Anticancer activity of nanocarriers loaded with cisplatin were more affective compared to free cisplatin in afatinib resistant CAL-51 cells.

Keywords: boltorn, dendrimer, nanomaterial, afatinib, hyaluronic acid

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1. INTRODUCTION

Cancer is the major cause of fatality worldwide which adversely affects the lifestyle of patients. Mostly the occurrence of cancer is because of the changes in genome of cells, which have both positive and negative impacts on the body, however, sometime these changes adversely affect the body and lead to serious consequences. These changes are effects of primary exposure to some deteriorating factors such as exposure to UV light, imbalance of oxidants level, unhealthy food or microbial infection. These factors affect the suppression or over expression of metabolic pathways in the cells. Which causes inflammation, cancer, Alzheimer, diabetics and other health issues.

Normally cells grow and become old, either damaged or died and replaced by new cell. Sometime cells go for uncontrol cell division and multiply faster than normal cell growth. Thus, it leads to the formation of clumps of cells which create their own microenvironment, vascular system for transporting nutrients and exchange of gases. This happens when cellular repairing systems fail to recognize the mutation in the cell or are unable to repair the mutation in the cell. Instead of going for apoptosis, the only aim of cell is to survive. This clump of cells either behave as benign (localized) or malignant tumor which spread in whole body during metastasis. In pharmacotherapy, bioactive material used for treatment of different diseases has some beneficial action, however it can also exhibit side effects. For example, cytotoxic drug used in chemotherapy kill targeted cell but cause undesirable side effect to other body cells by killing them. To counter this problem nanocarrier play significant contribution to minimizing the side effect of drug by making it more specific with high therapeutic efficiency.

Nanotechnology is an ingenious approach with tremendous potential in drug delivery systems. Nanotechnology with parallel recent breakthroughs in controlled manipulation of material at nanoscale and understanding of disease at molecular level offers exceptional power in diagnosis, prevention and targeted therapy. Nanomaterials are made up of a variety of organic materials such as lipids, dendrimers, biodegradable and non-biodegradable polymers and inorganic materials including nanocrystals, semiconductor materials, metallic nanoparticles etc (Ganta et al., 2008.; Moghimi et al., 2005; Torchilin, 2006) as shown in Figure 1.1. For therapeutic purposes, designing and synthesis of nanomaterials are mainly selected by the type of payload, route of administration, material safety profile, diagnostic and therapeutic goal (Ganta et al., 2006.; Moghimi et al., 2005; Torchilin, 2006). Moreover, intracellular stability of these

nanocarrier materials and targeting the specific biomarkers on the cell surface enhance the cellular uptake (Ganta et al., 2008).

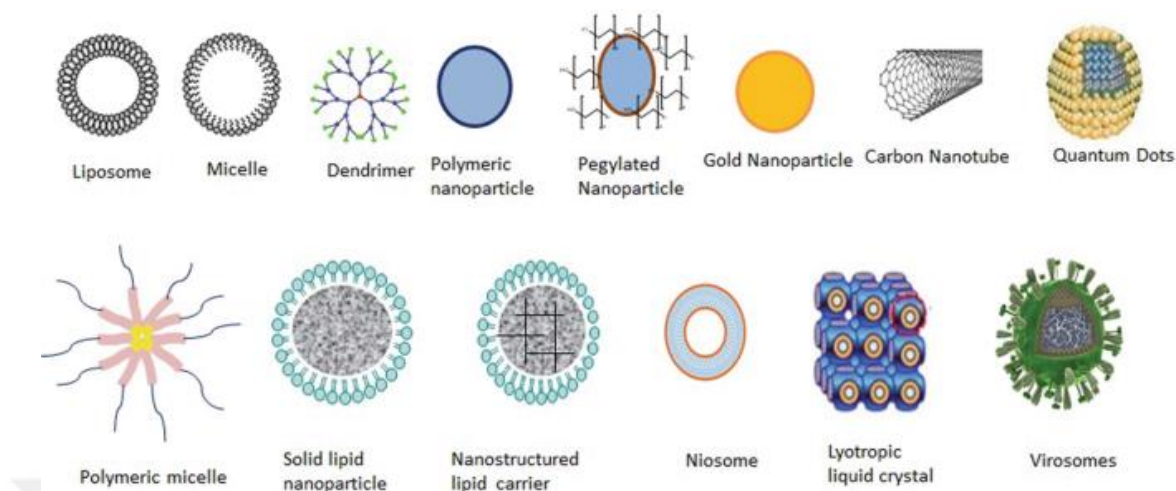


Figure 1.1. Different types of therapeutic nanocarrier for drug delivery (Singhvi et al., 2020).

Nowadays, biodegradable polymers have got much attention due to their least cytotoxicity effect and biocompatibility properties. Among organic biodegradable polymers, dendrimers are hyperbranched, synthetic polymeric macromolecules of nanometer dimensions. Branches of these polymeric molecules start from a central core and have multiple available functional groups on terminal end. Among them, Boltorn is a biocompatible, biodegradable hyperbranched dendrimer, having a central core and high extend of hydroxyl (-OH) surface groups. Boltorn is an interesting alternative in drug delivery application among dendrimers as this polyester based on bis-MPA needs tedious and multistep synthesis but allows easier access to larger quantities of delivery vehicles (Zeng et al., 2012). To enhance the cellular uptake and develop polarity to load more cargo in drug carriers allows researchers to design them with different ligands. It has been reported in literature that fluorinated molecules facilitate the interaction with cellular membrane, therewith enhancement of the cellular uptake. Fluoropolymers become ideal delivery vehicles for transfer of drugs and biomacromolecules. Biodegradable polymers are tailored with fluor groups to tackle the delivery challenges, which help by enhancing stability, permeability, serum tolerance and endosomal escape of DNA and protein/peptides (Song et al., 2022). In this study, core of the nanocarrier was designed by tailoring the Boltorn HX (X=40, 30, 20) with heptafluorobutyle chloride and flamma 774 isothiocyanate as a fluorescent molecule. The core molecule behaves as a carrier because

of its hyperbranched nature which give space to the cisplatin an anticancer drug. For affective targeting of cancerous cells, biomarker targeting based shell was designed with conjugation of folic acid and R8-c(RGD) peptide. Affinity of these targeting molecules are toward the receptors molecules over expressed on surface of cancer cells compared to normal cells. That is why these markers help in the uptake of carrier molecules inside the cell and unload its cargo. Which reduces the negative effect of active materials on normal body cells and significantly selects the cancerous cell.

The aim of this study is to synthesize a target specific nanocarriers which eliminate or reduce the side effect on normal cells. Therefore, the core element of nanocarrier is synthesized with Boltorn H (40, 30 and 20) a hyperbranched polymer. which is selected and functionalized with heptafluorobutyl chloride for endosomal escape and produces a hydrophobic characteristic. Moreover, it was further functionalized with Flamma 774 isothiocyanate, which can give signal by fluorescing, an indication of successful delivery of therapeutic materials to the targeted site. On the other hand, the shell of nanocarrier is designed to target folate receptors and integrin receptors on surface of cancerous cell line for targeted therapy. Moreover, nanocarrier is loaded with cisplatin are applied on TNBC cells have developed resistance against afatinib a tyro kinase inhibitor. The designed nanocarrier was successfully synthesized and applied on TNBCs during this study.

2. GENERAL INFORMATION

2.1. Cancer and Its Prevalence

Cells grow to normal size and divide repeatedly at specific times. Sometime, this behavior changes by the effect of external or internal stimuli, which results in a change in division period and ultimately cell multiply in numbers resulting in a clump of cells. This abnormal growth and uncontrolled cell division, that lead to metastasis by invading other body tissue is characterized as cancer. This progressive development in cancerous cells is due to some internal and external factors such as oxidative stress, free radicals, exposure to ultra-violet (UV) light and chemicals, which influence cell growth negatively as shown in Figure 2.1. Classification of cancer depends on the type of cells from which it forms, such as leukemia (blood tissue), multiple myeloma (plasma cells, myeloma cells), carcinoma (from epithelial cells), sarcoma (formed in bone and soft tissues) and lymphoma (formed in B or T lymphocytes) (Takabe ve Benesch, 2022). Cancer has been become leading cause of death globally causing nearly 10 million deaths in 2020.

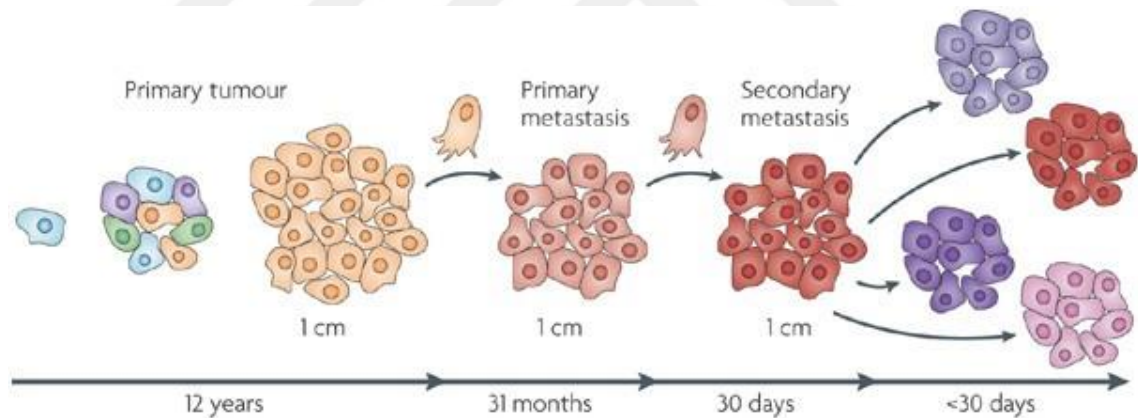


Figure 2.1. Cancer progression and metastasis (Klein, 2009)

Overall, prevalence of cancer increases worldwide; only in united state approximately 1,665,540 peoples are affected and 585,720 died in 2014 (Hassanpour ve Dehghani, 2017a). According to the statistical data of WHO about cancer, nearly 10 million deaths have been reported in 2020. Among them most leading cases reported were breast cancer with approximately 2.26 million followed by lung cancer of 2.21 million cases. While the most common cause of death due to cancer was reported in patients with lung cancer with around 1.8 million followed by colon and liver cancer. In patients with breast cancer, 2.3 million women were diagnosed with breast cancer. Moreover, 0.5-1%

cases of breast cancer reported in male only. However, 670,000 deaths have been reported globally only in 2022 in patients with breast cancer as shown in Figure 2.3.

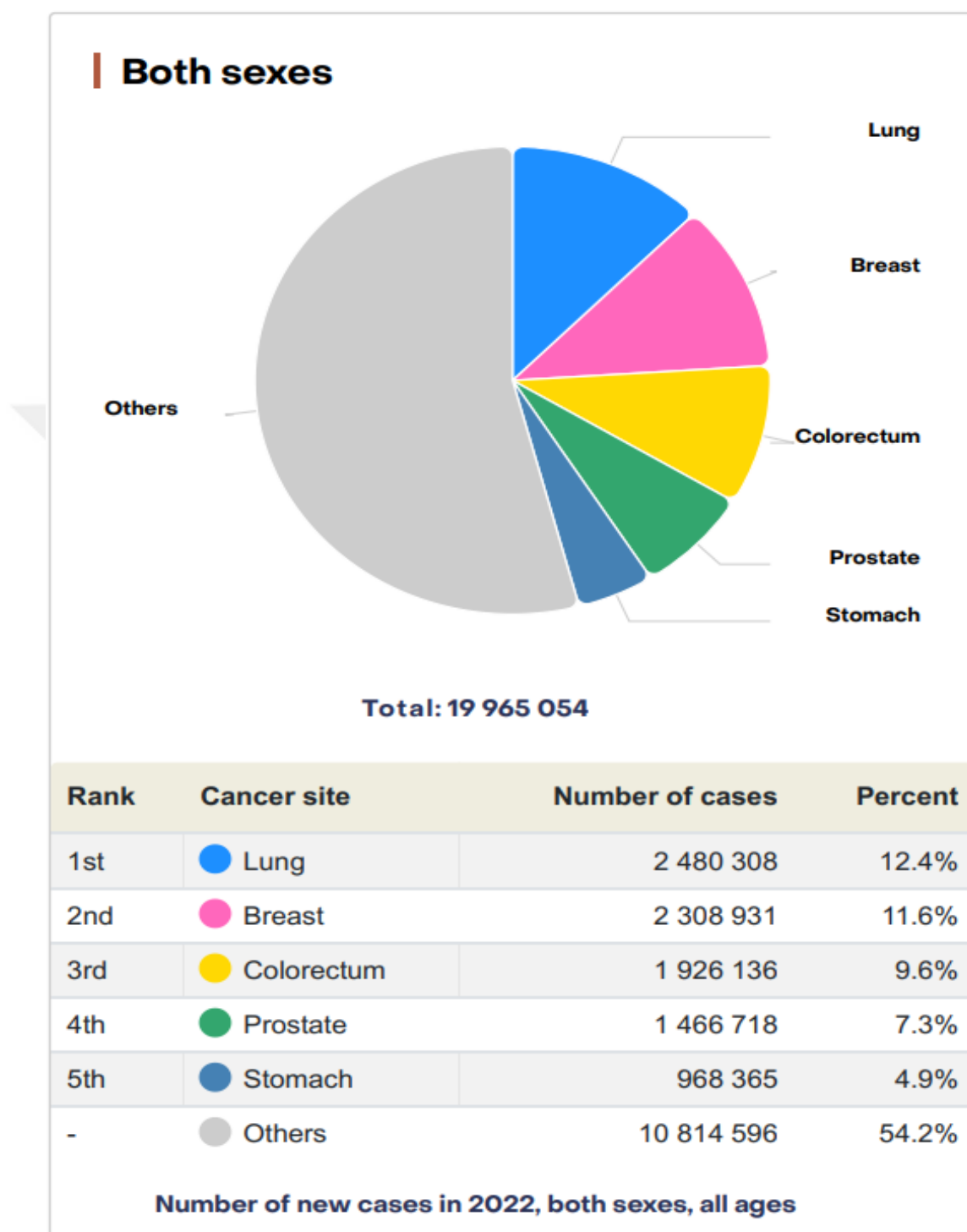


Figure 2.2. Statistical report of cancer cases by WHO (*Cancer*, 2025.)

The highest percentage of cancer types occur in man is prostate, lung, colon, urinary bladder and rectum cancer. However, its prevalence ratio in women is high in

breast, lung and broncos, colon and rectum cancer respectively. A series of successive mutations occur in genes, which lead to the development of cancerous cells, because of change of cellular functions. Exposure to various chemical compounds, environmental chemical substance, with some carcinogenic properties, viruses, bacteria, smoking and consumption of alcohol are directly or indirectly involved in mutation in genomes by affecting cytoplasm or nucleus of cell, lead to genetic disorders and mutation (Antwi et al., 2015; Hassanpour ve Dehghani, 2017b; Poon et al., 2014; Trafialek et al., 2014). In general, studies have shown that cancer disturbed the cellular mechanism by dysfunction vital genes, ultimately affecting the cellular functions and its metabolism. This dysfunction effect cell cycles, which triggers the proliferation of cells abnormally.

2.2. Breast Cancer and Its Types

With the elevation in occurrence of breast cancer, approximately 3.2 million new cases per year by 2050 has been predicted for female (Hortobagyi et al., 2005.). This elevation in numbers indicates the magnitude of incidence, its effects on societies, and the need for diagnostic, preventive and treatment measures worldwide. Breast cancer has found highly heterogeneous and most occurring types of cancer in women in terms of its etiology and pathological characteristics (Tao et al., 2015). Classification of breast cancer is based on clinical morphology, histological, genetic and epigenetic factors. Clinically, morphological characteristics of tumors include infiltrating ductal carcinoma of no special types and special types, such as infiltrating lobular carcinoma, mucinous, medullary, tubular and adenoid cystic carcinoma. Histologically, breast tumor was further divided by degree of differentiation, nuclear pleomorphism and mitotic count type classification. Furthermore, breast cancer has also been classified based on expression of cellular receptors such as Estrogen (ER), Progesterone (PR) and HER2. Among the expressions of these receptors on the surface of cell, ER positive found a more common type of cancer (Anderson et al., 2002). Moreover, they are further sub divided on the base of expression of receptors such as ER/PR-positive subgroups of luminal A and Luminal B. As well as three different ER-negative types; HER2 type, Basal-like type, and Normal like subtypes. These subtypes are characterized by elevated level of expression of HER2 receptors and associated genes. The second type of ER negative is associated with higher expression genes identified with myoepithelial or basal cell, while the third one shows varied gene expression and behave like normal-like subtypes (Perou et al., 2005.). Luminal and normal-like subtypes of cancer exhibit strong level of expression compared

to both HER2 and basal-like subtypes of ER negative breast tumors (Bauer et al., 2007). Some studies show that, some tumors did not overexpress Er, PGR and HER2 receptors on their cells, for why this type of tumors are classified as triple negative tumor. These tumors are very aggressive in nature and their prognosis become very difficult because of lack of receptors which also limited the effect of chemotherapy and treatment with trastuzumab etc (Nofech et al., 2009a).

2.2.1. Triple Negative Breast Cancer

Classically, breast cancer can be divided into 18 different subtypes based on its histological and morphological characteristics. However, only classification based on histo-morphology based characteristics failed to divide cancer type because of the difficulties in prognosis and treatment possibilities. Therefore, a new approach based on gene expression profile was applied by DNA microarray experiments to characterize the types of cancer. There was a major difference in expression of hormone receptor (HR) genes, which were either HR-positive or HR-negative (Sorlie., 2001). The HR-positive tumors clustered have shown expression patterns like luminal epithelial mammary cells, while HR-negative type was further divided into three types of subgroups on the base of their expression: normal mammary stromal cancer, characteristics with Her2 gene expression and basal/myoepithelial mammary cell type cancers (Sorlie., 2001). Studies showed that Her2 oncogene belongs to the epidermal growth factor receptor (EGFR) family which overexpresses almost 20% of the breast tumors fall in this category (Caddeo et al., 2013.; Huang et al., 2005.; Hudis, 2007). Studies shown that, patient with BRCA1 mutation, African-American women and younger women have generally higher incidence of TNBC (Dent et al., 2009., Morris et al., 2007) with a higher mortality rate of 40% within 5 years after diagnosis. TNBC develop in early age of life, however, most often occur in premenopausal women. Prognosis of the TNBC is very poor therefore TNBC patients suffer more compared to the other types of breast cancer (Carey et al., 2006; Nofech et al., 2009b; Rhee et al., 2008). TNBCs are larger, grade III type tumor with aggressive phenotype (Dent et al., 2006, Dent et al., 2009; Morris et al., 2012). The risk of estrogen receptor-positive tumor in women who gave birth to more than two children is 46% compared to the postmenopausal women with lower risk of 12% (Phipps et al., 2011.).

2.3. Diagnostics and Treatment of Breast Cancer

For the diagnosis of TNBC, significant challenges must be faced because of the aggressive nature and lack of specific targeting receptor sites on the surface of cancer cells. Therefore, accurate and timely detection of various methods have been developed including imaging examination, histological and immunohistochemical testing as well as expression of molecular biomarker (Jie et al., 2025a).

Imaging Examination:

For the diagnosis of TNBC, its characteristics and assessment of disease progress, visualization by imaging technique is very essential. In this context, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has shown promising results in detection of TNBC among various imaging methods. This technique provides information about tumor size, enhancement pattern and irregularity in margin which are vital for accurate diagnosis and treatment process (Chen et al., 2022). In addition, ultrasound has been used as early detection tool, especially in areas lacking advanced technique and traditional imaging provide results with low resolution (Yukun, 2021).

Histological and Immunohistochemically Testing:

In histological and immunohistochemical testing analysis tissue samples were obtained by biopsy to investigate the morphological characteristics and status of receptors on the surface of tumor cells. This methodology differentiates the TNBC from other subtypes of breast cancer by evaluating the overexpressed biomarkers such as Ki-67. High level of proliferating receptor Ki-67 expression indicates the aggressiveness of the TNBC tumor (Huang et al., 2000). In addition, immunohistochemistry tests to assess the immune check point markers give information about the microenvironment and its effect on treatment process. The PD-L1 expression in TNBC cells correlated with the efficacy of immunotherapy as a critical factor in therapeutic strategies (Patel et al., 2024.).

Role of Molecular Biomarker

It is a transformative approach to diagnose and manage TNBC by identifying potential indicators as biomarkers. Overexpression of PIM-1, PLAC8 and MiR-522 have been associated with TNBC and highlight as potential therapeutic target. Studies have shown that certain types of micro-RNA like MiR-5222 are linked to metastasis and enhance overall survival of TNBC cells in patients. That's why these receptors are utilized as prognostic markers in the diagnosis of TNBC (J. Chen et al., 2019; Patel et al., 2000.).

These biomarkers play a vital role not only in diagnosis but also help in the development of personalized medicine approaches.

2.4. Treatment of Triple Negative Breast Cancer

Due to lack of standard molecular targets, TNBC have historically very limited options for the treatment compared to other types of breast cancer, which is cytotoxic chemotherapy. In various clinical settings, taxanes (paclitaxel) and anthracycline (doxorubicin) have shown high efficacy as a traditional chemotherapy (Zhao et al., 2020a). Cyclophosphamide, Paclitaxel and Adriamycin in combination therapy have shown 35-45% outcomes as a standard neoadjuvant in chemotherapy against TNBCs (Bou et al., 2022). The National Comprehensive Cancer Network (NCCN) have recommended the systematic chemotherapeutic regimens for TNBS include Taxel/Docetaxel, Cyclophosphamide, Adriamycin, Fluorouracil (CAF), Epirubicin and Paclitaxel/Docetaxel (CEF-T). These DNA binding agents damage it and show promising anticancer activity, especially in cancer cell lines with germline BRCA mutation, as this protein plays vital role in repairing of damaged DNA (Byrski et al., 2010). Another study has shown the use of antibody-drug conjugates (ADCs) as a combinational therapy of trastuzumab emtansine and brentuximab vedotin for metastatic HER2+BC and Hodgkin lymphoma respectively (Fenn ve Kalinsky, 2019; Minckwitz et al., 2019; Younes et al., 2010). Similarly, sacituzumab govitecan (SG) is another ADC consisting of anti-trophoblast cell surface antigen 2 (Trop-2) combined with SN38 a antineoplastic drug and an active metabolite of irinotecan a Topoisomerase I inhibitor. This therapeutic strategy has shown promising activity in all types of cancers, particularly breast and lung cancer (Bardia et al., 2021; Tolaney, et al., 2021; Goldenberg et al., 2015; Zaman et al., 2019).

It was found that 25 % TNBC tumors have hyperactivation of PI3K/AKT/mTOR signaling pathways in mesenchymal and LAR subtypes. AKT inhibitors have shown promising efficiency in the treatment by disturbing the AKT/PTEN pathways. Another study investigated the expression of androgen receptors about 10-15% in TNBCs. A very encouraging results of non-steroidal androgen synthesis inhibitor TAK-700 was obtained in combination with antiandrogen enzalutamide (Niemeier et al., 2010.; Traina et al., 2018; Yamaoka et al., 2012.). It was observed that chemotherapy gives a high response in cancer patients in early-stage of disease. However, its efficacy is tempered by adverse side effects, such as anemia, gastrointestinal disturbance and neutropenia (Huang et al., 2021; Jie et al., 2025b; Zhao et al., 2020b). These side effects impact the quality of life

adversely, which leads to reduction in dose and delay in treatment in cancer patients. Researchers have started to understand the mechanism of chemotherapy resistance by investigating the role of genetic mutation and microenvironment of the tumor to mitigate these adverse effects by development of new strategies (Dai et al., 2019; Tan et al., 2023). To overcome the cytotoxic effect of chemotherapeutic agents, nanotechnology can play a significantly positive role. Nanocarriers can be designed for targeted therapy and control release. Their size and high surface area to volume ratio give positive age to enhance the efficacy of the therapeutic agent by reducing the side effects.

2.5. Cisplatin

Cisplatin or cisplatinum, chemically known as *cis*-diamminedichloroplatinum(II) ($\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$), is a first square planar metal (platinum) based coordination compound used as a chemotherapeutic agent (Hannon, 2007; Martinho et al., 2019). Cisplatin was discovered by Michele Peyrone in 1845, while its cell division inhibiting ability was discovered in 1965 by Dr. Barnett Rosenberg (Shah ve Dizon, 2009; Trimmer, 1997). Cisplatin and its derivatives have been sold with ~2 billion U.S. dollars worldwide as anticancer agent and almost 50% of the cancer patients are treated with cisplatin (Galanski et al., 2005). In late 1970s, cisplatin was used as antineoplastic agents for hematologic and solid tumor malignancies. Cisplatin was approved as a therapeutic agent for the treatment of ovarian cancer, testicular cancer and bladder carcinoma (Kondagunta et al., 2005; Ozols et al., 2003). Studies have shown that, cisplatin in combination with other medications such as doxorubicin, 5-FU and taxans have been applied for the treatment of breast cancer, ovarian and cervical cancers (Fleming et al., 2004; Silver et al., 2010; Whitney et al., 1999). Cisplatin was also found effective against metastatic, advanced and refractory cancer which include penile cancer, head and neck cancer, Hodgkin lymphoma, non-Hodgkin lymphoma, multiple myeloma and osteosarcoma.

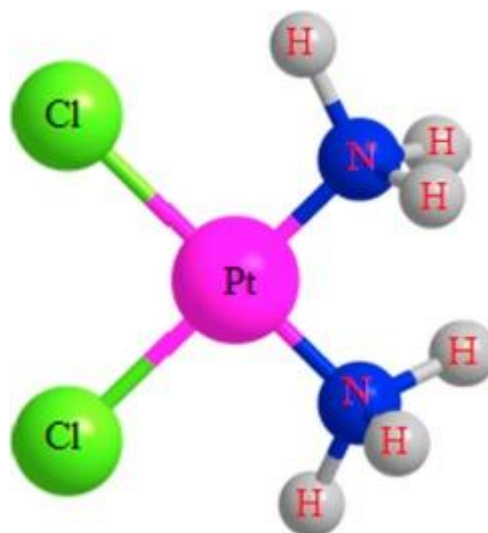


Figure 2.3. Structure of cisplatin (Florea et al., 2011.)

The mechanism of action of cisplatin works by binding of platinum covalently with purine bases (guanine and adenine) and causes non-cell cycle-specific cytotoxicity. This strong covalent binding with inter strand and intrastrand cross linking which leads to strand break. Thus, repairing mechanism of cell activates which lead cell toward apoptotic cell death because of permanent DNA, RNA and protein damage (Casini et al., 2018). Crosslinking to DNA causes the cell cycle arrest at S, G1, G2 and M phase, ultimately lead to apoptotic cell death. Another study explains the mechanism of action of cisplatin by targeting cells more than one sites, as it binds with both genomic DNA and mitochondrial DNA, creates DNA lesions and arrests the replication process and ultimately blocks the production of DNA, mRNA and proteins (Achkar et al., 2018; Ciccarelli et al., 1985; Jordan ve Carmo, 2000; Oncogene, 2003; Riddell et al., 2018.; Sigel et al., 2018).

2.5.1. Cisplatin in Breast Cancer

In recent years, cisplatin has gained attention in breast cancer therapy due to its effectiveness and potential role in precision medicine. Cisplatin induces damage in DNA in cancer patients. However, the patients with BRCA1 and BRCA2 mutation in genome exhibit highest sensitivity to cisplatin because of the damaged DNA repair system in damaged cancerous cells. It works by binding to DNA and forms intra-strand and interstrand crosslinks, which ultimately affect the replication and transcription process of cell and lead to apoptosis. In breast cancer cisplatin has been particularly investigated in TNBCs which often don't express targeting receptors molecules (Achkar et al., 2018;

Ciccarelli et al., 1985; Jordan ve Carmo, 2000; Oncogene, 2003; Riddell et al., y.; Sigel et al., 2018).

2.5.2. Mechanism of Action of Cisplatin and its Side Effects

Cisplatin works efficiently in fast growing malignant cells compared to slow-growing tumor (Aggarwal, 1993). It is stable at room temperature and pressure; however, it converts into its trans-isomer over time. It has molecular weight of 301gm/mol and a density of 3.74 g/cm³ with 2.35 g/L of solubility in water at 25 ° C. Cisplatin eliminated from the body by urination with 10 % of it in bile. The terminal half-life of cisplatin is about 24 hours; however, initial half-life is just 20-30 minutes. The mode of administration of cisplatin is generally intravenous by infusion with saline solution for the treatment of solid tumors. It is highly toxic therefore cancer patients need highly controlled intake as it causes serious side effects (Aldossary, 2019). Cisplatin treat malignancy, however it has very high cytotoxicity. If a patient is suspected of extravasation after treatment with cisplatin, the infusion of drug stopped immediately and sodium thiosulfate as antidot is administered to the patient (Ener et al., 2004). The secondary side effect in patients treated with cisplatin face leukemia which occurs years after completion of treatment (Barabas et al., 2008; Packer et al., 2013). It was observed that, tumor lysis syndrome also occurs in patients after cisplatin treatment as well as many common side effects like nausea, vomiting, diarrhea, hiccups, temporary hair loss, dark urine, dry skin and dehydration also occurs (Baeksgaard ve Sørensen, 2003; Barabas et al., 2008; Packer et al., 2013). Another study describes the major toxicities of cisplatin include nephrotoxicity, ototoxicity, neurotoxicity and hepatotoxicity (Astolfi et al., 2013; Dasari et al., 2014.; Manohar et al., 2018; Oun et al., 2018.).

2.6. Afatinib a Tyrosine Kinase Inhibitor

Afatinib or Gilotrif, is a second-generation anilinoquinazoline, a tyrosine kinase inhibitor used as a medication to treat locally advanced or metastatic non-small cell lung carcinoma. This tyrosine kinase inhibitor targets epidermal growth factor receptor (EGFR), which activate multiple downstream growth pathways (Keating, 2016). EGFR is a group of tyrosine kinase receptors also known as HER or ErbB, which include (HER1/ErbB1), (HER2/ErbB2), (HER3/ErbB3) and (HER4/ErbB4) receptors. A mutation in EGFR lead to overexpression of HER2 receptors on NSCLC tumors and adenocarcinomas. It has been investigated that L858R substitution mutation at exon 21

and exon 19 deletion mutation in EGFR is most common (Keating, 2016). In patients with HER2 positive breast cancer, afatinib has been investigated as mono therapeutic drug. Structural formula of afatinib is given below in Figure 2.2.

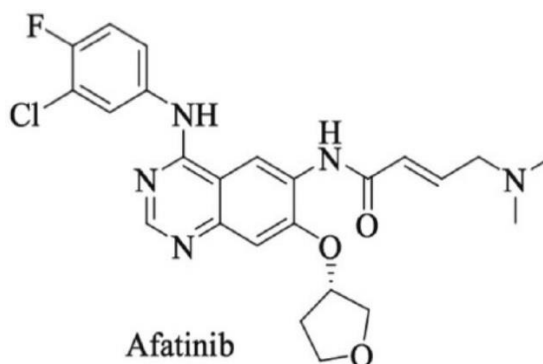


Figure 2.4. Structural formula of Afatinib (Ricciuti et al., 2018)

There are three different TKIs such as gefitinib, erlotinib and afatinib. Among them afatinib has been proven as most effective in treatment of NSCLC (Lin et al., 2012; Yang et al., 2017). According to the investigation of Aredo and coworkers, afatinib had shown limited activity in Osimertinib treated patients, which have developed resistance because of acquired EGFR mutation (Aredo et al., 2022).

The mechanism of action of afatinib involves irreversible inhibition of epidermal growth factor receptors (EGFR) and human epidermal growth factor receptors (HER 2 and HER 4). Both EGFR and HER2 receptors play significant role in proliferation and vascularization. These tyrosine kinase receptors overexpress in many types of cancer cells. It has been reported that afatinib exerts antineoplastic effects in squamous carcinoma cells of head and neck by suppressing mTORC1, an apoptosis initiator. However, combining therapy of using afatinib together with autophagy inhibitors has shown improved effect on squamous head and neck cells.

Afatinib irreversibly binds to an intracellular tyrosine kinase domain, resultingly inhibition of ErbB receptors downstream cascade messaging. It was investigated that cell resistant to erlotinib and gefitinib or cell line contain EGFR L858R/T790 M mutation shown significant response to afatinib as irreversible inhibitor (Wirth, 2015). Moreover, afatinib was also found effective to treat E709X, E709-T7delinsX and some exon (19 and 20) insertional mutation.

According to phase I clinical trials studies on afatinib, it was determined that daily oral administration of afatinib is 40-50 mg as a daily maximum tolerated dose (MTD).

The efficacy of afatinib studied on four patients with NSCLC having EGFR exon 19 deletion mutation, was determined by Response Evaluation Criteria in Solid Tumors (RECIST).

2.7. Pharmacokinetics and Dynamics Study of Afatinib

Drug metabolism and pharmacokinetics of afatinib was studied on number of animal species including minipigs, rats, mice and rabbits. To investigate the bioavailability of [^{14}C]-radiolabeled afatinib in rat and minipigs, a wide distribution of drug was observed in most tissues, however, decline was observed till 312 h in tissues. A large distribution of afatinib after intravenous dosing was 12.4 and 16.2 L/Kg observed in animal species including minipig and rat respectively (Wind et al., 2017).

2.8. Drug Resistance in TNBCs

Development of drug resistance in cancer cells during chemotherapy is the major obstacle in successful treatment of malignancies leading to 90% of death (Łukasiewicz et al., 2021). Drug resistance is common in all types of cancer which is not mutually exclusive. Its meaning, a tumor can be resistant to multiple treatment strategies, leading to therapeutic failure, relapses and poor patient prognosis (Bianco et al., 2012; Chen et al., 2012; Musgrove et al., 2009; Pohlmann et al., 2009.; Vu ve Claret, 2012). It can be categorized as intrinsic (pre-existing) or acquired (developed during treatment), however the exact mechanism is complicated and not fully understood. Drug resistance or multidrug resistance (MDR) hinders the efficacy of active pharmaceutical agents leading to relapse or metastasis. Cancer cells develop resistance by various genetical or molecular changes in cellular mechanism. The intrinsic drug resistance occurs because of pre-genetic changes before treatment. While, acquired resistance developed by activation of proto-oncogenes after treatment. In addition, genetic mutation causes alternation in gene expression, epigenetic markers and the changes in microenvironment of tumor (Holohan et al., 2013). Moreover, cancerous cells overexpress transporter P-glycoprotein (P-gp) which is involved in drug efflux, enhance gene mutation involved in DNA repair, epigenetic modification, senescence escape, epithelial to mesenchymal transition and tumor heterogeneity (Cosentino et al., 2021; Holohan et al., 2013). The ATP-binding cassette (ABC) transporter subfamily is behind the decrease in intracellular accumulation of drug. Among (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG) transporter, ABCB1, involved in overexpression of (p-gp) protein in cancers which is linked explicitly

with MDR (He et al., 2021; Kumar et al., 2017; Noel et al., 2020.; Vadlapatla et al., 2013) for example, SKBR3 and MCF-7 BC are paclitaxel-resistance cell lines have significantly overexpression of ABCB1 protein. In TNBC, overexpression of ABCC1 and ABCG2 protein was observed compared to other breast cancer types (Nedeljković ve Damjanović, 2019). On the other hand, cancerous cell show resistance by using DNA damage repair (DDR) pathways. Most of the anticancer drugs including cisplatin, 5-fluorouracil, gemcitabine, and methotrexate damage DNA in cancerous cell and lead to apoptosis. However, Poly (ADP-ribose) polymerase (PARP) plays significant role in repairing damaged DNA by activating DDR pathways. Therefore, scientists use Niraparib and anti PD-1 antibody pembrolizumab a PARP inhibitor combination therapy in the treatment of TNBCs (Henneman et al., 2015; L. Y. Li et al., 2021). In contrast to both drug efflux and DNA repair mechanism, cancerous cells also go through epigenetic modifications. In this process cancerous cells do gene silencing, histone modification, hypermethylation at CpG sites, hypomethylation and alteration in non-coding RNA expression. It was observed that, use of targeted therapy by tamoxifen in ER+BC patients leads to development of resistance after treatment. However, ER expression was decreased in cells, which indicated that mutation and epigenetic mutations are causes of acquired resistance against tamoxifen (Zhu et al., 2016).

2.9. Nanotechnology and Nanoscience

The realm of nanotechnology has surfaced as a notable promising area of study to engineer or manufacture materials by controlling size at atomic or sub-molecular scale (Farokhzad ve Langer, 2009). The term nanoscience and nanotechnology are often used interchangeably. However, nanoscience refers to understanding the activity of natural laws at nanoscale level while nanotechnology belongs to practical applications of this scientific knowledge. The focus of nanoscience is to develop nanomaterials at the range of 1-100 nm at least in one dimension in size. These nanomaterials are synthesized by top down and bottom-up approaches. Recent advancements in the field of nanotechnology impacted the area of medicine for diagnosis, treatment, and control of biological problems. Nanomedicine is a new era with possible applications of use of nanomaterials as delivery system for drugs, DNA, mRNA, and other imaging agents. Variety of nanomaterials are available as pharmaceutical vehicles for controlled and targeted release studies.

2.9.1. Nanomaterials

Nanomaterials have attained many attentions of researchers because of their multidimensional application in agriculture, industry, health, food, antimicrobial martials, diagnosis, sensor, pharmaceutical and semiconductor areas. Many types of nanomaterials play an important role in the field of nanomedicine as nanocarriers for delivery of active materials. For the delivery of pharmaceutical active ingredients, a variety of nanoparticles including polymeric, metallic, quantum dots, micelles, microcapsules, liposomes, niosomes, exosomes, solid lipid nanoparticles, dendrimers have been used directly or by designing for targeted delivery. The paradigm of using drug loaded nanocarriers is to enhance the bioavailability of drugs, increase there in-vivo efficacy, and targeted therapy (Farokhzad ve Langer, 2009).

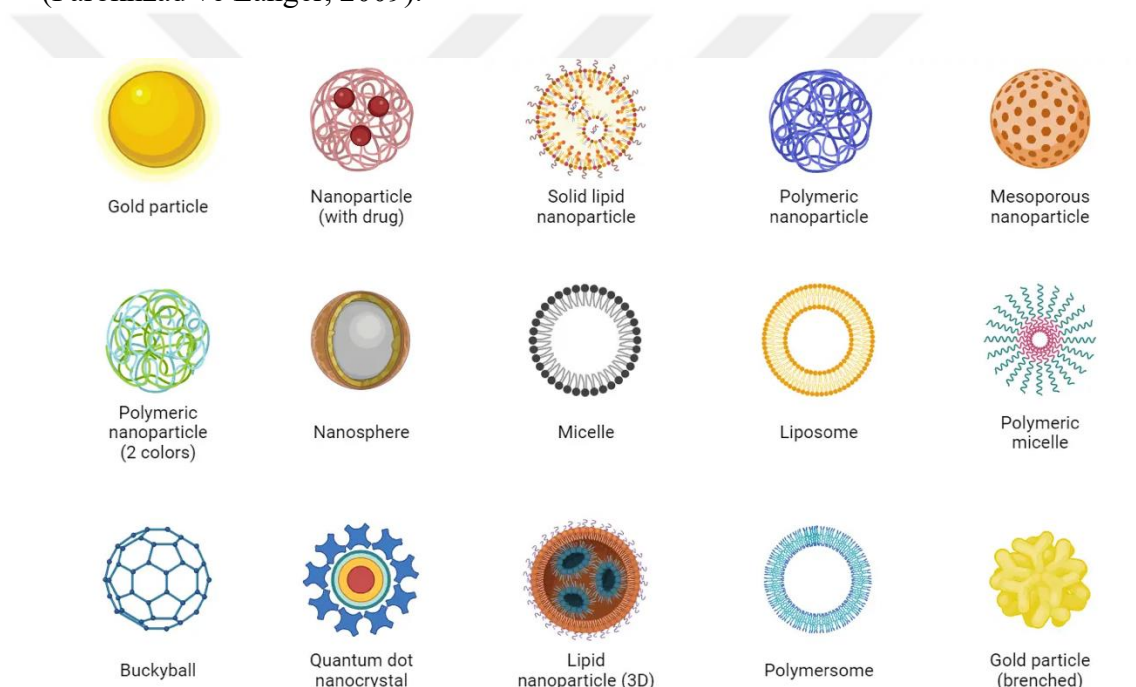


Figure 2.5. Nanoparticles of different shapes and characteristics as drug carriers
(*Nanoparticles* | *Encyclopedia MDPI*, 2025).

2.9.2. Drug Delivery

In modern pharmaceutical science, drug delivery is an important area of interest focusing on the development of new systems and strategies to deliver therapeutic agents to a specific area in the body with minimal side effects and maximum efficacy. Traditional drug administration methods have various limitations after intake by oral or intravenous routes. These active agents either face poor bioavailability or rapid degradation, inactivation or nonspecific distribution in the body, which reduce the efficacy or cause

significant side effects. To overcome these challenges, researchers have developed advanced drug delivery systems by using nanomaterials as carrier molecules such as dendrimers, hydrogels, mesoporous materials, liposomes, micelles, metallic nanoparticles etc (Farokhzad ve Langer, 2009). These nanocarriers provide stability, protect the drug from degradation, help in solubility and controlled release at targeted site which reduced the systematic toxicity of drug and dosing frequency in treatment of chronic diseases. Some of the nanocarriers also have their own side effects, therefore, scientists are focusing on designing of biodegradable nanocarriers with small particle size, high loading efficiency with prolonged circulation efficiency and accumulation at specific site in the body.

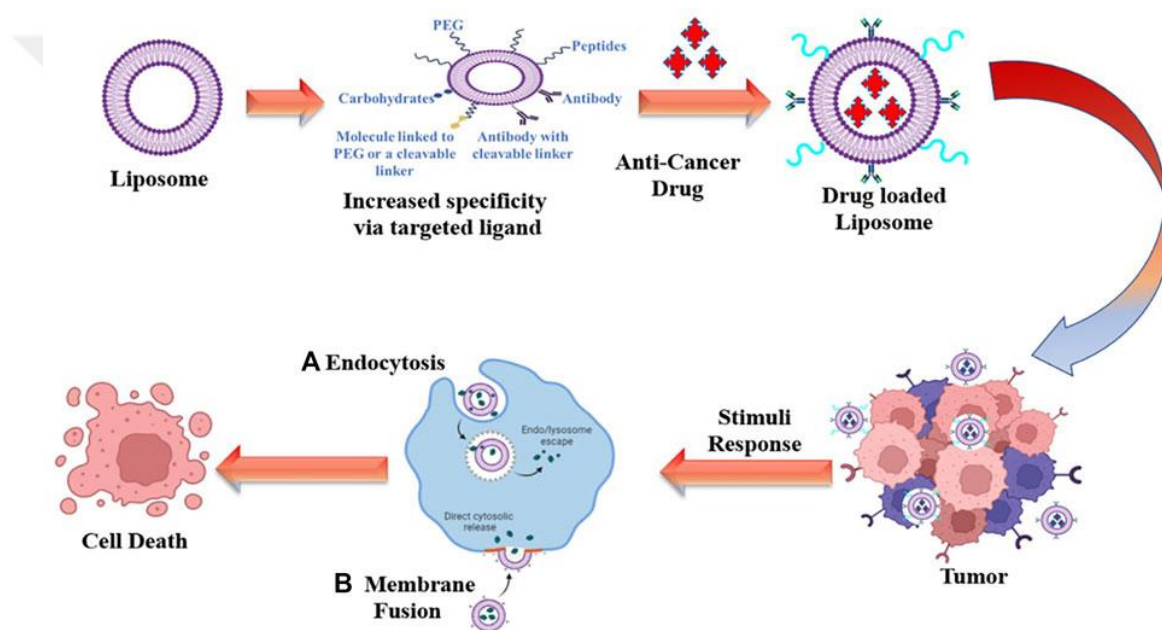


Figure 2.6. Representation of targeted therapy by using smart nanocarrier material
(Rana et al., 2023)

In current time, cancer is a principal cause of death with millions more new cases every year. The major obstacle in treatment of cancer patient is the side effect of nonspecific distribution of drug in the body. Nanocarriers play significant role for site specific delivery of cancer drugs. In early 1980s first liposome and protein-polymer conjugate nanocarrier was applied as clinical trials and later approved onto market in 1990s (Peer, et al 2000; Wang et al., 2019). these nanocarriers, reduced the disadvantages of conventional chemotherapy including unfavorable distribution, ineffective access to drug, less bioavailability multidrug resistance and regarded as “magic bullet”(Faustino et

al., 2007; Liu et al., 2017.; Mainardes et al., 2004.; *Wilhelm, 2000.*). Nowadays, natural polymers like solid lipid nanoparticles is applied because of their biocompatibility and biodegradable nature (Mainardes et al., 2004.).

2.10. Biodegradable Polymers as Drug Carrier

In the field of drug delivery, biodegradable polymers have emerged as groundbreaking molecules, offering sustainability, with the ability to design specific applications for targets effectively. These materials have attracted researchers because of their biocompatibility in nature and ability to break down in body without producing toxic products. Which later, eliminated from body without becoming burden on organ system compared to traditional drug delivery systems (Prajapati et al., 2019). Polymer is a Greek term which derived from two words “poly” mean many and “mer” mean unit. In the synthesis of polymer, small molecules or monomers are associated by condensation or addition reaction to build large molecules by polymerization. There are natural polymers which are biodegradable as well as synthetic polymers which can be categorized as both non-biodegradable and biodegradable polymers. Non-biodegradable synthetic polymers have drawbacks causing toxicity while accumulation into the body. However, biodegradable polymers have shown good biocompatibility, with less or no toxicity in the body. Therefore, these polymers and their composite are preferred over synthetic and non-biodegradable polymers in pharmaceutical and drug delivery applications.

Biodegradable polymers undergo *in vivo* biodegradation into harmless byproducts by enzymatic or non-enzymatic way and improve the pharmacokinetic of drug molecules. These biomaterials do not provoke constant inflammatory effect, have shown good medicinal properties and affective permeability with less toxic degradation products. Therefore, these polymer breakthrough in controlled drug delivery system, regenerative medicine, vaccine, peptide, protein, nucleic acid and anticancer drug delivery etc (Prajapati et al., 2019). As biomaterials have good biocompatibility and less or no toxicity, these polymers are used as potential material in implants, prostheses, stent, and drug delivery devices. For the delivery of active materials, different inorganic and organic materials have been utilized as carrier molecules, such as metal oxide, porous material and synthetic as well as natural polymers. Biodegradable polymers have local microstructure, degradation rate, and other ability of adjustable properties like stiffness and mechanical strength make their use in medicines successfully. Because of these properties, these polymers are used alone or as composite material. Nowadays, these

polymers are used to make medical devices, used for in-vivo applications, such as drug eluting stents, drug delivery devices and implants etc (*Akash: Natural and Synthetic Polymers as Drug Carriers...* - Google Scholar, n.d.; Asghari et al., 2017; Doppalapudi et al., 2014; Kesharwani et al., 2013). Recently colloidal systems containing micelles, nanocarriers, liposomes, aerosols, microemulsions, liquid crystalline phases have been developed, investigated, and applied in pharmaceutical and clinical areas.

2.11. Dendrimers

A dendrimer is a unimolecular micelle of covalently assembled polymeric macromolecule with hydrophobic interior and hydrophilic exterior. Dendrimers are prepared as different classes of fractal polymer having a central initiator core providing attaching site to generate tree-like branches, there for also known as cascade molecules. The first branch emerges from the heart core by the attachment of monomer. Each dendrimer molecule is globular in structure in nanometer dimension, monodisperse size and having modifiable surface for functionality. The word dendrimer is derived from Greek “dendron”, which mean tree and “meron” stand for part(Chabre ve Roy, 2010). Theoretical description of highly branched and polydisperse structure of dendrimers was first proposed by Paul J. Flory in 1952. He explained the formation of unimolecular, three-dimensional molecules by condensation of Abx-monomers, which provide a huge amount of surface group for further functionalization (Korake et al., 2021a). In 1978, Vogtle and co-workers published their work on the synthesis of polypropylene imine, which boosted the attention of researchers in area of drug delivery and tissue engineering (Korake et al., 2021b). These HBPs contain dendrons, uniformly dispersed or polydisperse as dendrimers, straight-dendritic compounds and hyperbranched polymer respectively. The heart core of dendrimer polymers provides space and shield to the drug molecules from exterior biological interaction, while terminal end containing various functional groups can be labeled with cell markers to make delivery specific and targeted. Various types of dendrimers have been used as drug delivery vehicles such as Boltorn H, Polyamide amine (PAMAM), Poly-L-lysine (PLL), and glycodendrimer etc.

2.11.1. Hyperbranched Polymer (HBPs) Boltorn HX

Dendrimers are perfectly branched monodispersed structures while hyperbranched polymers are imperfectly branched structures. Synthesis of dendrimers has been found complex, time consuming and economically non-sustainable because of multiple

intermediatory synthesis steps of deprotection, protection and purification at industrial scale and large-scale utilization. Conversely, synthesis of hyperbranched polymer is easy, economic because of the one-pot synthesis process even at large scale or bulk in amount. Boltorn HX are biodegradable, hyperbranched polymeric dendrimer containing terminal hydroxy groups. In this study Boltorn H (40, 30 and 20) was used, which contain (64, 32 and 16) hydroxyl groups respectively. Boltorn H (40, 30 and 20) particularly belonged to the class of polyester polyols containing branching unit of 2,2-bis (methylol) propionic acid (bis-MPA). Self-condensation of AB₂ monomer 2,2-bis (methylol) propionic acid (bis-MPA) with B₄ core molecule ethoxylated pentaerythritol (PP50) used to synthesize Boltorn H molecules highly soluble in organic solvents (Korake et al., 2021b). It has been reported in literature that, Boltorn H40 have globular structure with minimal size of 3 nm, carrying abundant amount of available functional groups with exceptionally imperative properties of biodegradability, stability, biocompatibility for bioactive conveyance and biological applications. Zagar and coworker have reported dissimilar core to monomer ratio of H20, H30 and H40 polycondensation polyester structure of (1/2, 1/8 and 1/60 respectively, and exhibit spherical configuration with less branches entanglement. The functionalization of surface hydroxyl group with different ligands offers opportunities for crafting and improving H40, H30 and H20's features to develop a useable product for numerous types of biological applications.

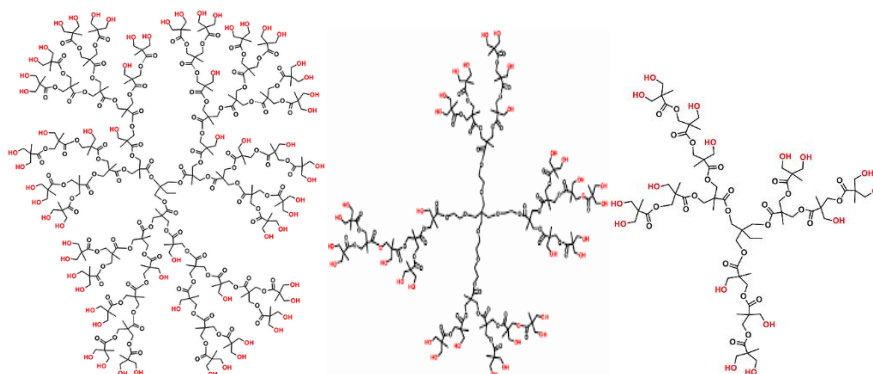


Figure 2.7. Structural representation of hyperbranched dendrimer (a) Boltorn H40, (b) Boltorn H30 and (c) Boltorn H20 (Azzouz et al., 2013; Evtugyn et al., 2013).

During the last decades, enormous attention has been gained by H40 molecules as a chemotherapeutic delivering agent because of unimolecular micelles construct, fixed core size, compactness of branches and governable dimensions (Korake et al., 2021c, 2021b). Moreover, amendment in surface function groups provides basics for the

fabrication of various organic and inorganic hybrid molecules. Transition metals incorporate into dendrimers allowing it to gain catalytic, optical, sensing, magnetic and electrical properties in HBPs.

2.12. Fluorinated Polymers

Polymeric modification with a variety of active materials becomes a powerful synthetic tool for the synthesis of a wide collection of materials with desirable properties. The chemical modification of polymer with fluorine affects many desirable physical properties such as enhancement of stability, hydrophobicity and chemical resistance of polymeric molecules (Dhara ve Banerjee, 2010; Imae, 2003; Leivo et al., 2004). Fluorine, known as the highest electronegative element, endows dipole moment and low polarizability in carbon-fluorine (C-F) bond which is one of the strongest bonds (~485 kJ/mol) in organic chemistry (Xin et al., 2024). Functionalization of fluorine atom onto side chain or backbone of polymer enhances its thermal and oxidative stability. Incorporation of fluorine on surface of molecules affects the hydrolytic activity in water and biological fluid and ultimately affects its hydrophobic and hydrophilic properties (Dhara ve Banerjee, 2010; Imae, 2003; Leivo et al., 2004).

In recent days, fluorinated polymers have attracted much attention of researchers especially in the field of nanomedicine and drug delivery. Addition of fluorinated segments such as heptafluorobutanoyl chloride, perfluoroalkyl chain, or trifluoromethyl groups on surface of the polymer help in the formation of highly stable micelles, vesicles or nanocarriers which enhance the circulation time in blood and drug loading efficiency (Vanparijs et al., n.d.). Most importantly, fluorinated molecules help in reduction of protein adsorption phenomena which help the nanocarriers to evade immune clearance and prolong systemic circulation. Recent studies highlight the fluorinated molecules interaction with membrane and cellular uptake, which can be potential tool for incoming research to reduce drug resistance (Dhara ve Banerjee, 2010; Imae, 2003; Leivo et al., 2004; Xin et al., 2024).

2.13. Hyaluronic Acid

Hyaluronic acid (HA) is a linear polysaccharide, also known as glycosaminoglycan (GAG), widely found in extracellular matrix of vertebrate and even in bacterial species. It found abundantly in skin, cartilage, in fetal umbilical cord and synovial fluids of joints (Stankovská et al., 2007). HA contains alternating β -1,4 and β -1,3 glycosidic linkage of

D-glucuronic acid (GlcUA) acid and N-acetylglucosamine (GlcNAc) repeating units. The chain length of HA varies from short as single disaccharide units to 25,000 disaccharide units. Therefore, there in-vivo distribution varies from few kilodaltons (KDa) to several kilodaltons (KDa) in molecular weight (Fraser et al., 1997; Stankovská et al., 2007). Hyaluronan synthase isoenzyme enzyme (HAS1–3) is responsible in synthesis of HA compared to other glycosaminoglycan which are being synthesized in Golgi apparatus (Fraser et al., 1997). HA is commonly found in dermis, vitreous humor, and within the ECM of all types of vertebrates. It is continuously excreted in significant amount by specialized cells such as fibroblast, keratinocytes, chondrocytes. The most important property of hyaluronic acid is its scavenging ability against free radical, unique characteristics of biocompatibility, degradation and drug delivery materials (Bot et al., 2008; B. Chen et al., 2014; Peter et al., 2009). The chain length, concentration and molecular weight of HA varies in body, which is associated with their functions and regulated by both synthesis and degradation mechanisms. Among high molecular weight HA (HMW-HA) and low molecular weight HA (LMW-HA), the molecule with HMW-HA of (>250 kDa) has been found as space filling molecules by retaining water in high hydration capacity to provide supporting framework to cells (Stern et al., 2006a). Moreover, it plays an important role as lubricant and shock absorber in particular cavity and regulates signal transduction upon binding to CD44 cell receptors (Yang et al., 2012).

Various biological processes are affected by HMW-HA production in cells, such as inhibition of proliferation, reduction in angiogenesis, suppression of immune response, inhibition of its own synthesis and phagocytosis, promotion of integrity and quiescence in tissues (Feinberg ve Beebe, 1983; Liu et al., 2023). Contrary to HMG-HA, synthesis and accumulation of LMW-HA and its oligosaccharide (<250 kDa) and (<10 kDa) respectively, are found associated with elevated effect in angiogenesis, immune and inflammatory response. Moreover, it was observed that HA of 200 kDa enhances aggressiveness in behavior of cancerous cells, produce inflammatory chemokines, impede the process of fibrinolysis and constitute microenvironment for tumor (Horton et al., 2000; Litwiniuk et al., 2016). In addition, it was found that HA level elevates significantly in breast cancer, lung cancer, pleural mesothelioma cancer, prostate cancer, ovarian and endometrial cancer. This elevation is results of hyperactivation of HA synthase isoenzyme and hyaluronidase. This correlation of over expression makes HA a prognostic marker (Nykopp et al., 2010) and utilized as drug delivering agent (Vasvani et al., 2020). It was observed that HA with chain length of 10 kDa are involved in

angiogenesis, stimulate cytokine production in dendritic cells, differentiation in endothelial cells and facilitate cell migration (Stern et al., 2006b). In addition, oligosaccharide fragment of HA consisting of 0.8–1.2 kDa have shown transcriptional activation of matrix metalloproteinases in fibroblast and tumor cells (Fieber et al., 2004).

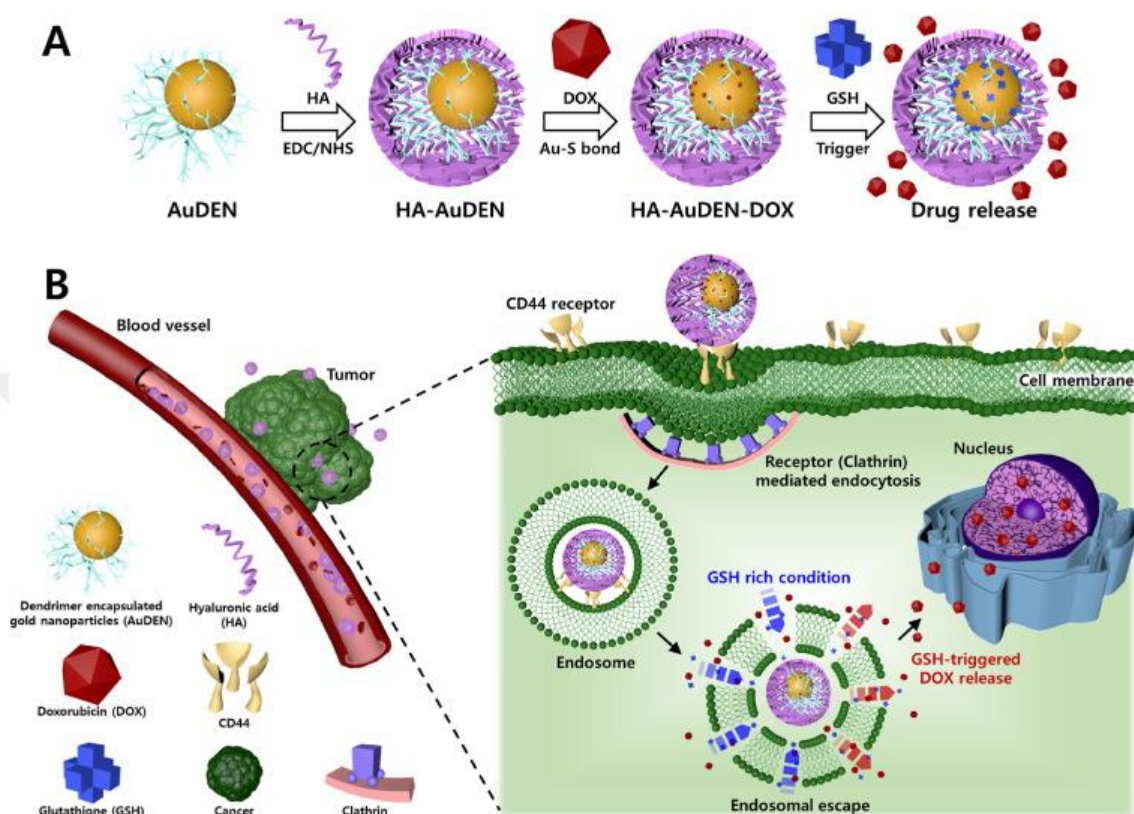


Figure 2.8. Hyaluronic acid decorated gold nanoparticle encapsulated dendrimer nanoparticles for targeted delivery for ovarian cancer (Lee et al., 2022)

2.14. Integrin Receptor and RGD Peptide

Integrin $\alpha 5 \beta 3$ and $\alpha v \beta 3$ are types of heterodimeric glycoprotein receptors, which play its role in cell-cell and cell-extracellular matrix interaction. In early stage of angiogenesis $\alpha v \beta 3$ integrin express at low level on mature endothelial cell and epithelial cells. However, it highly expresses on activated endothelial cell of tumor neo vasculature such as lung carcinomas, melanomas, breast cancer, osteosarcomas and neuroblastomas (*Arginine-Glycine-Aspartic Acid (RGD)-Peptide Binds to Both Tumor and Tumor-Endothelial Cells in Vivo* | *Cancer Research* | *American Association for Cancer Research*, 2025.; Van et al., 2000). A specific recognition site of integrin is RGD peptide, which is composed of three amino acids: arginine (Arg), glycine (Gly), and aspartic acid (Asp). These amino acids arrange linearly and form motifs of this peptide (Javid et al., 2024;

Sarin et al., 2005). RGD peptide is generally found in cell signaling and adhesion proteins such as fibronectin, vitronectin, and fibrinogen (Pang et al., 2023a; Ruoslahti ve Pierschbacher, 1987; Yamada et al., 2025) to facilitate cell adhesion. Moreover, it influences some biological processes including immune response, wound healing and angiogenesis. This peptide bind with a transmembrane integrin receptors on cellular surface, which is essential for signaling, motility and survival of cell (Bellis, 2011). The $\alpha 5\beta 3$ and $\alpha v\beta 3$ integrin have strong sequence similarities therefore both are recognized by RGD peptide in “side-on” and “one-side” interaction respectively (Delouvrié et al., 2012). Moreover, RGD also show affinity towards recognition of $\alpha 3\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 6$, and $\alpha v\beta 5$ receptors of the integrin family (Plow et al., 2000). In 1984, RGD sequence was first identified as a cell adhesion motif in the extracellular matrix. Overexpression of integrin on tumor neo vasculature and cancerous cell allow the binding of RGD peptide make it potential targeting agent for imaging, drug and gene delivery into cancerous cells for treatment. Therefore, scientists start to modify it to enhance its binding affinity and selectivity toward specific integrin receptors. To promote cell adhesion and tissue regeneration in tissue engineering, RGD incorporated materials were started to use in 1997. Later, RGD peptide was started to use as a targeting ligand in drug delivery system (Koivisto et al., 2014; Pang et al., 2023b; Schnittert et al., 2018; Y. Zhang et al., 2012). The latest research has shown that RGD peptide caught the attention of researchers and been used in scaffold and hydrogels for tissue engineering, incorporated in bioinks of 3D bioprinters for precise deposition of cells on printed structures.

In addition, scientists found that integrin also binds with native ligands which contain RGD sequence. This finding opened new ways in therapeutic and diagnostic areas to treat cancerous cells by utilizing RGD peptides as a targeting motif. Nowadays, significant interest has been gained in radiolabeling ligands, radiopharmaceuticals, fluorescence markers, drug conjugates, miscellas and nanoparticles for therapeutic and diagnosis of cancer.

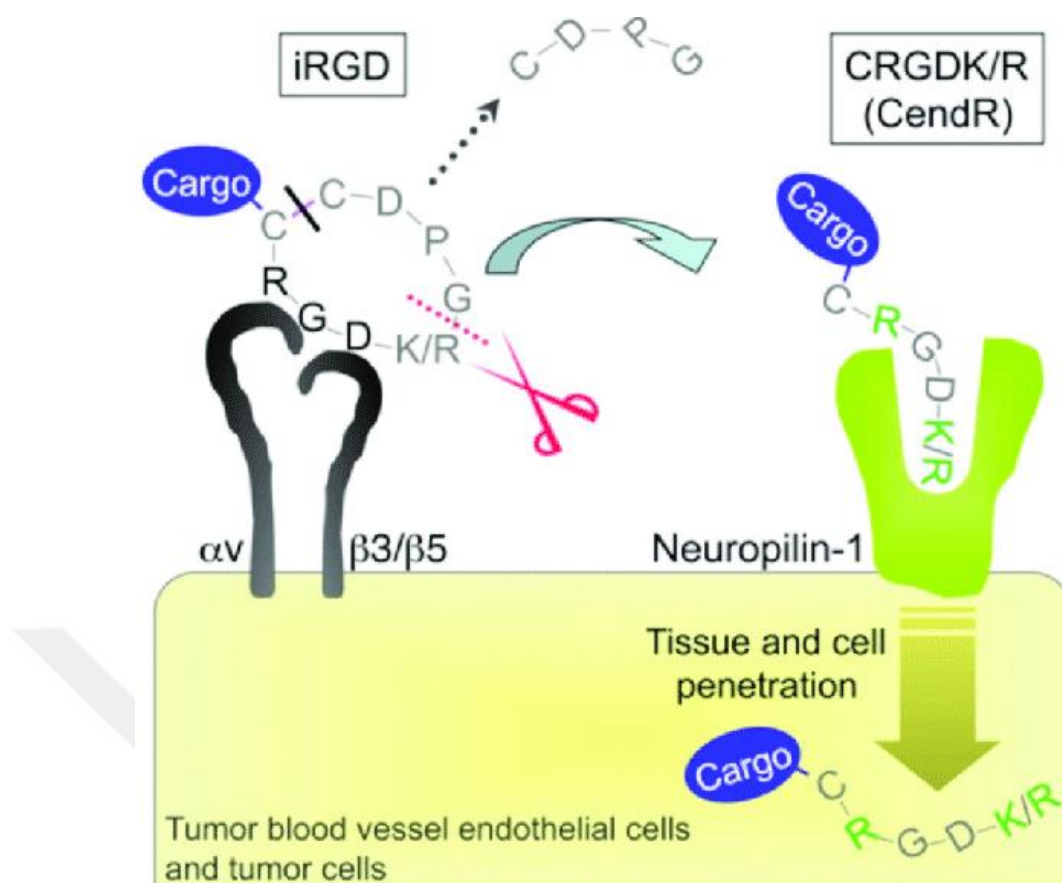


Figure 2.9. Signaling mechanism of integrin and neuropilin-1 for RGD peptide (Zhang et al., 2018)

2.15. Folic Acid and Folate Receptor

Folate receptors (FR) in eukaryotic cells are high-affinity receptors which mediate the cellular uptake of folic acid and its derivatives (Zheng et al., 2016). Compared to normal healthy cells, folate receptors over express on cancerous cells, therefore a desired option in targeted cancer therapy (Xia ve Low, 2010). Similarly, derivatives of folate having two carboxylic groups show similar affinity to FR (Q. Chen et al., 2018; Venkatasubbu et al., 2013). Thats why researchers have successfully synthesized folic acid conjugated small molecules, macromolecules and other nanocarriers (Chakraborty et al., 2011; Deb ve Vimala, 2018; Low et al., 2008). Folic acid, or folate is a water soluble, natural form of vitamin B9. It is mostly found in vegetative parts of plants such as dark leaves, citrus fruits and meat. As an essential nutrient, folate plays an indispensable role in nucleotide synthesis and cell maturation. It helps in methylation of uracil to thymine, which is required for replication and repairing process of DNA (Duthie, 1999; Reidy, 1987). As it helps in proliferation of cells, folate metabolism is essential for

both normal and cancerous cells. Some beneficial effects of folate have reported as it lowering homocysteine and protecting the body from cardiovascular diseases and stroke (Forman et al., 2005; Rimm et al., 1998). In contrast to previous study, it was also observed that folate elevates the progression of atherosclerosis. In addition, deficiency of folate is responsible of spine defects (spina bifida) and brain (anencephaly) (Czeizel ve Dudás, 1992; lancet ve 1991; Smithells et al., 1976). Similarly, insufficient folate serum level was found associated with cervical intraepithelial neoplasia (CIN) and cervical cancer (Gutierrez et al., 2021; Li et al., 2020). Several studies conducted on relationships of folate with cancer have shown intake of folic acid during preconception period and pregnancy reduce the risk of cancer by lowering the risk of chromosomal abnormalities (Cantarella et al., 2017; Gutierrez et al., 2021; Li et al., 2020).

However, excessive intake led to head and neck squamous cells carcinomas (Fan et al., 2017), as well as enhance the methylation of DNA in colonic tissues and leukocytes. In addition, high folate supplementation was found linked with exacerbate effect of cancer in smokers and people who have Kirsten rat sarcoma (K-Ras)-mutated tumors (Kherbek et al., 2022).

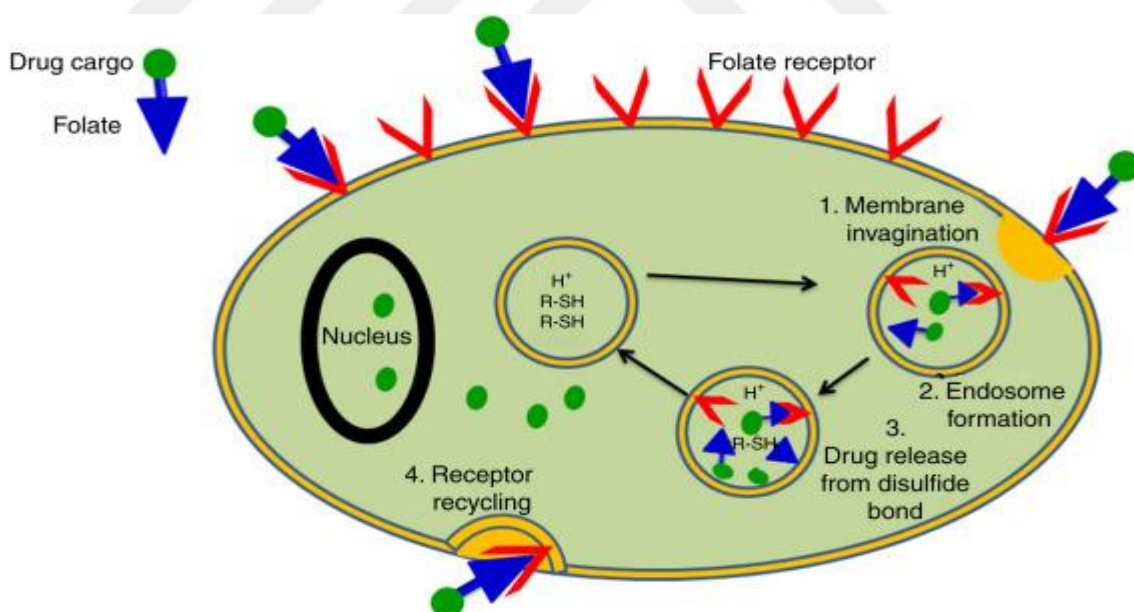


Figure 2.10. Phagocytotic mechanism of Folate receptors against folic acid linked cargo (Velikyan, 2014)

In this study, afatinib and plasmid carrying nanomaterial were synthesized using hyperbranched dendrimer as core molecule wrapped with target specific polysaccharide conjugated with biomarkers. Boltorn H (40, 30 and 20) hyperbranched polymer was

selected as core molecule and potential carrier. This biodegradable polymer was functionalized with HFBC to make it more stable and neutral to intracellular environment. Moreover, Flamma 774 isothiocyanate was conjugated at remaining end terminal of the BHX molecules as a fluorescent molecule to observe the targeted reach. To make nanocarrier target specific, folic acid and cyclic RGD peptide was functionalized on hyaluronic acid molecules. Folate and RGD peptide receptors have been observed over express on cancerous cells compared to normal cells. In each step of functionalization of both core and shell molecules, material was characterized to understand the successful conjugation by FT-IR, DSC, NMR spectroscopy. Furthermore, nanoparticles were also characterized by DLS, FTIR, NMR, MALDI-TOF, Fluorescence spectrometry, SEM and TEM studies. To find out the cytotoxicity of nanomaterial, MTT assay was done over different cell lines.

3. MATERIALS AND METHODS

3.1. Materials

For the synthesis of dendrimer nanoparticles, highest purity grade hyaluronic acid (Sigma Aldrich), Boltorn H40 regular (BH 40, polymer factory), Boltorn H30 regular (BH 20, polymer factory), Boltorn H20 regular (BH 20, polymer factory), N, N-Dicyclohexylcarbodiimide (DCC) (Sigma Aldrich), N, N-Dimethylformamide (DMF) (MERCK), Ethanol (Merck) was purchased.

For the functionalization of polymeric dendrimer, Heptafluorobutyryl chloride (Sigma Aldrich), Sodium Azide (Sigma Aldrich), N-Hydroxysuccinimide (Sigma Aldrich), Flamma-774 NHS ester (Bioact) was bought with highest purity grade to use in this study.

For the functionalization of shell molecule of nanocarriers, Folic acid (Merck), Dimethyl sulfoxide DMSO (Merck), Diamine (polyethylene glycol) methyl ether (Sigma Aldrich), cys-RRRRRRRR-c(RGDfK) peptide (Genescript) were bought and used in this study.

For determination of amine group on the surface of polymeric dendrimer, Ninhydrine (Sigma Aldrich), Hydrindantin (Sigma Aldrich), 2-methoxy ethanol (Sigma Aldrich) was used in this study.

In the *in-vitro* studies, CCD18CO and CAL51 cell line were used. During cell culturing, RPMI (Gibco), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), Fetal bovine serum (Gibco), Penicillin-Streptomycin (10,000 U/mL) (Gibco), Trypsin 0.25% and EDTA 0.1% solution (Sigma Aldrich), Afatinib (Abc), Cisplatin (v pharma) were purchased and used under sterile conditions.

3.2. Instruments and Equipment

Nanocarriers were characterized by FT-IR of Shimadzu company (IR Affinity-ISI model) was used to understand the available functional group on the surfaces of the polymers. KBr pellet was used to do measurement in the range of 4000-400 cm^{-1} wavenumber. On each step after functionalization, functionalized polymers were further characterized at 600 MHz, 14.1 tesla magnetic powered, NMR spectrometry of Agilent (Premium compact model). For thermogravimetric analysis, TG/DTG/DTA combined system was performed by Seiko SII TG/DTA 7200, while DSC measurements were carried out by Perkin Elmer DSC 400 instrument. For the analysis of particle size and its

zeta potential, Malvern Nano ZS90 Zetameter/Particle Sizer was used. To further characterize the size and morphology of the nanocarriers, the nanocarriers were examined using a Scanning Electron Microscope (SEM, JEOL JSM-7100F) and a Transmission Electron Microscope (TEM, JEOL JEM-1400 PLUS). UV-Vis spectrometric analysis during this study was done on the T60 model of Spectrometric Analyzer PG Instrument. After each step polymer was dialyzed and then freeze dried using ALPHA1-2LD PLUS model instrument. For the calculations of molecular weight of the final polymeric nanocarriers after functionalization with different molecules, MALDI-TOF Mass spectrometry was used by Bruker Microflex LT of model and Nitrogen laser MNL106, 337 nm laser type of instrument. In cell culturing and MTT assay, inverted microscope of OLYMPUS CKX41 model was used, while UV-spectrometer of 96 plate reader from Epoch company and Biotech model was used.

3.3. Experimentation

3.3.1. Synthesis of Amin Group Functionalized BHX-NH₂

BHX-NH₂ was synthesized according to the method described in the literature (Rajesh et al., 2005), the terminal hydroxyl groups (-OH) of BHX (40, 30, 20) were conjugated with amine (-NH₂) groups. BHX [BH40 (0.437 mmol), BH30 (0.443 mmol), and BH20 (0.457 mmol)] were dissolved in 15 mL of DMF under magnetic stirring. Then, hydrazoic acid (0.219, 0.222, and 0.219 mmol) was added to the corresponding solutions of BHX (X=40, 30, 20), respectively and stirred. As a catalyst, the following amounts of di-isopropyl azodicarboxylate (1.5 mmol.L⁻¹ solution) were added to the mixtures: 0.132 mL to the mixture prepared with BH40, BH30 and BH20. Subsequently, a triphenylphosphine (3.0 mmol.L⁻¹ solution) was added as follows: 52.4 μL to the mixtures of BHX. Later, mixtures were heated at 50°C for 3 hours to allow the reaction to proceed. After that, 100 μL of HCl solution (1.0 mmol.L⁻¹) was added dropwise to the mixture, and the reaction was continued for 3 hours. Once the reaction was completed, the liquid phase was dialyzed using cellulose tube membrane (MWCO=12000 Da) in deionized water to remove the unreactive material and solvent. BHX-NH₂ were taken from dialyzing membrane and dried by freeze-dryer under vacuum. A schematic representation of the amine-functionalized BHX-NH₂ synthesis is presented in Figure 3.1.

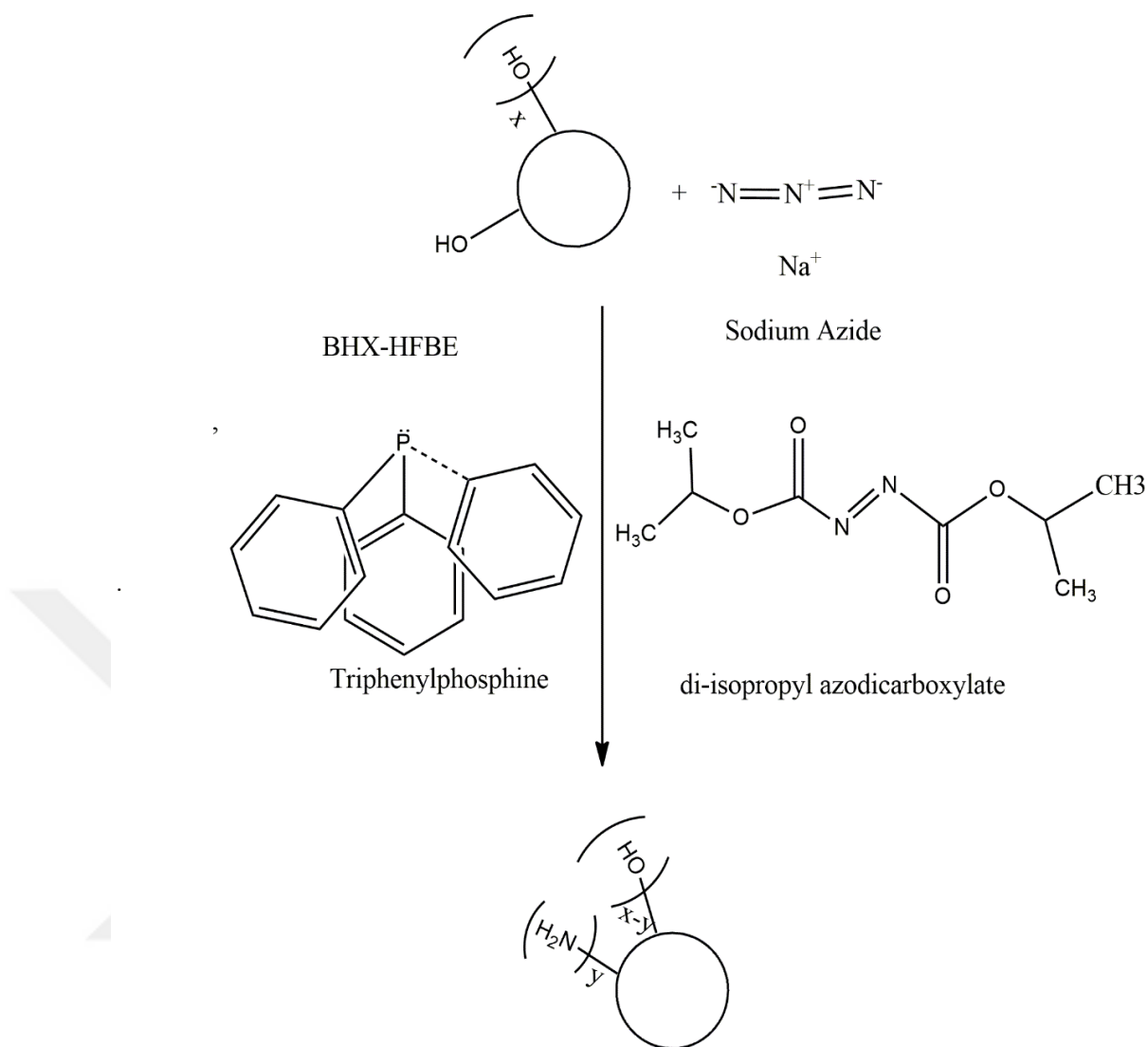


Figure 3.1. Schematic representation of amine-functionalized BHX-NH₂ synthesis

3.3.2. Synthesis of Fluorinated Boltorn BHX-NH₂ (BHX-HFBE-NH₂)

50 mg of the synthesized BH40-NH₂ (0.225 mmol), BH30-NH₂ (0.221 mol), and BH20-NH₂ (0.215 mol) were placed in a round-bottom flask and dissolved in 10 mL of anhydrous methanol by stirring with a magnetic stirrer in an ice bath. A constant-pressure dropping funnel was mounted onto the round-bottom flask. The BHX-NH₂ solution was mixed with a solution of heptafluorobutyryl chloride (HFBC) prepared in 10 mL of anhydrous methanol at different molar ratios (1: 0.25; 1: 0.2; 1: 0.15). The resulting solution was then transferred to a separatory funnel and fixed onto the round-bottom flask. Heptafluorobutyryl chloride was added to the BHX solution at a rate of 3 drops per second with vigorous stirring while in the ice bath. After the addition was completed, the ice bath was removed, and the mixture was left for 48 hours. At the end of this period, the liquid

phase in the flask was removed. The separated liquid phase was dialyzed against double-distilled water for 3 days using a cellulose tube membrane (MWCO=12000 Da) with the water being changed, 3 times a day. After dialysis, the liquid phase was separated and lyophilized for 2 days. The resulting solid phase was weighed and is being stored at -20°C. A schematic representation of the BHX-HFBE-NH₂ synthesis is presented in Figure 3.2.

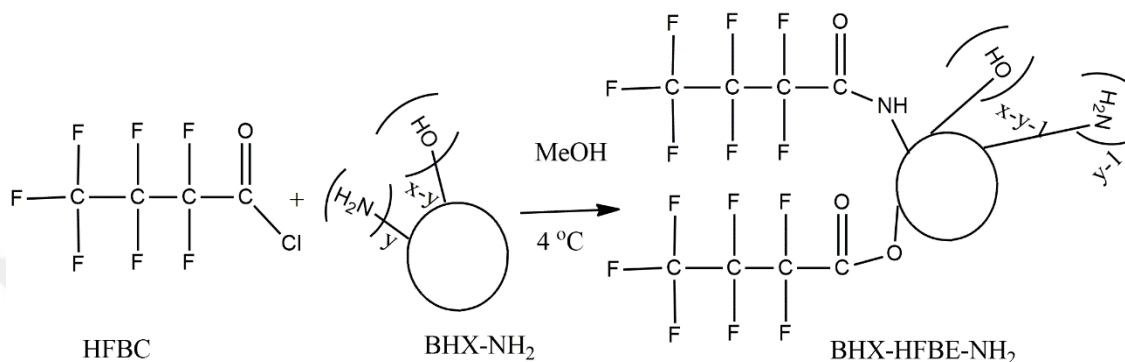


Figure 3.2. Schematic representation of BHX-HFBC-NH₂ synthesis

According to the data obtained from theoretical calculations', when reacted with HFBC at different molar ratios (1: 0.25; 1: 0.2; 1: 0.15), the amount of HFBC that will bind to BH40-NH₂ will be 0.0548, 0.0438, and 0.0329 mmol; the amount of HFBC bound to BH30-NH₂ will be 0.055, 0.044, and 0.033 mmol; and the amount of HFBC bound to BH20-NH₂ will be 0.0573, 0.045, and 0.034 mmol.

3.3.3. Conjugation of BHX-HFBE-NH₂ with Fluorescent Dye (BHX-HFBE-NH-F774)

To evaluate the binding and biological distribution of nanocarriers in CCD18CO and CAL 51 cells, BHX-HFBE-NH₂ was conjugated with the Flamma-774™ isothiocyanate dye. 100 mg of BHX-HFBE-NH₂ was dissolved in 5 mL of dimethyl sulfoxide (DMSO), and 0.849 nmol of Flamma™-774 ($\lambda_{\text{abs}}=778$ nm, $\lambda_{\text{em}}=808$ nm) was added to a 100 μ L portion of the resulting solution. The mixture was stirred in the dark for one day. To remove DMSO and unreacted reagents, the solution was dialyzed against deionized water using a cellulose tube membrane (MWCO=12000 Da). It was then lyophilized and dried. The reaction scheme is presented in Figure 3.3.

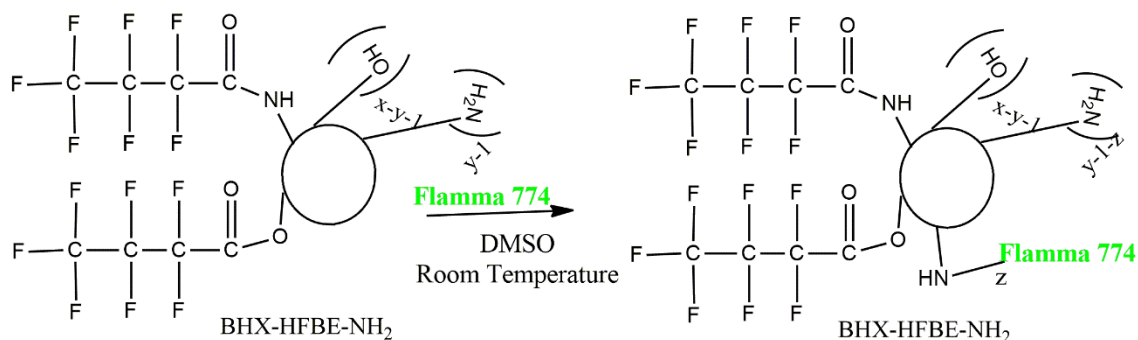


Figure 3.3. Schematic representation of conjugation of flamma-774 to synthesize BHX-HFBE-NH₂

3.3.4. HA-FA Synthesis

HA-FA was synthesized according to the method described in the literature (Tong et al., 2020). Folic acid (FA) was dissolved in DMSO. Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were added to the FA solution and stirred at 30°C for 5 hours. The precipitate was then filtered and removed. In the next step, hyaluronic acid (HA) was dissolved in formamide and added dropwise to the FA solution. The mixture reacted for 48 hours at 40°C while protecting it from light. The liquid phase was dialyzed against deionized water for 48 hours using a dialysis membrane (MWCO= 3500 Da). The product was then lyophilized, and the solid product (HA-FA) was obtained. The reaction flow chart for the synthesis of folic acid-functionalized hyaluronic acid is presented in Figure 3.4.

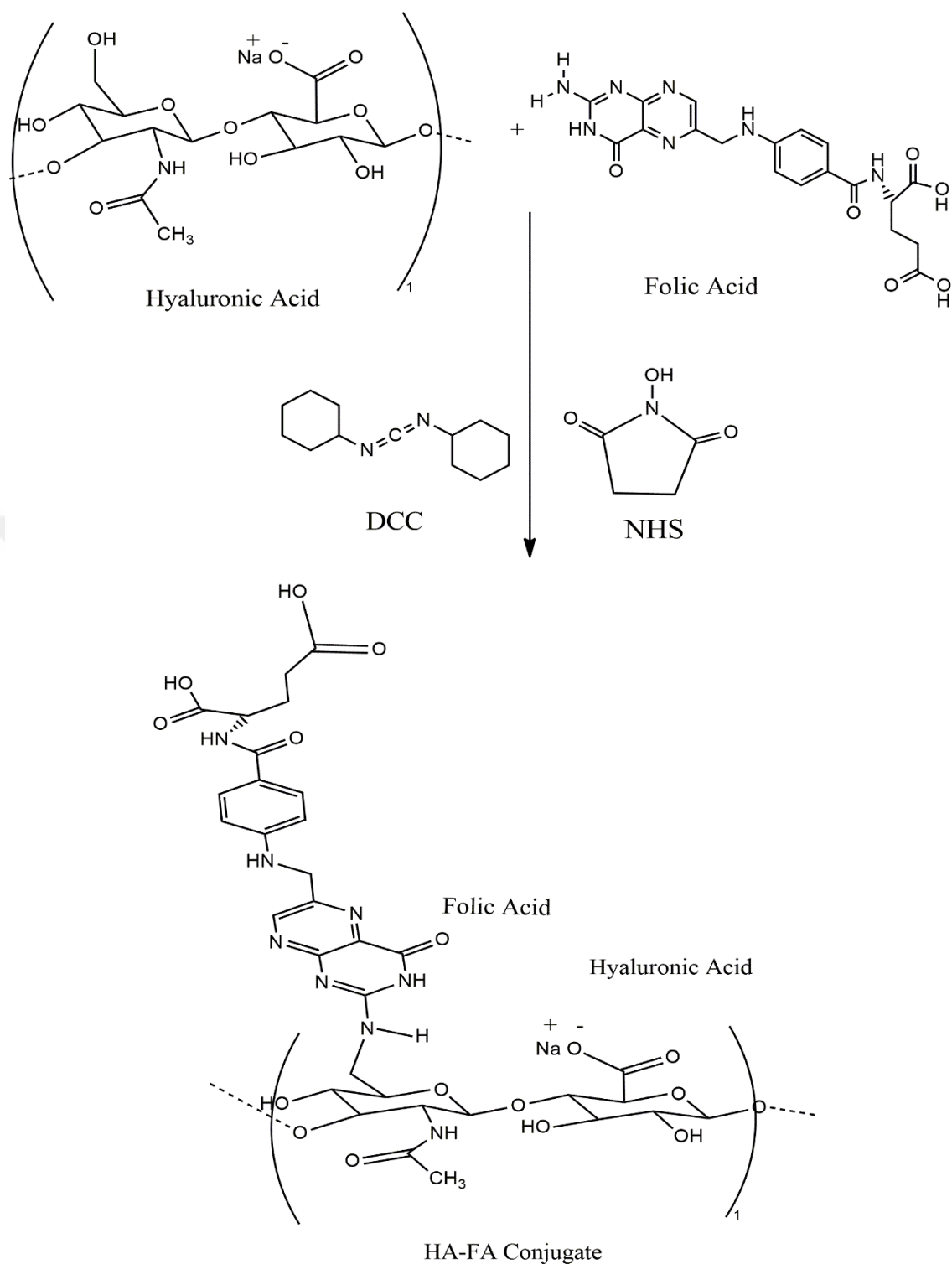


Figure 3.4. Schematic representation of folic acid functionalization with hyaluronic acid

3.3.5. RGD-R8-PEG-HA-FA (RRPHF) Synthesis

20 mg of HA-FA was dissolved in 20 mL of 2-(N-morpholino)ethanesulfonic acid (MES) buffer, and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDCI) (1.2 mg)

and N-hydroxysuccinimide (NHS) (0.74 mg) were added, allowing the reaction to proceed at room temperature for 2 hours. In this way, the carboxyl groups of HA were activated. Then, 21.37 mg of poly(ethylene glycol) 2-aminoethyl ether acetic acid (NH₂-PEG-COOH) was added to introduce the carboxyl group into the structure.

In the first step of the synthesis, poly(ethylene glycol) 2-aminoethyl ether acetic acid (NH₂-PEG-COOH) was dissolved in DMSO. Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were added to the (NH₂-PEG-COOH) solution, and the mixture was stirred at 30°C for 5 hours. The precipitate was then filtered and removed. Following this, HA-FA was dissolved in formamide, and the (NH₂-PEG-COOH) solution was added dropwise. The mixture was allowed to react at 40°C for 48 hours while protected from light. The synthesis scheme is shown in Figure 3.5.



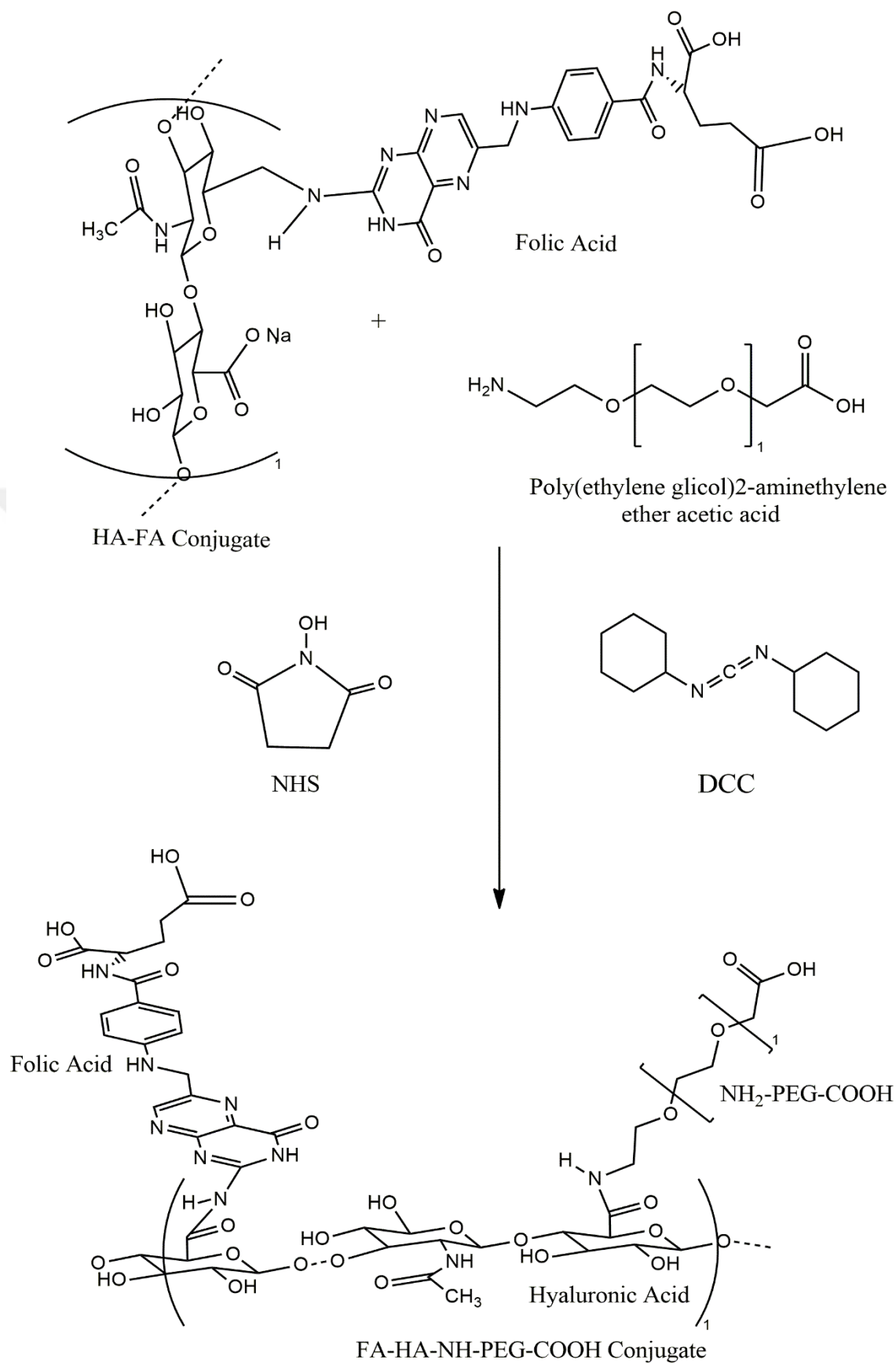


Figure 3.5. Synthesis Scheme of Hyaluronic Acid Functionalized with Folic Acid and Poly(ethylene glycol) 2-aminoethyl ether Acetic Acid

The mixture was stirred at room temperature for 24 hours. The resulting liquid phase was dialyzed against PBS for 3 days and against deionized water for 3 days using a dialysis membrane (MWCO= 3500 Da), then lyophilized.

Then, 3.92 mg of [c(RGDfK)-RRRRRRRR-Cys] RGD-R8 was added to the obtained phase, and the reaction was proceeded for another 48 hours. The final product was dialyzed against PBS for 2 days and against deionized water for 1 day, then lyophilized for 2 days. The resulting RRP HF will contain HA and FA terminal groups capable of dual targeting cancer cells. The scheme related to this synthesis is presented in Figure3.6.

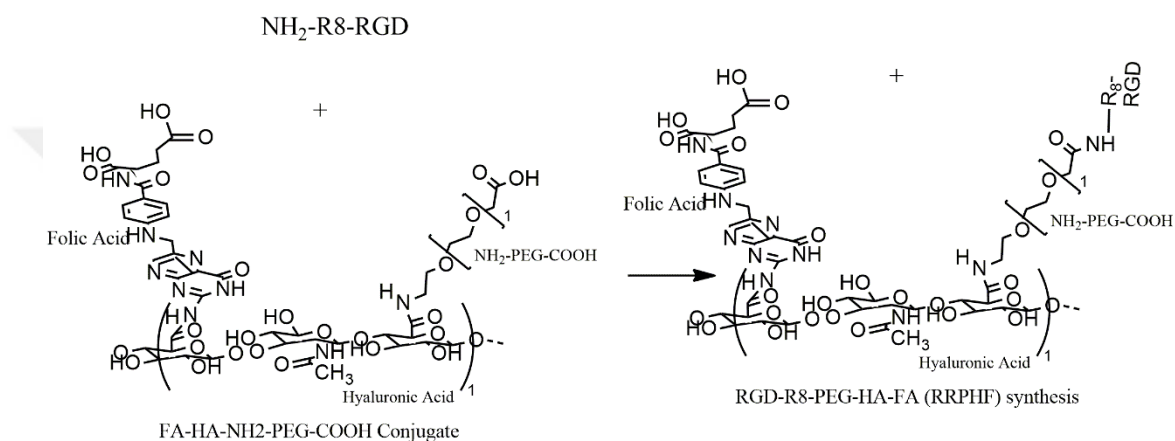


Figure 3.6. Synthesis Scheme of Hyaluronic Acid Functionalized with Folic Acid and Poly(ethylene glycol) 2-aminoethyl ether Acetic Acid and RGD-R8-PEG

3.4. Ternary Complex Synthesis

The synthesized BHX-HFBE-NH-F774 100 mg and RGD-R8-PEG-HA-FA 15 mg were mixed and incubated for 1 hour at room temperature in PBS solution. After incubation, the ternary complex RRP HC (1-9) based on HFBC concentration were freeze dried and characterized by Zeta sizer. RRP HC (1, 4 and 7) was selected based on zeta potential results for further characterization with MALDI-TOF, Thermogravimetry, SEM and TEM analyzers.

3.5. Drug Loading and Release Study

Afatinib is a tyrosine kinase inhibitor, and its receptors sites lie in cellular membrane. The aim of study was to overcome drug resistance by using nanocarriers. Therefore cisplatin (SN: 1638276906585 from Vero pharma) was used as model drug because cisplatin interacts with double strand of the DNA and leads to apoptosis.

Therefore, 12 mg of RRPNC nanocarriers (**1, 4 and 7**) were weighed and 0.66 mL (50mg/100 mL) of cisplatin as a model drug was added onto nanocarriers and incubated for 12 hours at room temperature. After the completion of incubation period, 3 mg of hyaluronic acid functionalized with folic acid and RGD peptide was added and incubated further for 1 hour after gentle shaking. The nanocarriers loaded with cisplatin was centrifuged and pellet was collected for drug release study.

Drug loading efficiency was measured by the formula:

$$DLE(\%) = \frac{\text{Weight of drug in nanocarriers}}{\text{Total weight of drug+Loaded Nanocarrier}} \times 100 \quad (3.1)$$

Similarly, the encapsulation efficiency of nanocarriers was also calculated by formula given below:

$$EE(\%) = \left(\frac{\text{weight of drug encapsulated in nanocarriers}}{\text{Amount of drug initially added}} \right) \times 100 \quad (3.2)$$

Here DLE is drug loading efficiency while EE is drug loading encapsulation efficiency.

For drug release study, 25 ml of PBS buffer with pH of 7.4 and 6.5 was taken into 50 ml beaker. Pellet of cisplatin loaded RRPNC **1, 4 ve 7** was added into (MW: 1KDa) dialysis membrane. For each sample reading was taken by UV spectrometer after different time intervals of (0 minute, 30 minutes, 1 h, 2h, 4h, 8h, 16h, 32 and 67h). Amount of cisplatin release against time was measured by comparing the absorption value of calibration curve.

3.6. Cell Culture

The CCD-18CO, MCF-10A and CAL51 cell lines used in the study were cultured under sterile conditions, utilizing low passage number cells. The MCF-10A and CAL51 cell line was cultured in RPMI (Roswell Park Memorial Institute 1640) medium and CCD-18CO in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin.

3.6.1. Afatinib Application and Determination of Afatinib Resistance

All cell lines were cultured under sterile conditions in 100 mm plates in a 5% CO₂ atmosphere at 37°C. When the culture density reached 70-80%, the cells were passaged using 0.25% trypsin/1 mM EDTA. Afatinib was dissolved in DMSO and applied to the cells with a final DMSO concentration of 0.1%. Once both cell lines reached sufficient density, 5000 cells per well were seeded in a 96-well plate for counting. Afatinib was applied to both cell lines over a range of 10 nM to 10 μM for 1-5 days. DMSO-treated

cells were used as a control group. After the afatinib treatment, cell viability analysis was performed, and IC₅₀ values were calculated (Table 1). Each of these steps was repeated three times for both CCD-18CO (normal cell line) and CAL51 (Afatinib-resistant cell line). The results obtained were calculated as average value. The aim was to determine the ideal drug concentration and duration for the Afatinib-resistant CAL51 cell line and to demonstrate that the normal cell line CCD-18CO does not have drug resistance properties.

3.6.2. Obtaining Afatinib-Resistant Cells

There are numerous studies in the literature that have established drug resistance in breast cancer and other cancer cell lines (Dermott et al., 2014). For this study, the IC₅₀ value of afatinib for the CAL51 cell line was determined to be 2.4. Therefore, for the development of afatinib resistance CAL51 cell line, seeded cells were continuously cultured in 6 μ M of afatinib for 5 days and then cultured again without afatinib for two days. This process was repeated multiple times and calculated the number of surviving cells. All analyses were conducted in triplicate.

3.6.3. Cell Viability Assay

To evaluate the cytotoxicity of cisplatin without nanoparticles and cisplatin-loaded RRPMHC **1, 4 and 7**, nanoparticles. A cell viability assay was performed on two different cell lines: CCD-18Co (normal human colon fibroblasts) and afatinib drug resistance CAL-51 (human breast cancer cells). Cells were cultured in DMEM (for CCD-18Co) and RPMI-1640 (for CAL-51) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under standard conditions (37°C, 5% CO₂).

Cells were seeded into 96-well plates at a density of 1×10^3 cells per well and allowed to adhere overnight. The following day, cells were treated with cisplatin-loaded RRPHMC **1, 4 and 7** nanoparticles and free cisplatin at varying concentrations ranging from 1 μ g/mL to 40 μ g/mL. After 24 and 48 hours of incubation, 10 μ L of resazurin solution was added to each well and incubated for 3 hours at 37°C.

Following incubation, cell viability was measured by multiplate reader UV visible spectrometer at 560 nm of wavelength. All experiments were performed in triplicate, and results were expressed as mean $\pm$ standard deviation (SD).

4. RESEARCH FINDINGS AND DISCUSSION

4.1. FTIR Studies

4.1.1. FTIR Analysis of Synthesized BHX and its Derivatives (BHX, BHX-NH₂, BHX-NH₂-HFBE ve BHX-HFBE-NH-Flamma 774)

Due to the high number of polar -OH groups in the structure of Boltorn HX polymers (for example, BH-40, BH-30, and BH-20 contain 64, 32, and 12 -OH groups, respectively), they tend to form hydrogen bonds with each other and with other groups in the hyperbranched polymer. Most of the -OH groups are engaged in hydrogen bonding. The hydrogen bonds can occur between OH...OH or C=O...OH. Due to the globular compact surface of the hyperbranched polymer, a very limited number of hydroxyl and carboxyl groups are accessible, and these groups do not participate in hydrogen bonding (Žagar ve Grdadolnik, 2003; Žagar ve Žigon, 2011).

The FT-IR spectra of the polymers BH40, BH40-NH₂, BH40-NH-HFBE1, and BH40-HFBE-NH-Flamma 774 are presented in Figure 4.1.

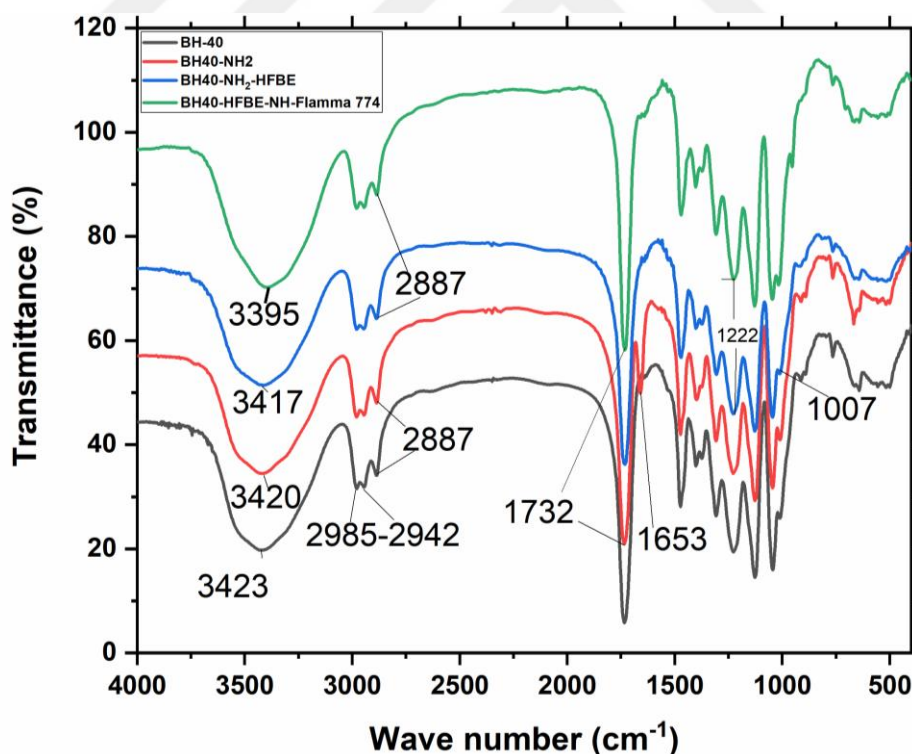


Figure 4.1. FT-IR spectrum of BH40 and Functionalized polymer: BH40 (Black), BH40-NH₂ (Red), BH40-NH₂-HFBE1(Blue), BH40-HFBE-NH -Flamma 774 (Green).

In the FT-IR spectra of the polymers BH40, BH40-NH₂, BH40-NH₂-HFBE1, and BH40-HFBE-NH-Flamma 774, the strong and broad band observed at 3425 cm⁻¹ is attributed to the intramolecular bond stretching vibrations of the -OH group. However, in BH40-NH₂, BH40-NH₂-HFBE, and BH40-HFBE-NH-Flamma 774 polymers, the peaks for -OH and -NH₂ stretching vibrations overlap in the range of 3395-3420 cm⁻¹ and cannot be distinguished. The asymmetric stretching vibrations of the aliphatic C-H bond in BH40 and the functionalized polymers are observed at 2985 and 2942 cm⁻¹, while the symmetric stretching peak is seen at 2887 cm⁻¹. C=O stretching vibrations appear at 1732 cm⁻¹. Additionally, the N-H bending vibration observed at 1653 cm⁻¹ indicates the formation of the BH40-NH₂ structure.

The strong C-F peaks associated with heptafluorobutyryl chloride, which are expected to be observed in the range of 1400-1000 cm⁻¹, overlap with the peaks of BHX (*Heptafluorobutyryl Chloride*, n.d.-a). The C=O peaks in the HFBC molecule also overlap with the peaks at 1732 cm⁻¹. The peak observed at 1222 cm⁻¹ in BH40-NH₂-HFBE1 and BH40-HFBE1-NH-Flamma 774 can be attributed to the C-F stretching vibration, overlapping with the peaks of the unmodified BH40 and BH40-NH₂ polymers. These findings are consistent with the literature (Su et al., 2018). Since the BHX-NH₂-HFBE compound was functionalized with 9.9x10⁻⁷ mol of Flamma 774 to yield BHX-NH₂-HFBE-Flamma 774, the functional groups in Flamma 774, such as Ar-H, S-O, and C=N, will be observed with very low intensity. Therefore, the peaks expected for Flamma 774 have overlapped with those of the BHX-NH₂-HFBE compound, and these peaks cannot be distinguished in the FT-IR spectrum. The conjugation of Flamma 774 to the structure has been confirmed by fluorescence, DSC, and ¹H-NMR analyses.

FT-IR spectrum of BH30, BH30-NH₂ and BH30-NH₂-HFBE1 polymers are given in Figure 4.2.

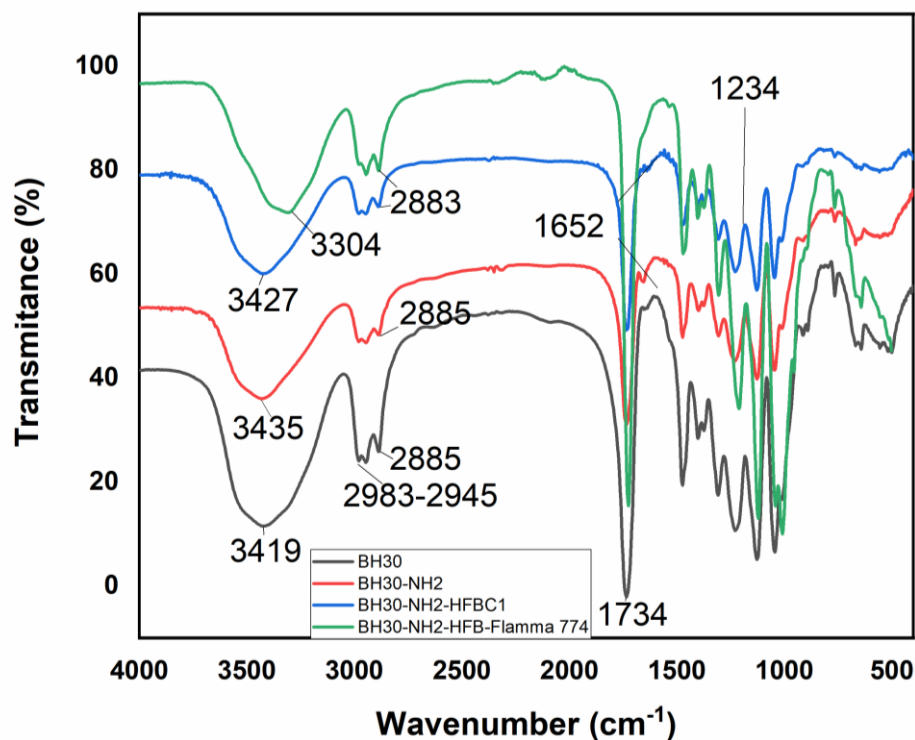


Figure 4.2. FT-IR spectrum of BH30 and functionalized polymers, BH30 (Black), BH30-NH₂ (Red), BH30-NH₂-HFBE1(Blue)

The peaks observed at 3419, 3435, and 3427 cm⁻¹ in the FT-IR spectrum of the polymers can be attributed to -OH groups in the structures of BH30, BH30-NH₂, BH30-NH₂-HFBE1, and BH30-HFBE-NH-Flamma 774, respectively. In the FT-IR spectra of BH30-NH₂, BH30-NH₂-HFBE1, and BH30-HFBE-NH-Flamma 774, it has been noted that the -OH and NH₂ stretching vibrations expected at 3435, 3437, and 3304 cm⁻¹ overlap, resulting in a broad band. The asymmetric stretching vibrations of the aliphatic C-H bond appear at 2983 and 2945 cm⁻¹, while the symmetric stretching vibrations are observed at 2885 cm⁻¹. The C=O stretching vibrations are noted at 1734 cm⁻¹. The peak observed at 1652 cm⁻¹ is attributed to N-H bending vibrations, indicating the formation of the BH30-NH₂ structure. The intense C-F peaks expected to be observed in the range of 1400-1000 cm⁻¹ overlap with the peaks of BH30 (*Heptafluorobutyryl Chloride*, n.d.-b). The C=O peaks of the HFBC molecule also coincide with the peak at 1734 cm⁻¹. In BH30-NH₂-HFBE1 and BH30-HFBE1-NH-Flamma774, the C-F stretching vibrations at 1234 cm⁻¹ overlap with the vibrational peaks of BH30. These findings are consistent with the literature (Su et al., 2018). Since the BH30-NH₂-HFBE compound was functionalized with 9.9x10⁻⁷ mol of Flamma 774 to obtain -BH30-NH₂-HFBE-Flamma 774, the peaks corresponding to functional groups such as Ar-H, S-O, and C=N in the Flamma 774

structure were observed at very low intensity. The vibrational peaks observed between 2310-1944 cm^{-1} are attributed to the benzene ring. Therefore, the peaks expected for Flamma 774 overlapping with those of BHX-NH₂-HFBE and BHX-NH₂-HFBE-Flamma 774 compounds, and these peaks were not separately observed in the FT-IR spectrum. The binding of Flamma 774 to the structure has been confirmed by fluorescence and ¹H-NMR analyses.

The FT-IR spectra of the BH20, BH20-NH₂, BH20-NH₂-HFBE1 and BH20-NH₂-HFBE-Flamma 774 polymers are presented in Figure 4.3.

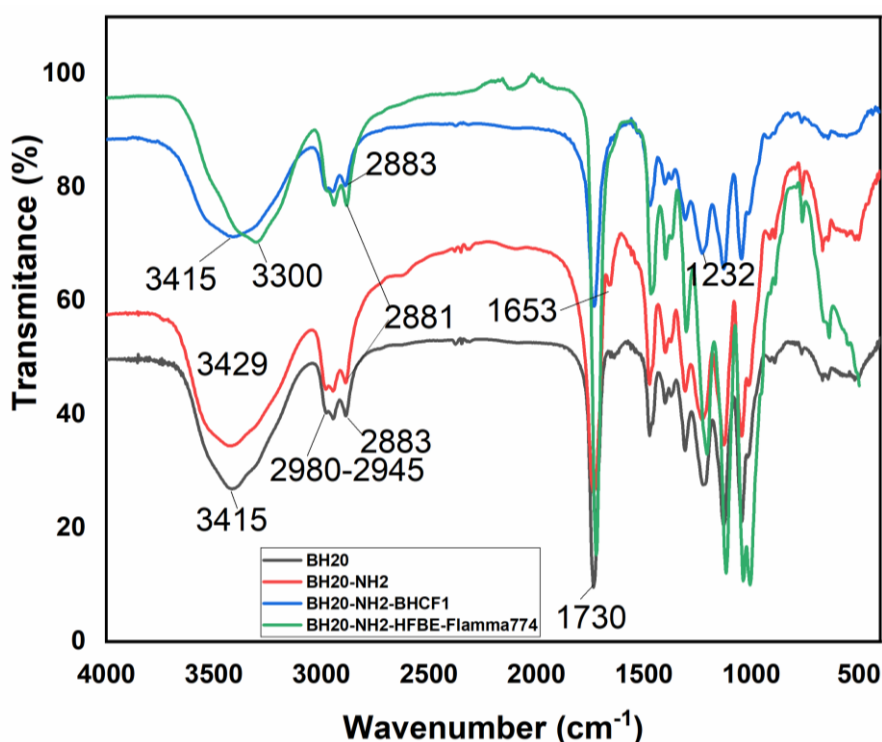


Figure 4.3. FTIR spectrum of BH20 and functionalized polymer, BH20 (Black), BH20-NH₂ (Red), BH20-NH₂-HFBE1 (Blue)

The peaks observed at 3415 cm^{-1} , 3429 cm^{-1} , and 3300 cm^{-1} in the spectra of BH20, BH20-NH₂, BH20-NH₂-HFBE1, and BH30-HFBE-NH-Flamma 774 correspond to -OH groups. In the spectra of BH20-NH₂ and BH20-NH₂-HFBE1, as well as BH30-HFBE-NH-Flamma 774, the -OH and NH₂ stretching vibrations overlap, appearing as a broad band. The asymmetric stretching vibrations of the aliphatic C-H bond are seen at 2980 cm^{-1} and 2945 cm^{-1} , while the symmetric stretching vibrations are observed at 2881 and 2883 cm^{-1} . The C=O stretching vibrations are noted at 1730 cm^{-1} , and the N-H bending vibration observed at 1653 cm^{-1} indicates the formation of the BH20-NH₂ structure. The

C-F stretching vibrations at 1232 cm^{-1} overlap with the vibrational peaks of BH20. The peaks corresponding to functional groups such as Ar-H, S-O, and C=N in the BH20-HFBE-NH-Flamma 774 structure are observed at very low intensity. Because these expected peaks overlap with those of the BHX-NH₂-HFBE and BH20-HFBE-NH-Flamma 774 compounds, they were not separately observed in the FT-IR spectrum. However, in the BH20-HFBE-NH-Flamma 774 polymer, the vibrational peaks in the range of $2220\text{--}1944\text{ cm}^{-1}$ are attributed to the benzene ring in Flamma 774. Additionally, fluorescence and ¹H-NMR analyses have confirmed the binding of Flamma 774.

4.1.2. FT-IR Spectrum Analysis of Functionalized Hyaluronic Acid.

The FT-IR spectra of hyaluronic acid (HA) conjugated with folic acid and RGD-R8 peptide (HA-NH-PEG-COOH) are presented in Figure 4.4.

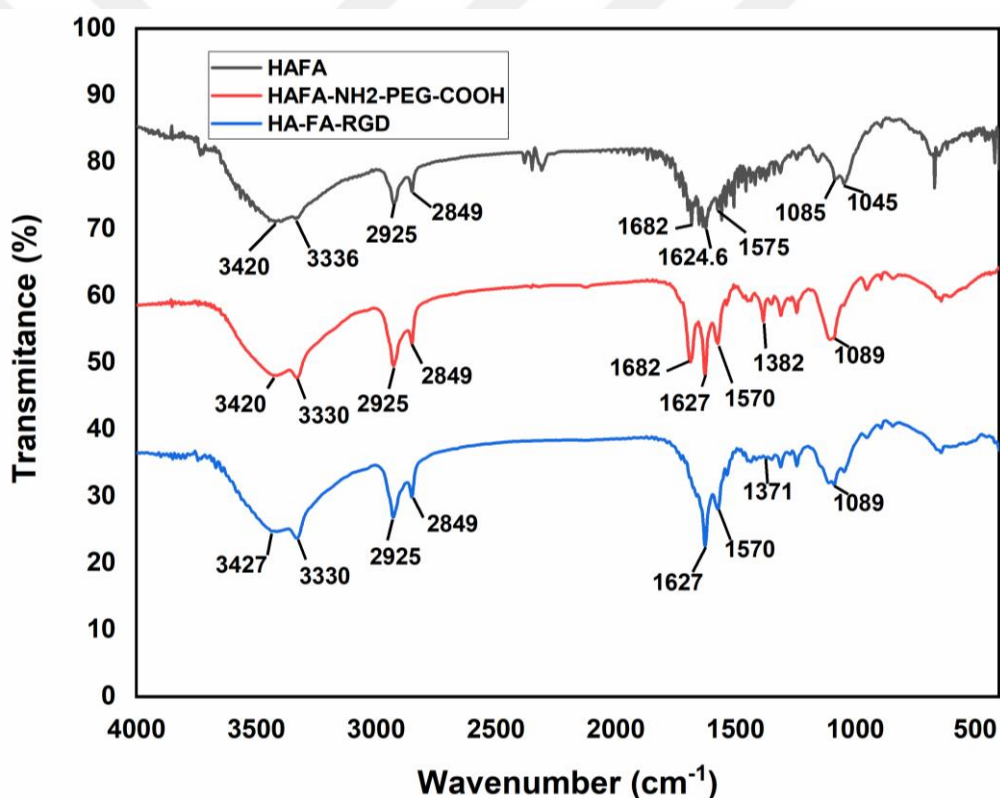


Figure 4.4. FT-IR spectra of hyaluronic acid functionalized with Folic Acid and RGD-R8

In the FT-IR spectra, the N-H stretching vibrations observed at 3427 cm^{-1} , 3336 cm^{-1} , and in the range of $3420\text{--}3330\text{ cm}^{-1}$ overlap with a strong broad peak from the -OH group. The spectral peaks at 2925 , 2846 cm^{-1} and 2849 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of aliphatic CH groups. A strong peak at 1682 cm^{-1}

is attributed to the symmetric stretching vibration of the C=O group. Additionally, the peaks at 1682, 1627, 1624, and 1570 cm^{-1} can be assigned to the bending vibration of -CO-NH- from primary amines. The peaks at 1382 cm^{-1} and 1371 cm^{-1} arise from the bending vibrations of the OH in the COOH group. The strong peak at 1109 cm^{-1} is attributed to the C-O stretching vibrations in the C-OH group, while the strong peaks at 1096 and 1081 cm^{-1} correspond to C-O-C stretching vibrations. The FT-IR spectra of HA-FA and HA-RGD are similar due to the presence of the same functional groups in both structures.

4.2. Differential Scanning Calorimetric (DSC) Analysis

The thermal properties of modified and unmodified Boltorn HX ($x = 40, 20, 30$) polymers were investigated using differential scanning calorimetry (DSC). The scanning was conducted at a rate of 10 $^{\circ}\text{C}/\text{min}$ over a temperature range of -50 $^{\circ}\text{C}$ to 400 $^{\circ}\text{C}$, and the results are presented in Figures 4.5, 4.6 and 4.7.

In Figure 4.5, endothermic peaks observed at 77 $^{\circ}\text{C}$, 54 $^{\circ}\text{C}$, 71 $^{\circ}\text{C}$, 87.5 $^{\circ}\text{C}$, and 79.4 $^{\circ}\text{C}$ are attributed to the breaking of -H bonds and the formation of new bonds in the BH-40, BH40-NH₂, and functionalized BH40-NH₂ with HFBC at three different molar ratios of (1: 0.25; 1: 0.2; 1: 0.15) were coded as BH40-NH₂-HFBE1, BH40-NH₂-HFBE2, and BH40-NH₂-HFBE3 polymers respectively, respectively. Additionally, two weak endothermic peaks at -29 $^{\circ}\text{C}$ and -42 $^{\circ}\text{C}$ are seen for the BH40-NH₂. The broad endothermic peaks in the range of 50-80 $^{\circ}\text{C}$ are attributed to hydrogen bonding.

The shift observed in the TG graph arises from the different terminal groups at the ends of the hyperbranched polymer. This shift also results from a decrease in the number of hydrogen bonds after binding with -OH groups. Zagar et al. interpreted the heating above T_g as leading to the formation of more stable hydrogen bonds due to the rearrangement in the hydrogen bonding network (Žagar et al., 2005). The newly formed hydrogen bonds are more stable; while fewer in number, they are thought to occur between linear arrangements within the hyperbranched polymer structure. It has been reported that this bond network is not only reorganized intermolecularly but also contributes to the intramolecular folding of branches towards the interior of the hyperbranched structure (Žagar et al., 2005). As the temperature increases, the hydrogen bonding network between (-OH...OH-) and (-OH...O=C) weakens, resulting in another endothermic peak being observed after 28 $^{\circ}\text{C}$.

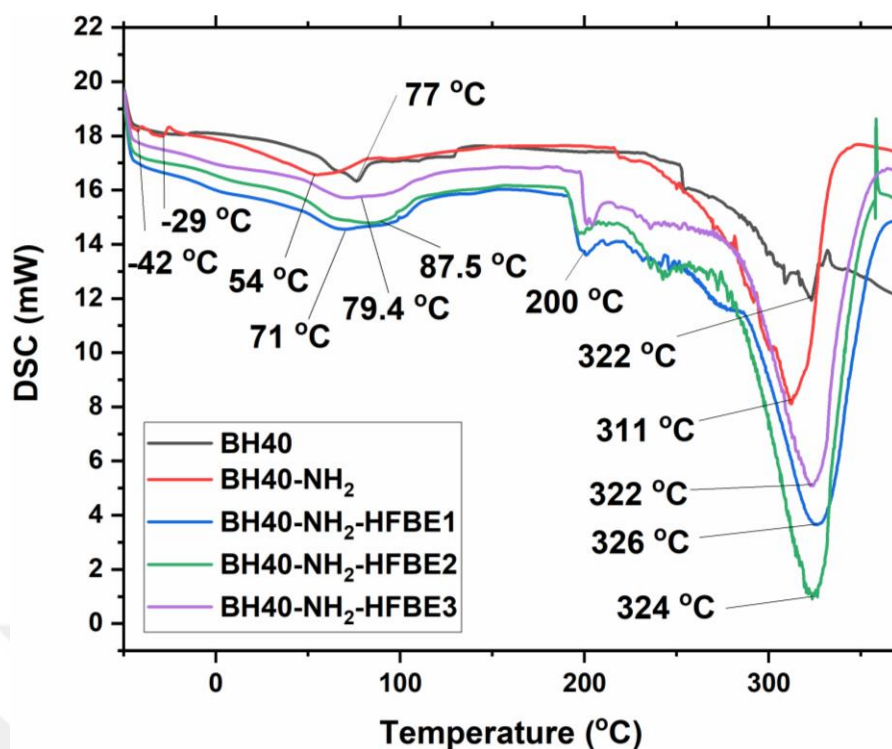


Figure 4.5. DSC curve of Boltorn 40 (Black), BH40-NH₂ (Red), BH40-NH₂-HFBE1 (Blue), BH40-NH₂-HFBE2 (Green) and BH40-NH₂-HFBE3 (Purple) polymer

In the BH40-NH₂-HFBE1, BH40-NH₂-HFBE2, and BH40-NH₂-HFBE3 polymers, an endothermic peak is observed at 200 °C, while this peak is not seen in the BH40 and BH40-NH₂ polymers. Additionally, the endothermic peak for BH40 is observed at 322 °C, shifting to 311 °C for BH40-NH₂. For BH40-NH₂-HFBE1, BH40-NH₂-HFBE2, and BH40-NH₂-HFBE3, this peak shifts to 226 °C, 324 °C, and 322 °C, respectively, and is observed as a more intense endothermic peak.

These results indicate that the BH40 polymer has been successfully modified. The DSC curves for Boltorn 30, BH30-NH₂, BH30-NH₂-HFBE1, BH30-NH₂-HFBE2, and BH30-NH₂-HFBE3 polymers are presented in Figure 4.6.

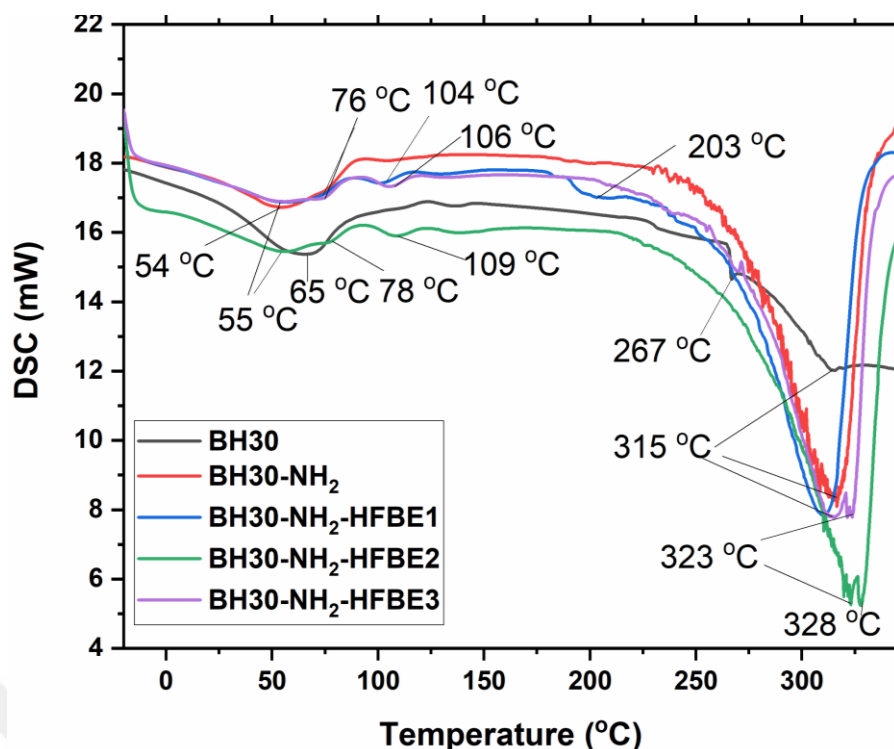


Figure 4.6. DSC curve of Boltorn 30 (Black), BH30-NH₂ (Red), BH30-NH₂-HFBE1 (Blue), BH30-NH₂-HFBE2 (Green) and BH30-NH₂-HFBE3 (Purple) polymer

The endothermic peaks observed at 54 °C, 55 °C, 65 °C, 76 °C, and 78 °C for the BH-30, BH30-NH₂, BH30-NH₂-HFBE1, BH30-NH₂-HFBE2, and BH30-NH₂-HFBE3 polymers can be attributed to the breaking of hydrogen bonds and the formation of new ones. The additional endothermic peak at 100 °C in the BH30-NH₂-HFBE1, BH30-NH₂-HFBE2, and BH30-NH₂-HFBE3 polymers are not observed in the BH30 and BH30-NH₂ polymers. Furthermore, the endothermic peak for BH30 observed at 315 °C is seen as a more intense peak for BH30-NH₂, BH30-NH₂-HFBE1, and BH30-NH₂-HFBE3, with the peak shifting to 323 °C and 328 °C for BH30-NH₂-HFBE2 and BH30-NH₂-HFBE3, respectively, indicating that the polymer has been modified.

The DSC curves for BH20-NH₂, BH20-NH₂-HFBE1, BH20-NH₂-HFBE2, and BH20-NH₂-HFBE3 polymers are presented in Figure 4.7.

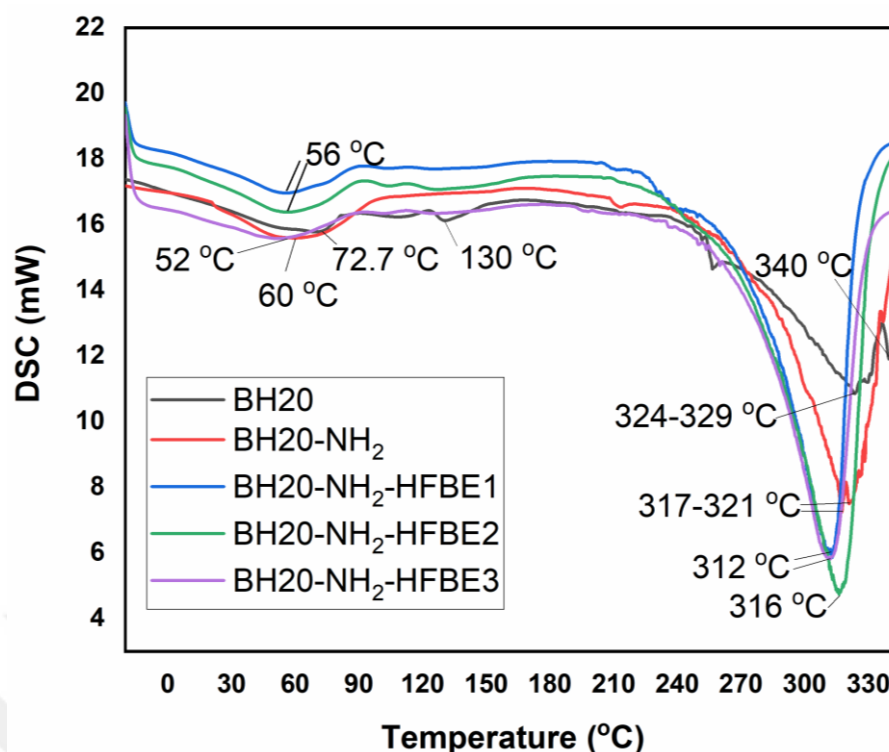


Figure 4.7. DSC curve of Boltorn 20 (Black), modified with NH_2 group (Red), functionalized with fluorine BH-20- NH_2 -HFBE1 (Blue), BH-20- NH_2 -HFBE2 (Green) and BH-20- NH_2 -HFBE3 (Purple) polymer.

In the BH20 polymer, an endothermic peak is observed at 72.7 °C while an extra endothermic peak is observed at 130 °C. Moreover, some peaks are noted at 60 °C, 56 °C, and 52 °C for the BH20- NH_2 , BH20- NH_2 -HFBE1, BH20- NH_2 -HFBE2, and BH20- NH_2 -HFBE3 polymers, respectively. These peaks can be attributed to the breaking of hydrogen bonds in the polymer structures and the formation of new ones. Additionally, the endothermic peak observed for BH20 at 324-329 °C is seen as more intense for BH20- NH_2 , BH20- NH_2 -HFBE1, BH20- NH_2 -HFBE2, and BH20- NH_2 -HFBE3 at 317-321 °C, 312 °C, and 316 °C, respectively. These results indicate that the polymer has been modified.

4.3. NMR Studies

4.3.1. ^1H -NMR Spectrum of BHX- NH_2 -HFBE

^1H -NMR spectrum of amine functionalized BHX- NH_2 -HFBC polymers is given in Figure 4.8. Figure 4.8 (a) showed the conjugation of $-\text{NH}_2$ group at $-\text{OH}$ of hyperbranched BH40- NH_2 -HFBC polymer. Surrounding environment of the neighboring hydrogen

atoms also have been changed. $^1\text{H-NMR}$ (DMSO-d_6) δ (ppm) a: (1.04 $-\text{NH}_2$), (1.07-1.26 CH_3), (2.52-2.53 DMSO), (3.34-3.51 CH_2-OH), (4.06-4.18, 4.63 CH_2-OR), (4.95 OH). The $^1\text{H-NMR}$ spectrum of the amino-functionalized BH-30 and BH20 polymer is presented in Figure 4.8(b) and Figure 4.8(c), where NH_2 molecules are bonded to the $-\text{OH}$ groups of the hyperbranched BH-40 polymer. The environment of the hydrogen atoms has also changed due to the neighboring atoms. The $^1\text{H-NMR}$ (DMSO-d_6) chemical shifts (δ ppm) are as follows: (1.04 $-\text{NH}_2$), (1.07-1.26 CH_3), (2.52-2.53 DMSO), (3.34-3.51 CH_2-OH), (4.06-4.18, 4.63 CH_2-OR), and (4.95 OH).

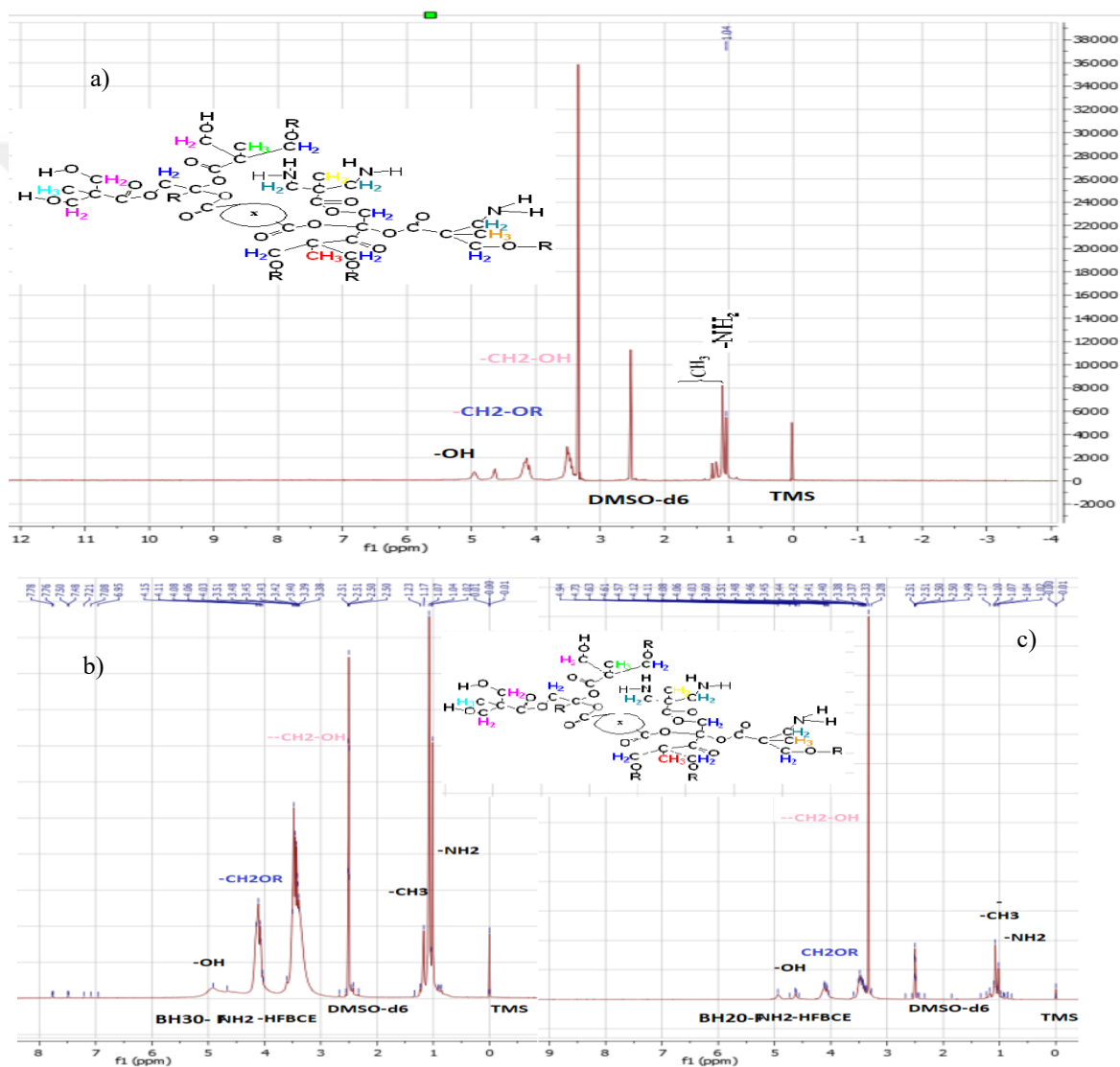


Figure 4.8. $^1\text{H-NMR}$ spectrum of Hyperbranched a) BH40-NH₂-HFBE, b) BH30-NH₂-HFBE, c) BH20-NH₂-HFBE polymer

4.3.2. ^1H -NMR Spectrum of BHX-HFBE-NH-F774

^1H -NMR spectrum of Hyperbranched BH40-HFBE-NH-F774 polymer is given in Figure 4.9.

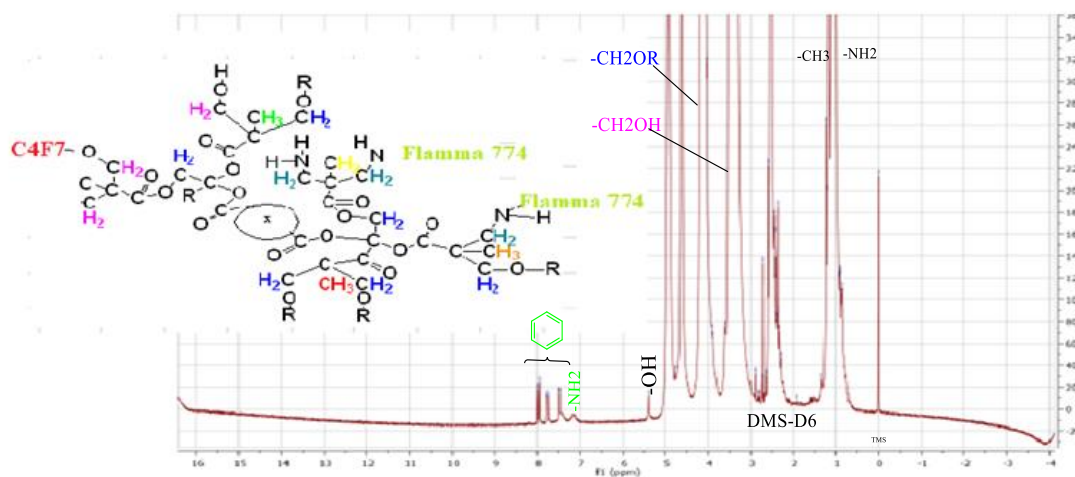


Figure 4.9. ^1H -NMR spectrum of BH40-HFBE-NH₂-F774

^1H -NMR (DMSO- d_6) δ (ppm) a (1.02-1.24 CH₃), (1.04 -NH₂), (2.37-2.59 DMSO), (3.335-3.48 CH₂ -OH), (4.06-4.11CH₂-OR), (4.62-4.93 CH₂-OH), (5.38 -OH) Boltorn H40 ve (7.175 -NH), (7.482, 7.76-7.99 -benzene ring) peaks are attributed to Flamma 774. The peaks observed between 0.5-1.17 ppm originate from the -H of the -CH₃ groups in linear, terminal, and dendritic positions. ^1H -NMR spectrum of hyperbranched BH30-HFBE-NH-F774 polymer is given in Figure 4.10.

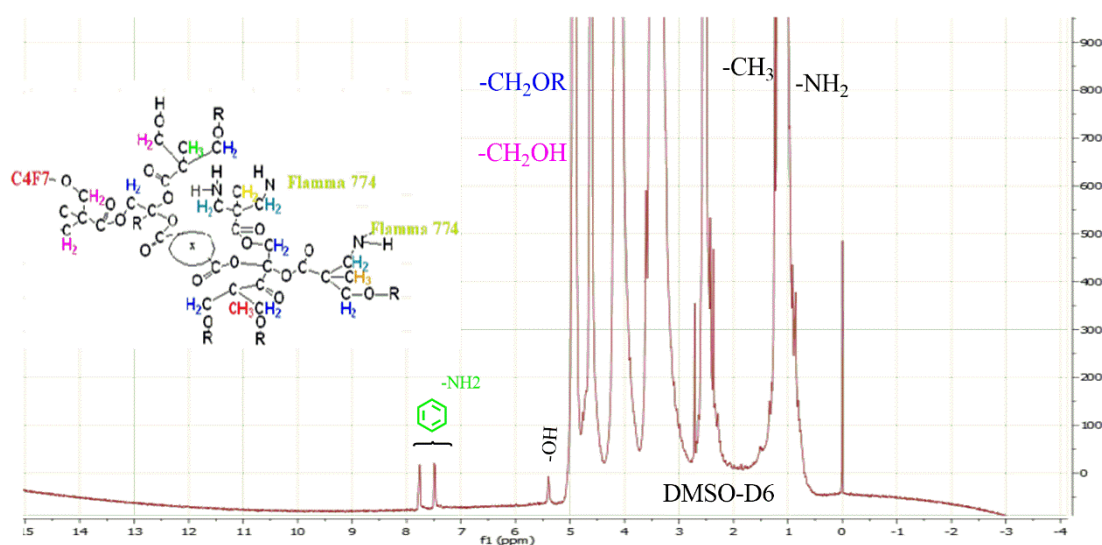


Figure 4.10. ^1H -NMR spectrum of BH30-HFBE-NH₂-F774

$^1\text{H-NMR}$ (DMSO-d_6) δ (ppm) (1.02-1.24 CH_3), (1.04 $-\text{NH}_2$), (2.37-2.59 DMSO), (3.335-3.48 CH_2-OH), (4.06-4.11 CH_2-OR), (4.62-4.93 CH_2-OH), (5.388 $-\text{OH}$), (7.482, 7.76 -benzene ring) pikes belong to Flamma 774. The peaks observed between 0.5-1.17 ppm are due to the protons of the $-\text{CH}_3$ group at linear, terminal, and dendritic positions.

$^1\text{H-NMR}$ spectrum of hyperbranched BH20-HFBE-NH-F774 polymer is given in Figure 4.11.

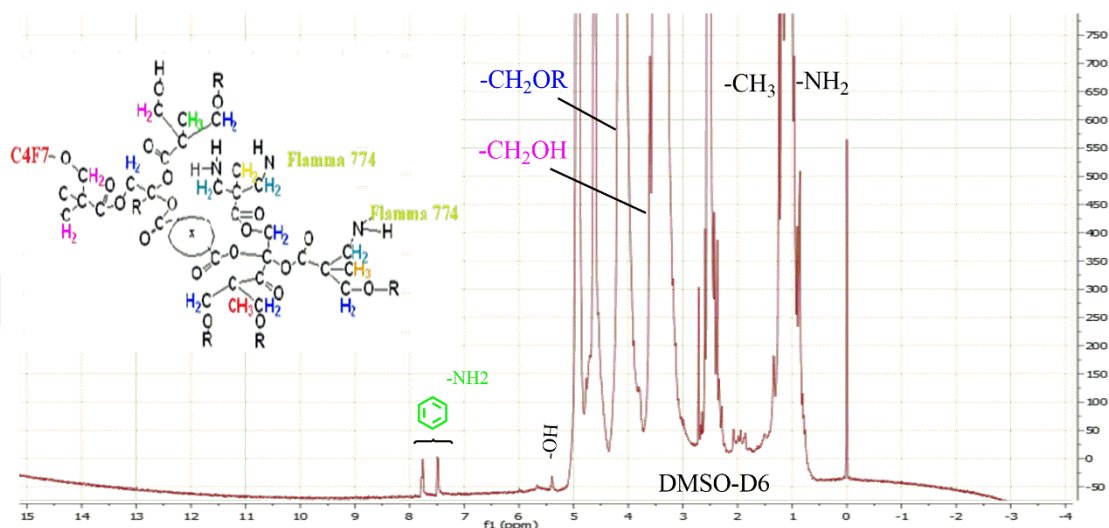


Figure 4.11. $^1\text{H-NMR}$ spectrum of BH20-HFBE-NH₂-F774

$^1\text{H-NMR}$ (DMSO-d_6) δ ppm a (1.02-1.24 CH_3), (1.04 $-\text{NH}_2$), (2.37-2.59 DMSO), (3.335-3.48 CH_2-OH), (4.06-4.11 CH_2-OR), (4.62-4.93 CH_2-OH), (5.388 $-\text{OH}$), (7.482, 7.76 - benzene ring) peaks belong to Flamma 774 (Figure 4.14). The peaks observed in the range of 0.5-1.17 ppm originate from the protons of $-\text{CH}_3$ groups in linear, terminal, and dendritic positions.

4.3.3. $^1\text{H-NMR}$ Spectrum of HA-FA Conjugate

The $^1\text{H-NMR}$ spectrum of folic acid conjugated with hyaluronic acid dissolved in D_2O is shown in Figure 4.12, where the different position of the $-\text{H}$ atoms in the HA-FA conjugate can be observed. The $^1\text{H-NMR}$ (D_2O) chemical shifts (δ ppm) are as follows: in hyaluronic acid, (1.25-1.27 $-\text{NH}_2$), (1.61-1.96 COCH_3), (2.934-4.050 $\text{H}(\text{CH}_2)$ and CH); (4.39-4.902 D_2O), (6.77 $-\text{CH}$), (7.60-7.62 $-\text{NH}$), (7.99 $\text{C}=\text{O}$ -bound $-\text{NH}$), (8.68 $-\text{CH}$). All peaks in the range of 6.77-8.68 ppm originate from different hydrogen atoms of folic acid.

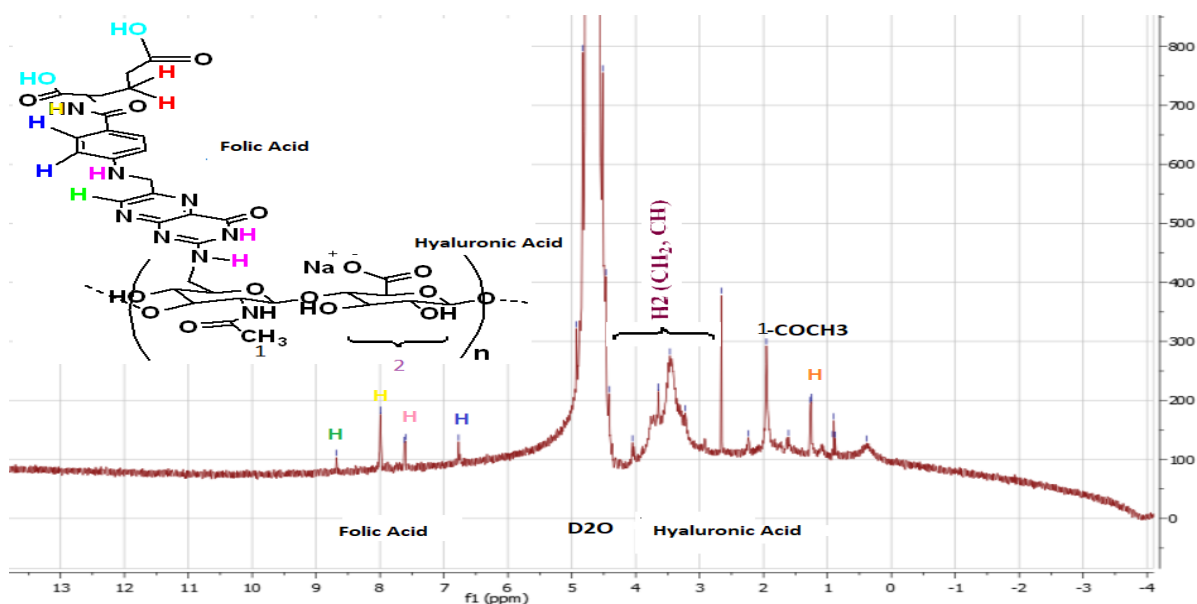


Figure 4.12. ^1H -NMR spectrum of Folic acid functionalized with hyaluronic acid

4.3.4. ^1H -NMR Spectrum of Folic Acid, Poly(ethylene glycol)2-aminoethyle Ether Acetic Acid Conjugated with Hyaluronic Acid

The ^1H -NMR spectrum of folic acid dissolved in D_2O and hyaluronic acid conjugated with poly(ethylene glycol) 2-aminoethyl ether acetic acid is presented in Figure 4.13.

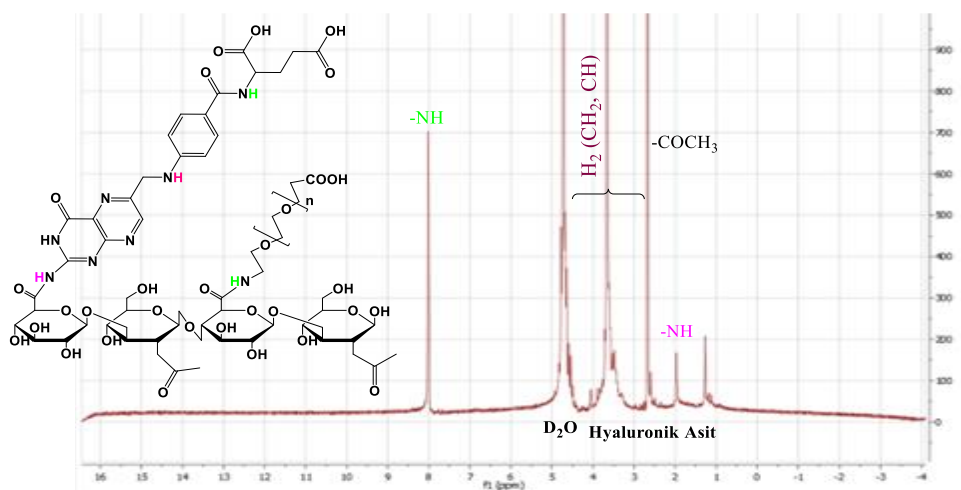


Figure 4.13. ^1H -NMR spectrum of hyaluronic acid functionalized with folic acid, poly(ethylene glycol) 2-aminoethyl ether acetic acid

The different positions of the $-\text{H}$ atoms in the HA-NH-PEG-COOH conjugate can be observed. The peaks between 1.12 and 1.29 ppm correspond to protons of $(-\text{CH}_3)$ group in hyaluronic acid. A peak at 1.97 ppm, associated with the $-\text{COCH}_3$ protons in hyaluronic

acid, overlaps with the peak for the -NH proton linked to the benzene ring of folic acid, appearing at 1.99 ppm. Protons belonging to the -CH₂ groups in hyaluronic acid are observed between 2.592 and 2.73 ppm. The peaks seen at 2.934-4.050 ppm correspond to CH₂ and CH protons. However, after conjugation with poly(ethylene glycol)2-aminoethyl ether acetic acid, the peak at 3.660 ppm appears as a more intense peak. Peaks between 4.39 and 4.902 ppm are attributed to D₂O. The peak at 8.01 ppm corresponds to the -NH proton of the poly(ethylene glycol)2-aminoethyl ether acetic acid linked through C=O. These results indicate that folic acid and poly(ethylene glycol) 2-aminoethyl ether acetic acid have been successfully conjugated with hyaluronic acid.

4.3.5. ¹H-NMR Spectrum of Folic Acid and RGD Peptide Conjugated with Hyaluronic Acid

The ¹H-NMR spectrum of folic acid dissolved in D₂O and conjugated with RGD-R8 peptide is presented in Figure 4.14.

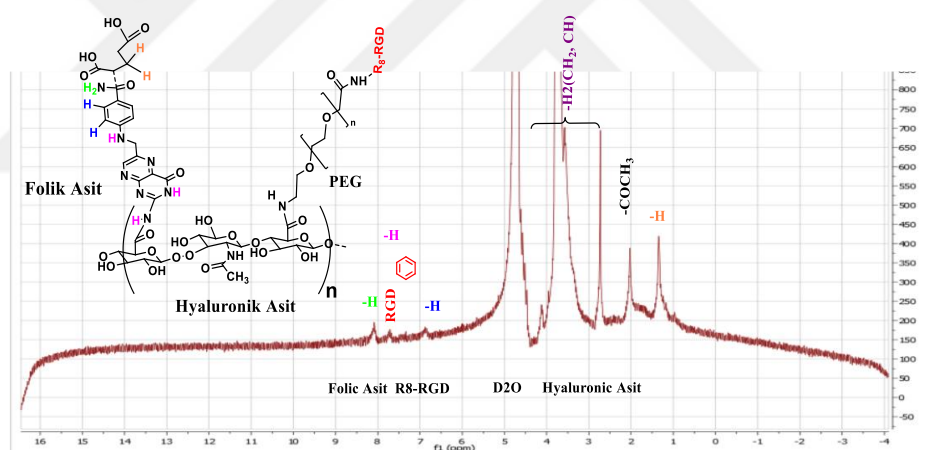


Figure 4.14. ¹H-NMR spectrum of folic acid and RGD peptide conjugated with hyaluronic acid

The different positions of the -H atoms in the HA conjugate can be observed. The peaks in the range of 1.12-1.29 ppm correspond to the -CH₃ protons of hyaluronic acid. A peak at 1.97 ppm, attributed to the -COCH₃ protons in hyaluronic acid, overlaps with the peak from the -NH group proton attached to the benzene ring in folic acid, appearing at 1.99 ppm. The protons of the -CH₂ groups in hyaluronic acid are observed at 2.592-2.73 ppm. Peaks observed at 2.934-4.050 ppm correspond to the CH₂ and CH protons. However, after conjugation with polyethylene glycol 2-aminoethyl ether acetic acid, the

peak at 3.660 ppm appears as a more intense peak. The peaks between 4.39-4.902 ppm belong to D₂O. The peak observed at 6.84 ppm corresponds to the protons of the heterocyclic rings present in folic acid and RGD. The peaks at 7.33 and 7.71 ppm are associated with the aromatic rings in folic acid and the protons of the -NH₂ group linked to these rings. The peak at 8.01 ppm corresponds to the -NH proton of the polyethylene glycol 2-aminoethyl ether acetic acid attached via C=O. The results indicate that folic acid and RGD-R8 peptide have been successfully conjugated with the polyethylene glycol 2-aminoethyl ether acetic acid chain and hyaluronic acid.

4.3.6. ¹⁹F-NMR Study of BH40-NH₂-HFBE-Flamma 774 Polymer

Fluor-19, a stable isotope, is found in small amounts in nature, but due to its good nuclear properties, it is used in ¹⁹F-NMR. ¹⁹F-NMR is faster and more sensitive than ¹H-NMR in measurements and can identify fluorine even at a minimal concentration of 0.1%. In heptafluorobutyryl chloride, ¹⁹F-NMR gives a single peak, while after functionalization, it splits into three peaks. The ¹⁹F-NMR spectra of hyperbranched BHX-HFBE-NH-F774 (x=40, 30, 20) polymers are presented in Figures 4.15, 4.16, and 4.17. The ¹⁹F-NMR spectrum obtained after the polymer is functionalized shows three peaks at -126.29, -116.36, and -80.13 ppm, as presented in Figure 4.15. A strong coupling is observed between heptafluorobutyryl-BH40 and its nearest neighbors. The peak at -126.29 ppm is attributed to the fluorine adjacent to the -COO group, while the peaks at -116.2 and -80.13 ppm correspond to the typical CF₂ and CF₃ groups. The ¹⁹F-NMR spectra of the BH30- HFBE-NH-F774 and BH20- HFBE-NH-F774 polymers show peaks at the same positions, but the intensity of the peaks is lower in the hyperbranched BH20- HFBE-NH-F774 polymer (*Heptafluorobutanoyl-Chloride - Optional[¹⁹F NMR] - Chemical Shifts - SpectraBase, 2025*)...

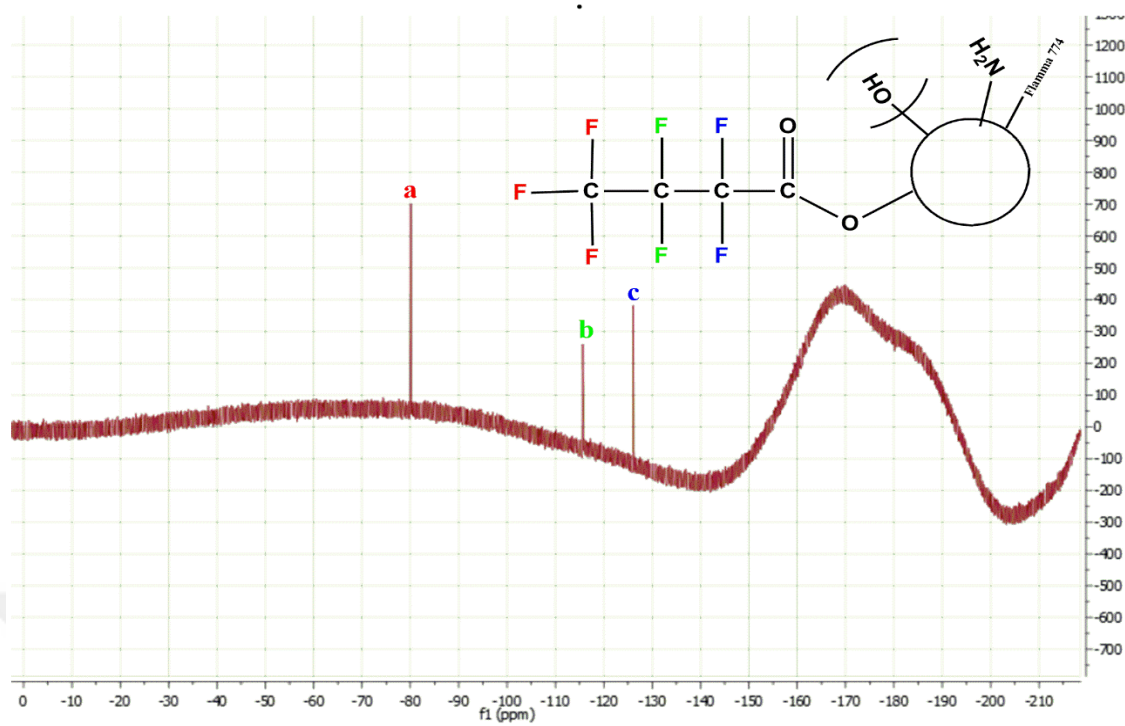


Figure 4.15. ^{19}F -NMR spectrum of BH40-HFBE-NH₂-F774

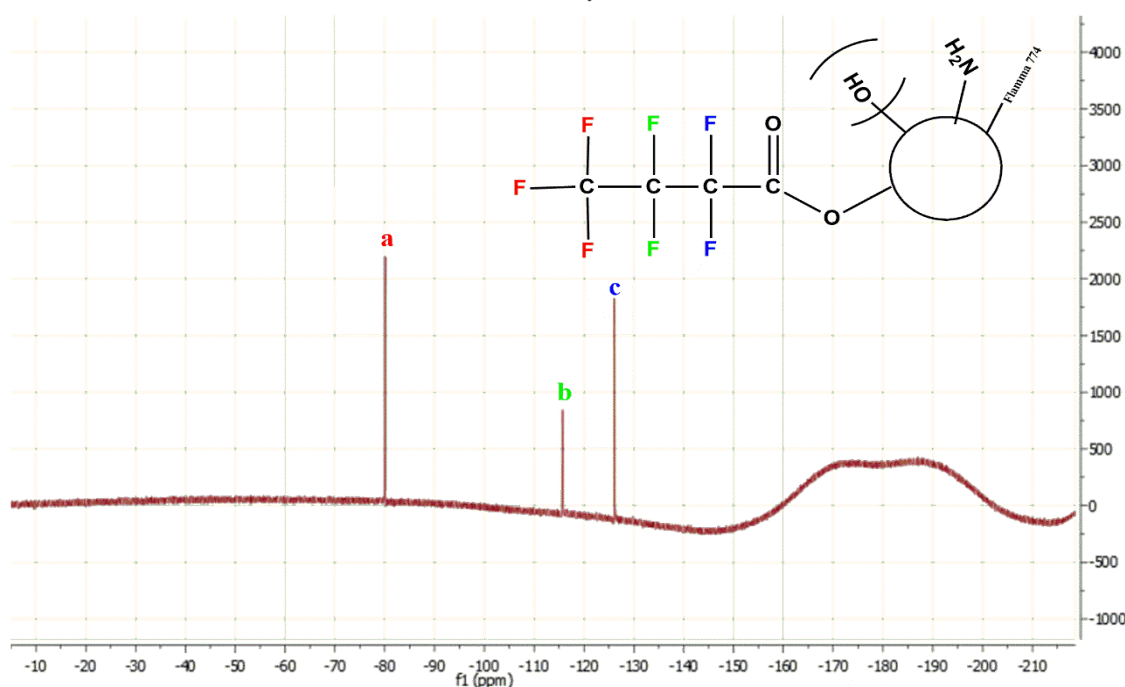


Figure 4.16. ^{19}F -NMR spectrum of BH30-HFBE-NH₂-F774

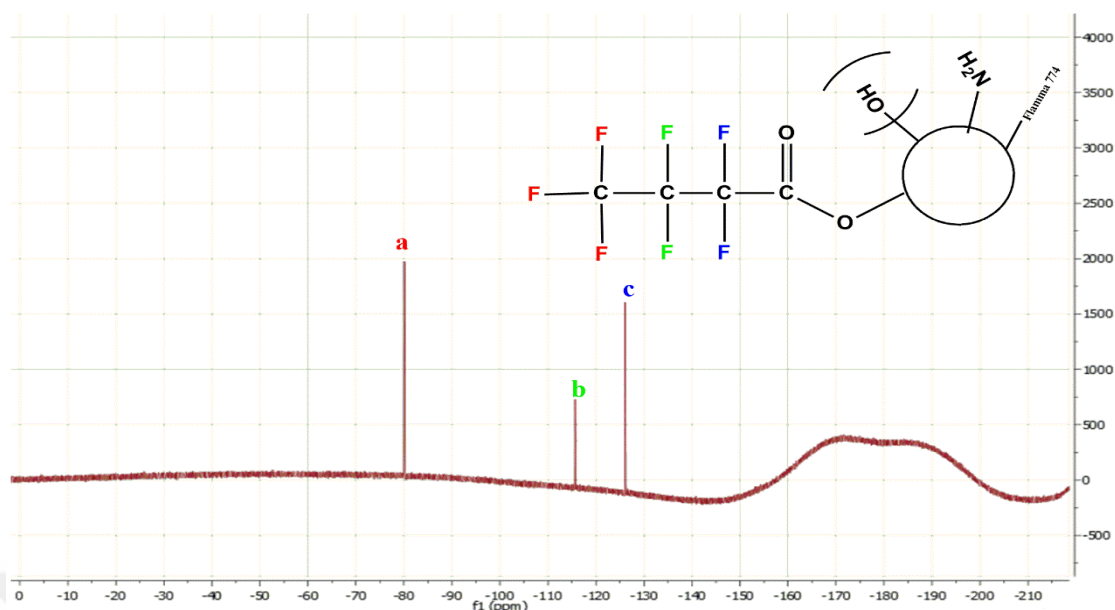


Figure 4.17. ^{19}F -NMR spectrum of BH20-HFBE-NH₂-F774

4.3.7. ^{13}C -NMR Spectrum of BHX-HFBE-NH-F774

^{13}C -NMR provides information about the surrounding Chemical groups and structures of carbon atoms, making it an important analytical tool for the identification of compounds and elucidation of structures. However, the peaks in the ^{13}C -NMR spectrum generally appear weaker compared to the proton NMR spectrum (Gurd ve Keim, 1973) . The ^{13}C -NMR spectrum of the hyperbranched BH40-HFBE-NH₂-F774 polymer is presented in Figure 4.18.

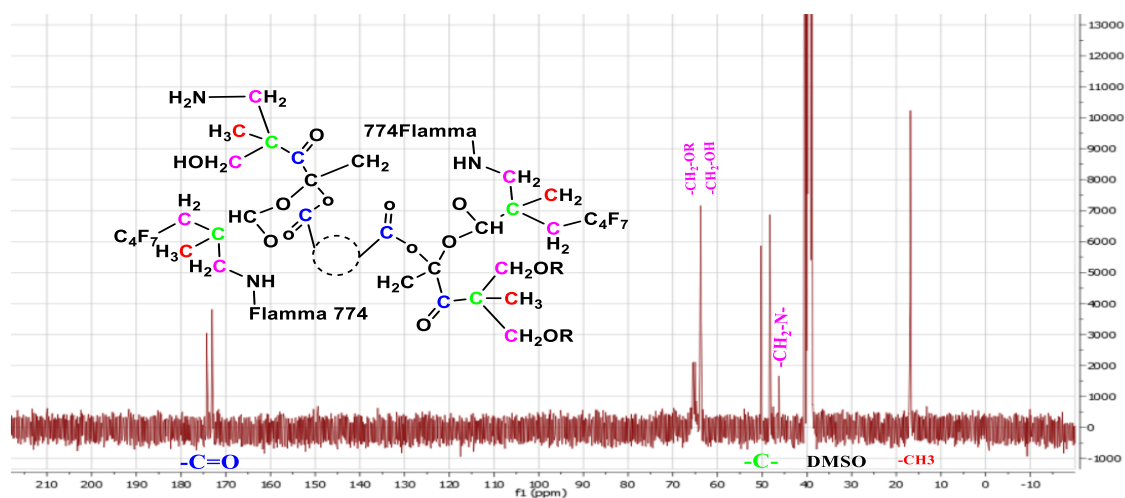


Figure 4.18. ^{13}C -NMR spectrum of BH40-HFBE-NH₂-F774

^{13}C -NMR (DMSO- d_6) δ ppm values are as follows: (16.76 and 16.84 -CH₃), (38.93, 39.13, 39.34, 39.55, 39.76, 39.97, 40.18, 40.46, 40.47 DMSO, -NH₂), (46.23, 48.25, 50.23 NH₂, -C-, C-O-C), (63.74, 63.89, 65.05, 65.52 CH₂-OH, CH₂-OR), (173.04, 173.17, 174.31 C=O). These peaks belong to BH40-HFBE-NH₂-F774; however, the very low molecular weight functionalized C-F peaks were not observed in the ^{13}C -NMR spectrum of BH40-HFBE-NH₂-F774, although the presence of CF groups was confirmed by ^{13}C -NMR. Additionally, C-F peaks were very weakly observed in the ^{13}C -NMR spectra of the BH30-HFBE-NH₂-F774 and BH20-HFBE-NH₂-F774 polymers. The peaks related to Flamma overlap with the peaks of BH40 in the ^{13}C -NMR spectrum. The ^{13}C -NMR spectrum of the hyperbranched BH30-HFBE-NH₂-F774 polymer is presented in Figure 19.

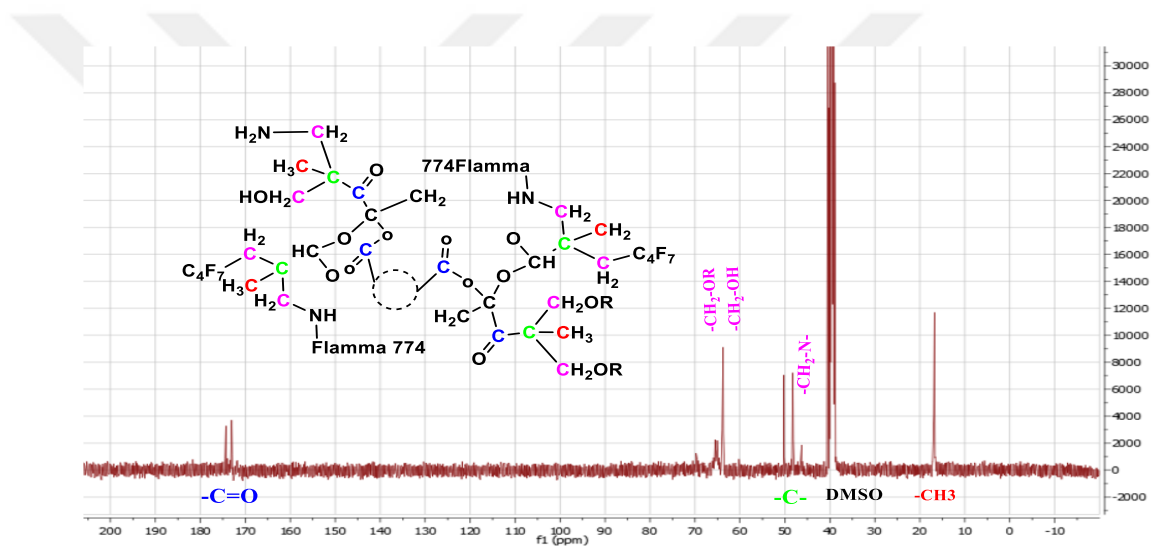


Figure 4.19. ^{13}C -NMR spectrum of BH30-HFBE-NH₂-F774

The ^{13}C -NMR peaks of BH30-HFBE-NH₂-F774 obtained in DMSO- d_6 solvent are as follows (δ ppm): (16.70, 16.75, 16.84 -CH₃), (38.94, 39.14, 39.35, 39.56, 39.77, 39.98, 40.19, 40.47 DMSO, -NH₂), (46.26, 48.25, 50.22 NH₂, -C-, C-O-C), (60.09 CH₂-OH), (63.76, 63.88, 65.49 CH₂-OR), (68.75, 69.87, 70.17 C-X), (173.04, 173.17, 174.31 C=O). The ^{13}C -NMR spectrum of BH30-HFBE-NH₂-F774 confirms that the polymer has been functionalized. The ^{13}C -NMR spectrum of the hyperbranched BH20-HFBE-NH₂-F774 polymer is presented in Figure 4.20.

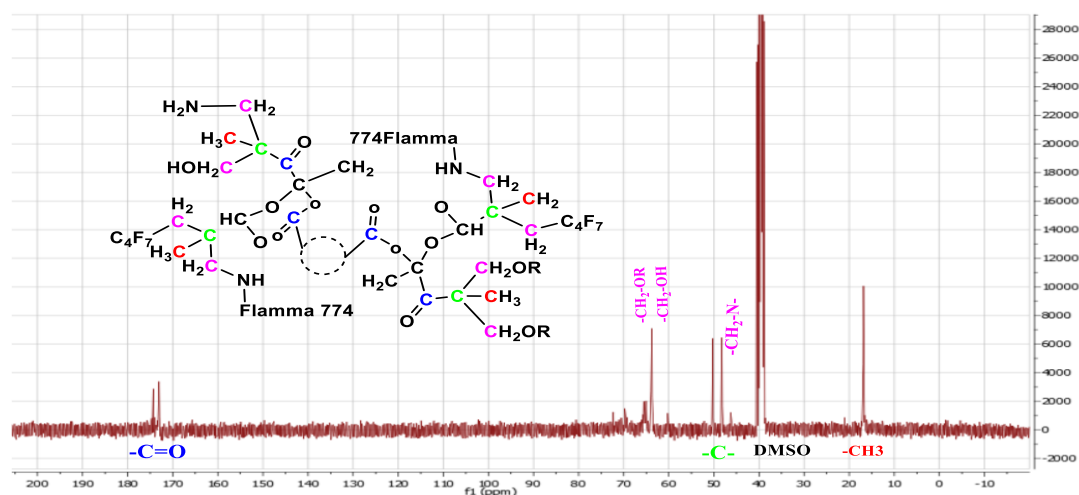


Figure 4.20. ^{13}C -NMR spectrum of BH20-HFBE-NH₂-F774

The ^{13}C -NMR peaks of BH20-HFBE-NH₂-F774 obtained in DMSO-d₆ are as follows (δ ppm): (16.75, 16.84, 20.49 -CH₃), (38.94, 39.14, 39.35, 39.56, 39.77, 39.98, 40.19, 40.47 DMSO, -NH₂), (46.094, 46.29, 48.26, 50.23 NH₂, -C-, C-O-C), (60.09 CH₂-OH), (63.75, 63.90, 65.07, 65.52 CH₂-OR), (69.27, 69.59, 69.96, 70.19, 72.20, 72.43 C-X), and (173.04, 173.17, 174.31 C=O). The ^{13}C -NMR spectrum of BH20-HFBE-NH₂-F774 indicates that the polymer has been functionalized.

4.4. Ninhydrin Test

The reaction of ninhydrin (triketohydrindene hydrate), used for the qualitative and quantitative determination of amino acids, produces a purple-colored product for all amino acids containing an α -amino group. However, amino acid molecules containing a secondary amino group, such as proline and hydroxyproline, yield a yellow-colored condensation product. The intensity of the color of the resulting product varies linearly with the concentration of the amino acid. Proteins and peptides containing free amino groups at the α -carbon react in the ninhydrin test to form a blue-purple colored product. Thus, the number of free amino groups can be determined based on the intensity of the color produced (Parkinson et al., 2008.).

To facilitate the binding of Flamma 744, 500 mg samples of BHX (40, 30, 20) were functionalized with -NH₂ groups (corresponding to 0.0022 mol, 0.00022 mol, and 0.00045 mol, respectively). A ninhydrin test was performed to determine the amount of bound Flamma 774, or in other words, the number of remaining amine groups at the terminal ends of BHX. For this purpose, 170 mg of ninhydrin and 30 mg of hydrazine were dissolved in 20 mL of 2-methoxyethanol. A 50 μL aliquot of this solution was mixed

with a sodium acetate buffer (0.2 mol. L⁻¹, pH=5.4). Different concentrations of 50 µL BHX-HFBE-NH₂ were added to this mixture and incubated in boiling water for 10 minutes. After cooling to room temperature, 150 µL of an ethanol/water (v/v=60:40) mixture was added. The absorbance of the samples was measured spectrophotometrically at a wavelength of 570 nm. The calibration curve for BH40 was prepared based on the absorbance-concentration relationship and is presented in Figure 4.21.

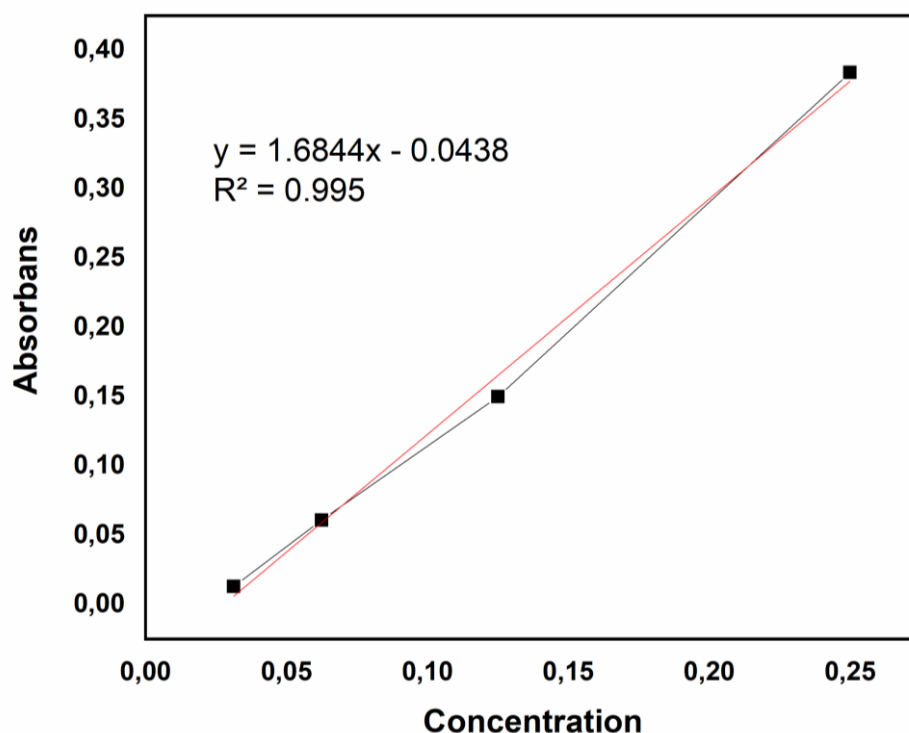


Figure 4.21. Calibration curve of BH40-NH₂ at different concentration values

The obtained calibration curve had an R² value of 0.995. The calibration curve, expressed by the equation $y = 1.6844x - 0.0438$, showed that the absorbance value of BH40-HFBE-NH-Flamma 774 at a wavelength of 571 nm was 0.228. By substituting this absorbance value into the calibration curve formula, it was determined that the number of -NH₂ groups not bound to Flamma 774 was 0.190 mmol, which corresponds to a concentration of 0.25 mol.L⁻¹.

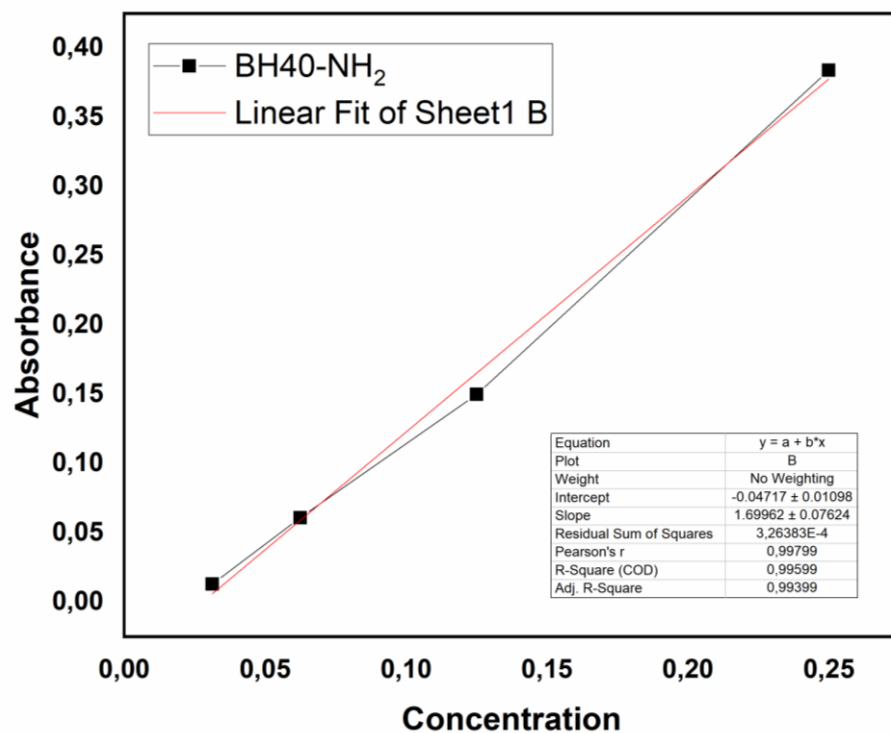


Figure 4.22. Calibration curve for the absorbance changes of BH40-NH₂ versus concentration

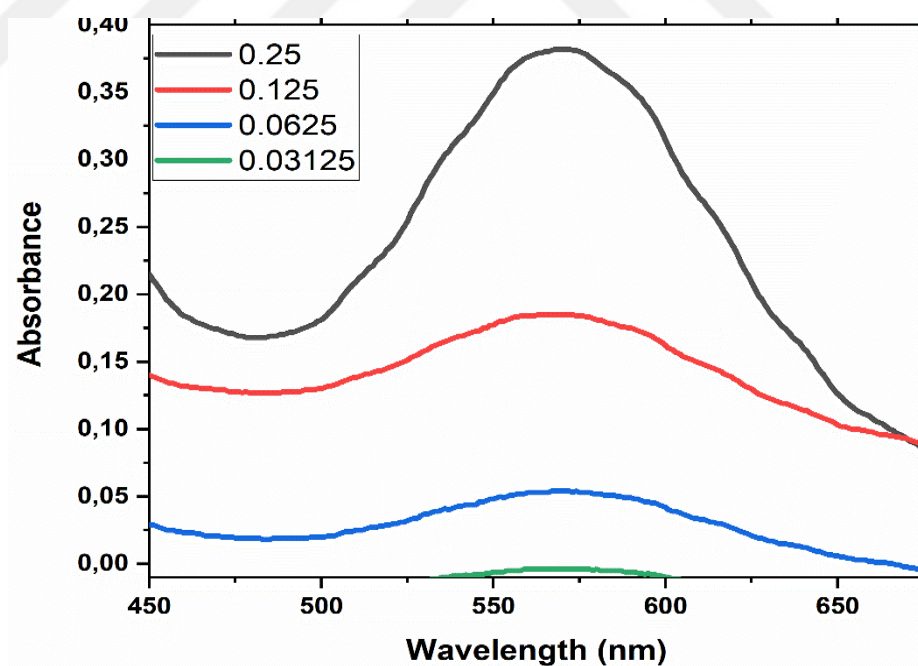


Figure 4.23. UV-vis spectra of BH40-NH₂ showing absorbance change versus concentration

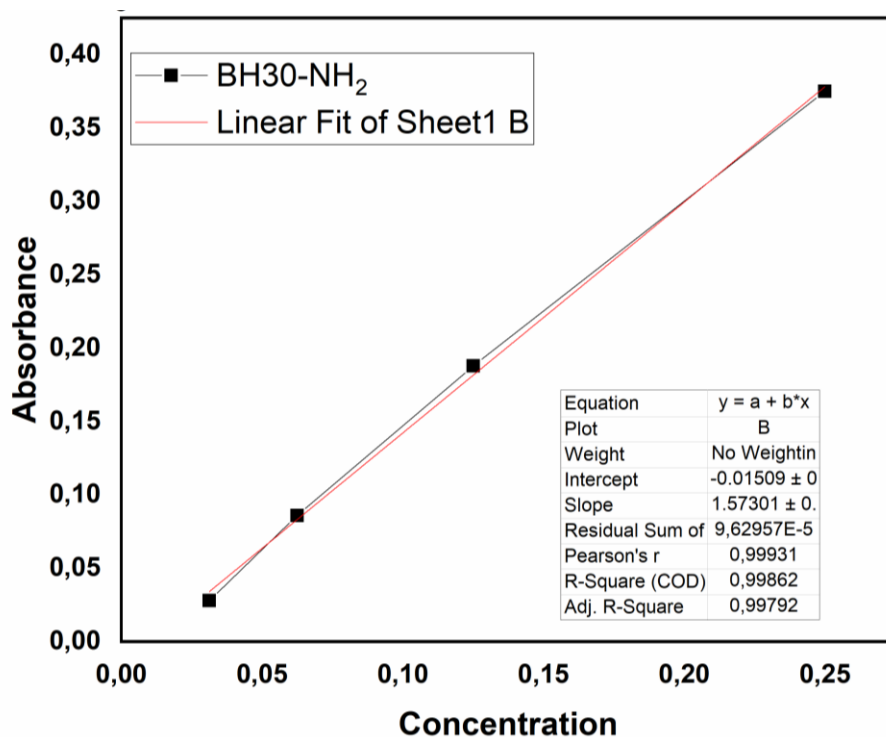


Figure 4.24. Calibration curve for the absorbance changes of BH30-NH₂ versus concentration

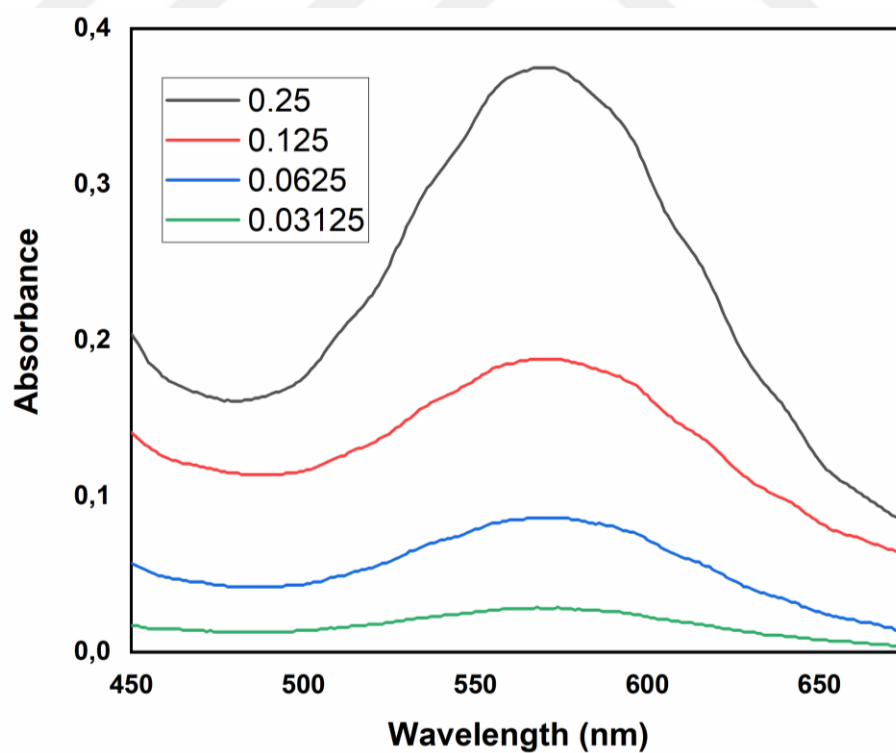


Figure 4.25. UV-vis spectra of BH30-NH₂ showing absorbance change versus concentration

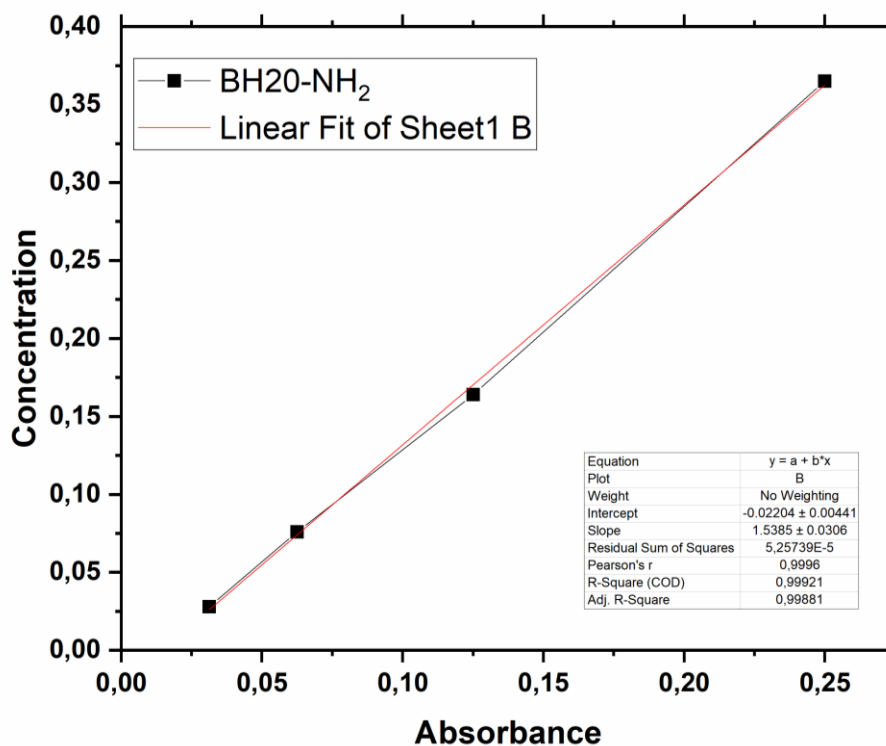


Figure 4.26. Calibration curve for the absorbance changes of BH30-NH₂ versus concentration

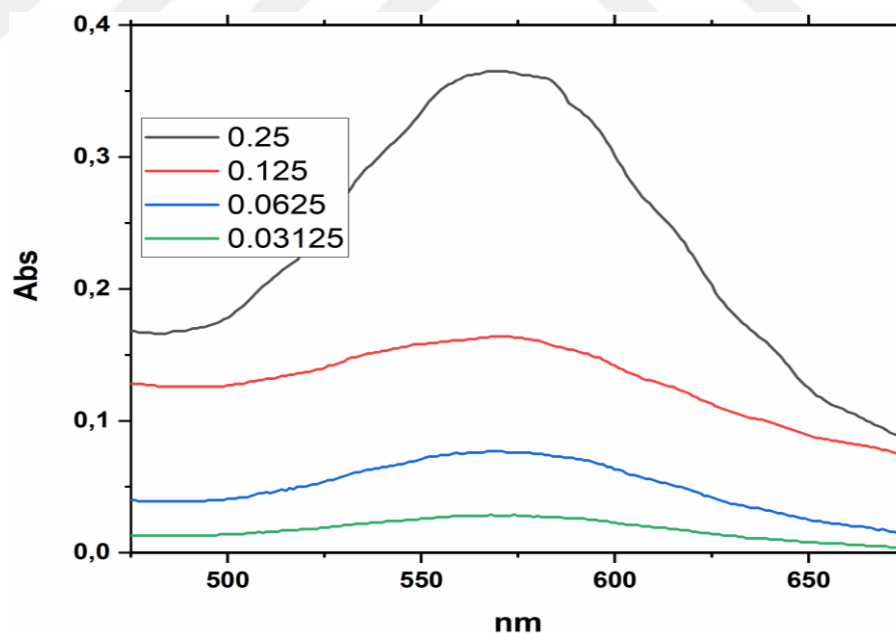


Figure 4.27. UV-vis spectra of BH20-NH₂ showing absorbance change versus concentration

The R^2 values of the calibration curves presented in Figures 4.22-4.27 were found to be 0.995, 0.998, and 0.999, respectively. The high R^2 values indicate a high accuracy

of the method used. The equations obtained for these curves are respectively $y = 1.69962x - 0.04717$, $y = 1.573x - 0.01509$, and $y = 1.5385x - 0.01763$.

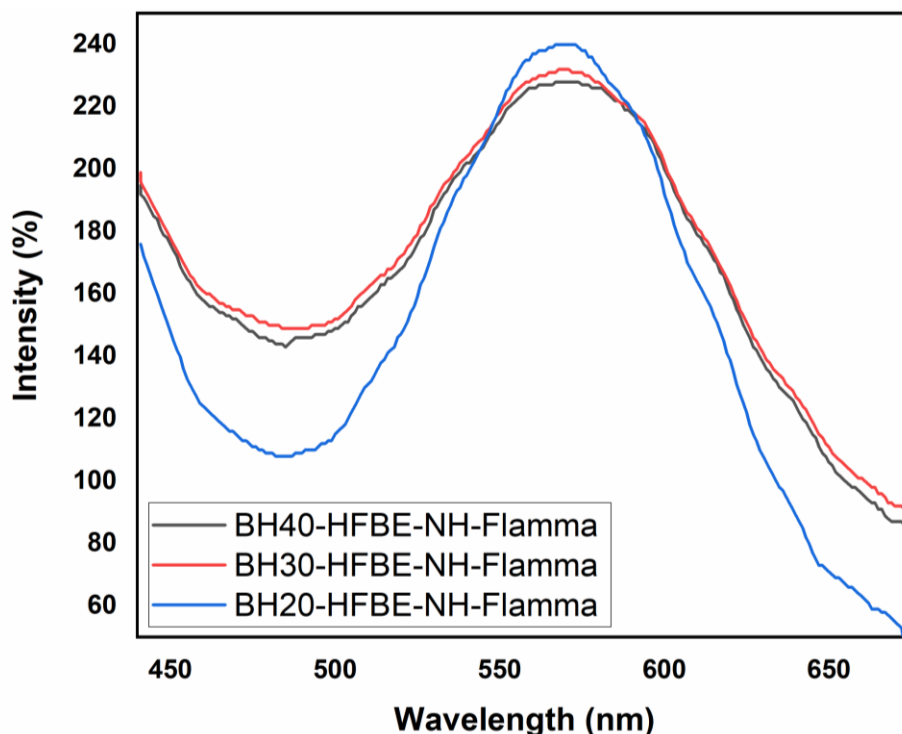


Figure 4.28. Absorption spectra of BH40-HFBE-NH-Flamma 774 (black), BH30-HFBE-NH-Flamma 774 (red), and BH20-HFBE-NH-Flamma 774 (blue)

In these studies, the absorbance values of BH40-HFBE-NH-Flamma 774 at a wavelength of 571 nm were observed to be 0.228, 0.232, and 0.236. By substituting these absorbance values into the calibration curve formula, the amounts of $-NH_2$ groups that did not bind to Flamma 774 were determined to be 0.161 mmol, 0.157 mmol, and 0.164 mmol, respectively as given in Figure 4.28.

4.5. Zeta Size Analysis of Ternary Complex (RRPHFC)

Dynamic Light Scattering (DLS) is a technique used to measure the sizes of particles in a liquid. This technique is based on the principle of light scattering because of thermal motion, which causes Brownian motion of the particles. DLS is particularly suitable for measuring the sizes of nanoparticles and macromolecules. Table 4.1 presents the zeta size and zeta potential results for BH40-HFBE-NH₂-Flamma 774.

Table 4.1. Zeta size and zeta potential results of BH40-HFBE-NH₂-Flamma

No.	Sample	Size	Charge	Stability
1	BH40-HFBE1-NH ₂ - Flamma 774	32.7	-3.54	Rapid aggregation or flocculation
2	BH40-HFBE2-NH ₂ - Flamma 774	50.7	-0.671	Rapid aggregation or flocculation
3	BH40-HFBE3-NH ₂ - Flamma 774	37.8	-0.210	Rapid aggregation or flocculation
4	BH30-HFBE1-NH ₂ - Flamma 774	37.8	-4.23	Rapid aggregation or flocculation
5	BH30-HFBE2-NH ₂ - Flamma 774	32.7	-0.384	Rapid aggregation or flocculation
6	BH30-HFBE3-NH ₂ - Flamma 774	28.2	-0.23	Rapid aggregation or flocculation
7	BH20-HFBE1-NH ₂ - Flamma 774	37.8	-6	Rapid aggregation or flocculation
8	BH20-HFBE2-NH ₂ - Flamma 774	28.2	-4	Rapid aggregation or flocculation
9	BH20-HFBE3-NH ₂ - Flamma 774	32.7	-0.273	Rapid aggregation or flocculation

Zeta size and zeta potential of BH40-HFBE-NH₂-Flamma 774 polymeric nanocarrier are given in Figure 4.29 zeta potential Figure 4.30, 4.31 and 4.32 respectively.

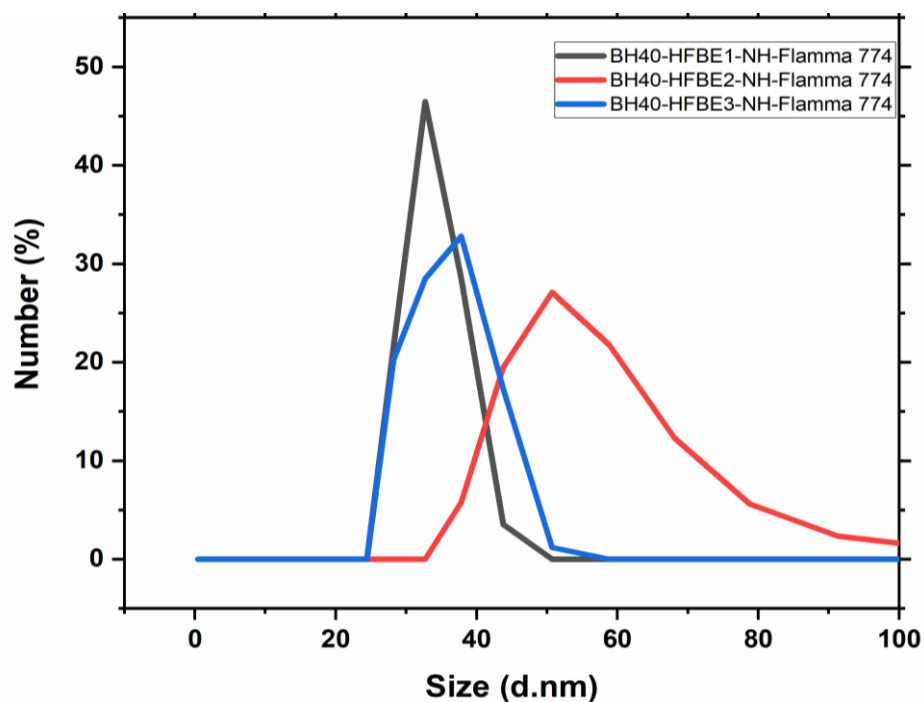


Figure 4.29. Zeta size spectrum of BH40-HFBE-NH₂-Flamma 774 polymers

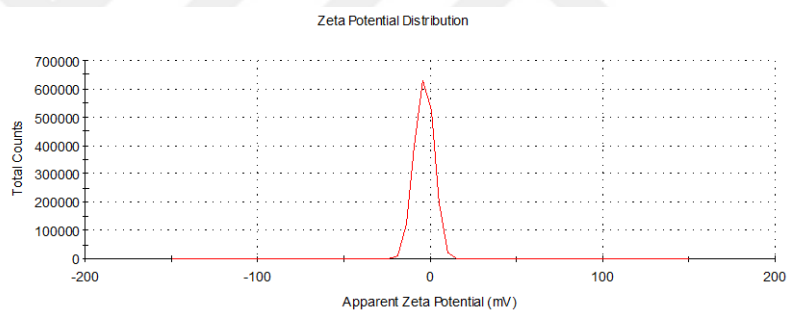


Figure 4.30. Zeta potential spectrum of BH40-HFBE1-NH₂-Flamma 774 polymers

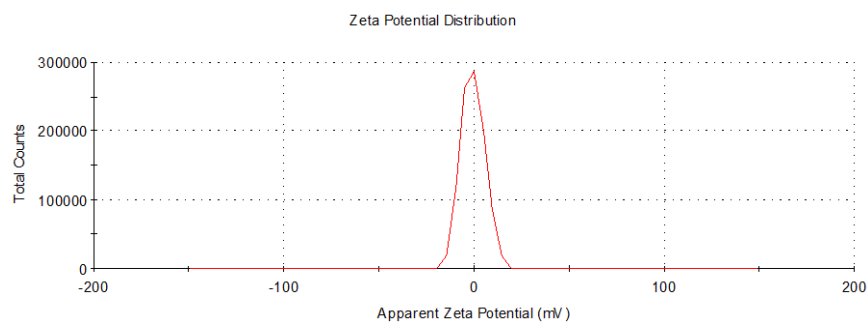


Figure 4.31. Zeta Potential spectrum of BH40-HFBE2-NH₂-Flamma 774 polymers

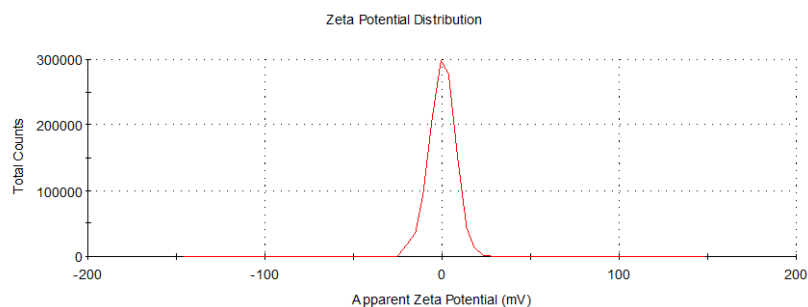


Figure 4.32. Zeta potential spectrum of BH40-HFBE3-NH₂-Flamma 774-3 polymers

Polymers containing fluorine atoms in their molecular structures are more electronegative than many other atoms concerning charge-related properties. This electronegative characteristic can influence the overall charge distribution and charge-carrying capacity of the polymer. Fluorinated polymers exhibit more hydrophobic properties, indicating increased durability and stability. They typically have low surface energy (H. Liu et al., 2019; Peng, 2019).

As a result, they have a hydrophobic structure and high oil affinity. These properties can also significantly affect surface interactions and charge accumulation. The size of the functionalized BH40-HFBE-NH₂-Flamma 774 polymer has been reported to be between ~35 and 40 nm, with zeta potentials ranging from -0.2 to -3.5 mV. This indicates a direct correlation with the amount of HFBC, suggesting an increased stability of the polymer. Zeta size and Zeta potential of BH30-HFBE-NH₂-Flamma 774 polymeric nanocarrier are given in Figure 4.33 zeta potential Figure 4.34, 4.35 and 4.36 respectively.

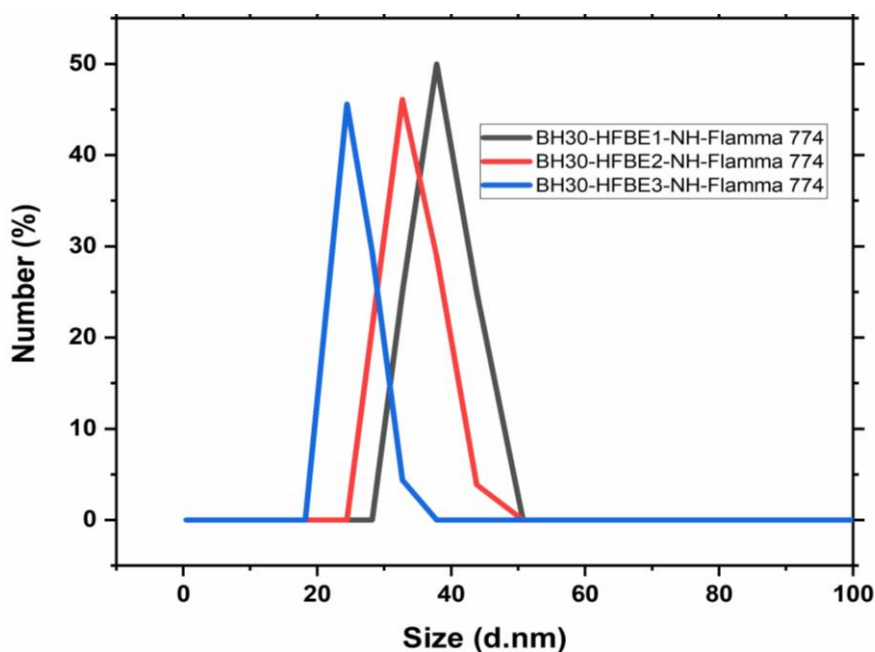


Figure 4.33. Zeta size spectrum of BH30-HFBE-NH₂-Flamma 774

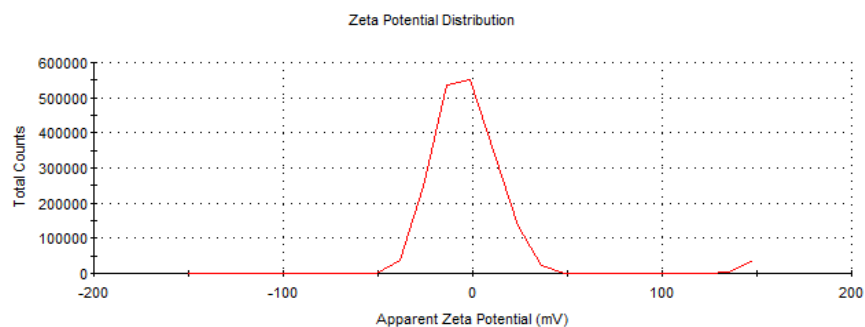


Figure 4.34. Zeta potential spectrum of BH30-HFBE1-NH₂-Flamma 774

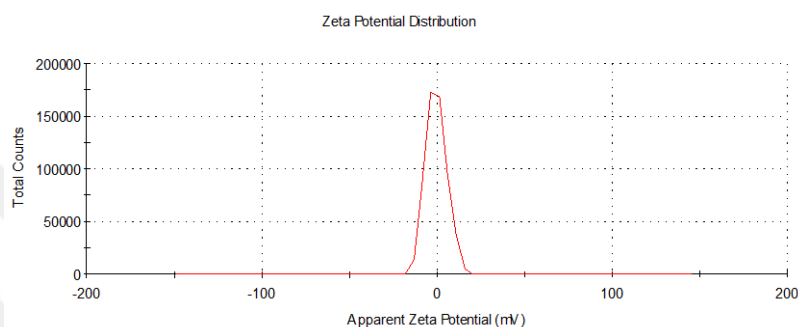


Figure 4.35. Zeta potential of BH30-HFBE2-NH₂-Flamma 774

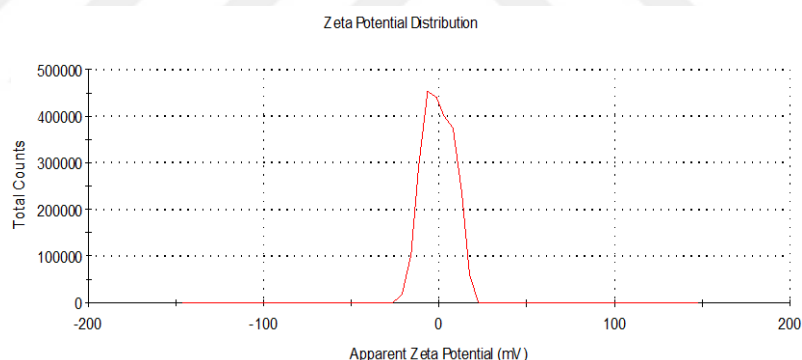


Figure 4.36. Zeta potential of BH30-HFB3-NH-F774

The zeta size of the RRPFC4 polymer is shown in Figure 4.37, and the results of the zeta potential studies are presented in Figures 4.38, 4.39, and 4.40. According to DLS measurements, the sizes of the BH30-HFBE-NH-Flamma 774 polymers range from 20 to 45 nm. Additionally, the zeta potential of the BH30-HFBE-NH-Flamma 774 polymer has been determined to be in the range of -0.2 to -4.3 mV. The zeta size of the BH20-HFBE-NH-Flamma 774 polymer is shown in Figure 4.40, and the zeta potentials are presented in Figures 4.41, 4.42, and 4.43.

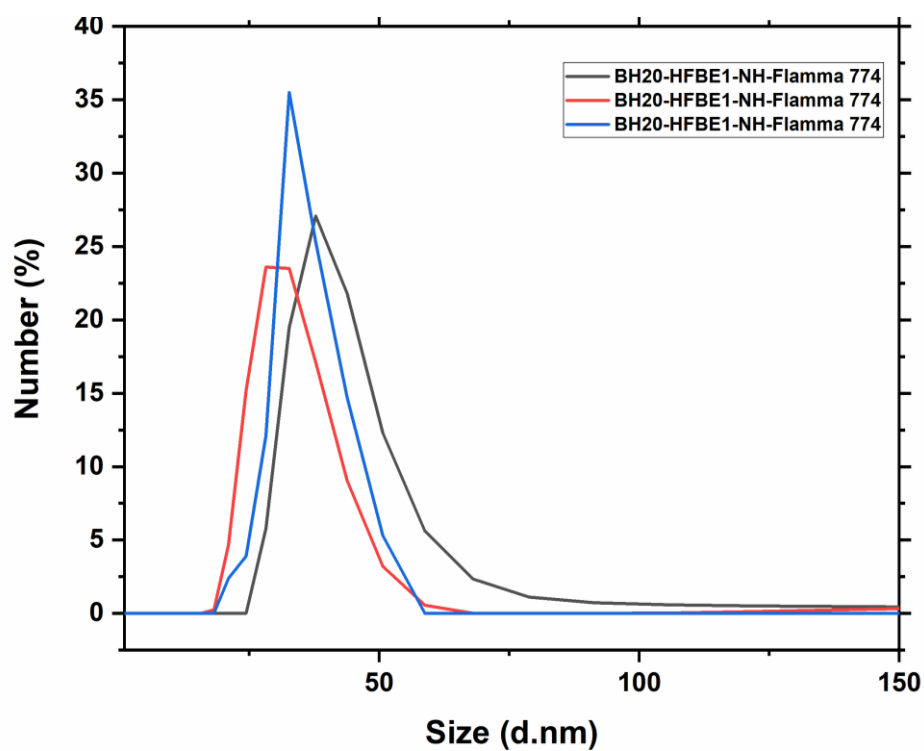


Figure 4.37. Zeta size spectrum of BH20-HFBE1-NH1-Flamma 774

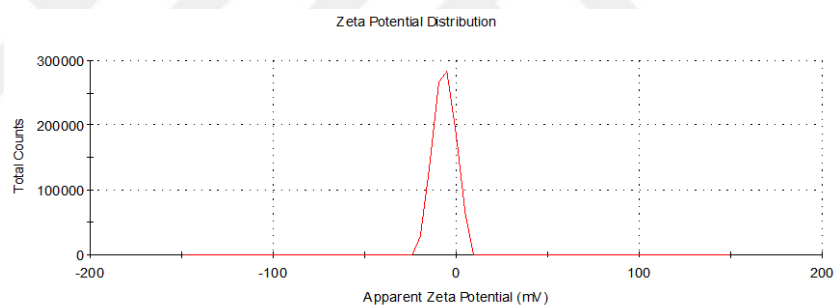


Figure 4.38. Zeta potential of BH20-HFBE1-NH-F774

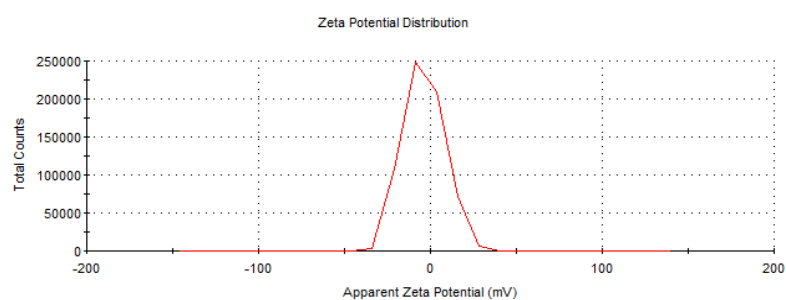


Figure 4.39. Zeta potential of BH20-HFBE2-NH-F774

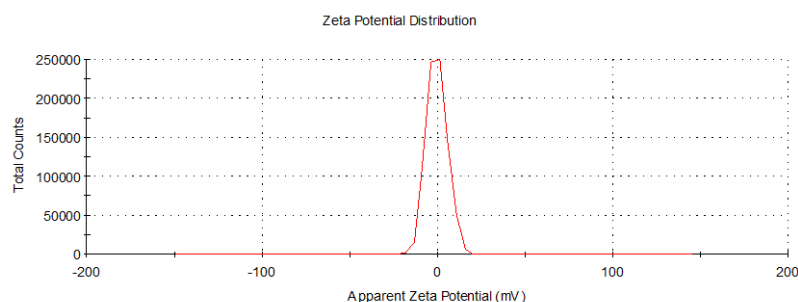


Figure 4.40. Zeta potential of BH20-HFBE3-NH-F774

The average particle size was found to be approximately between 50 nm and 200 nm. Additionally, a core-shell structure was prepared using BHX-HFBE-NH-Flamma 774 functionalized with folic acid and RGD-R8 peptide along with hyaluronic acid. After the core-shell structure was formed with functionalized hyaluronic acid and BH20-HFBE-NH-Flamma 774, it was observed that the particle size of the resulting nanocarriers increased. At the same time, there was a negative increase in the surface zeta potentials of the particles. Table 4.2 presents data on the changes in zeta size and potential of the nanocarriers.

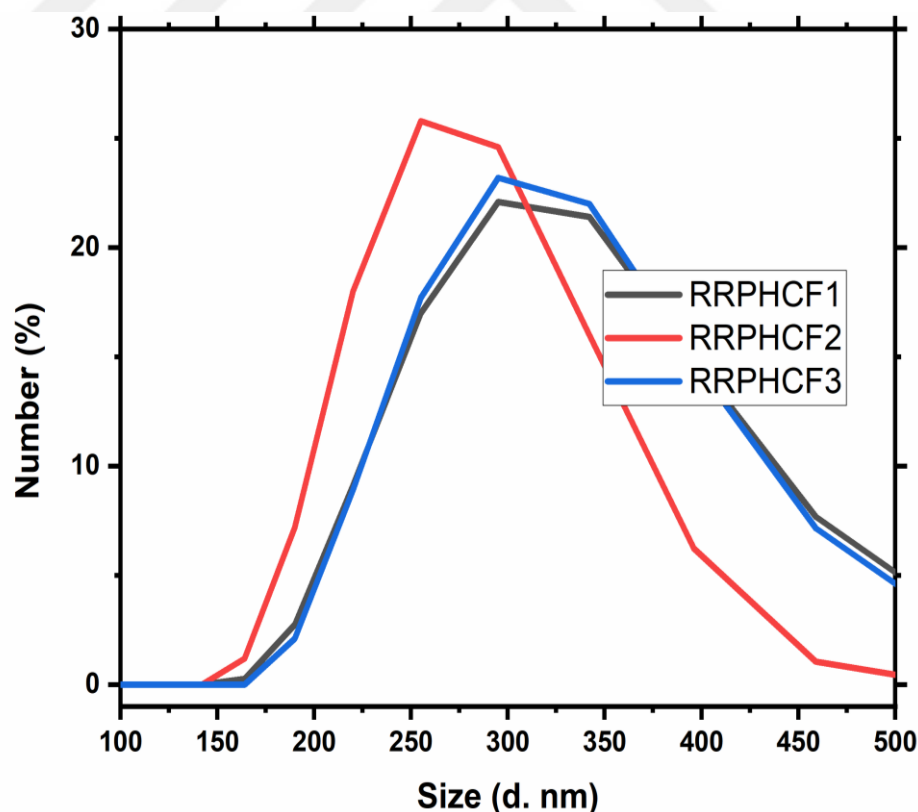


Figure 4.41. Zeta potential of RRPHFC1, 2 and 3 nanocarriers

Table 4.2. Zeta size and zeta potential results of synthesized nanocarriers

No.	Sample	Size	Charge	Status
1	RRPHFC1	295	-19.3	Onset of instability
2	RRPHFC2	255	-17.5	Onset of instability
3	RRPHFC3	295	-10	Onset of instability
4	RRPHFC4	255	-26	Onset of instability
5	RRPHFC5	255	-17.6	Onset of instability
6	RRPHFC6	295	-11	Onset of instability
7	RRPHFC7	295	-30	Onset of instability
8	RRPHFC8	342	-25	Onset of instability
9	RRPHFC9	295	-13.5	Onset of instability

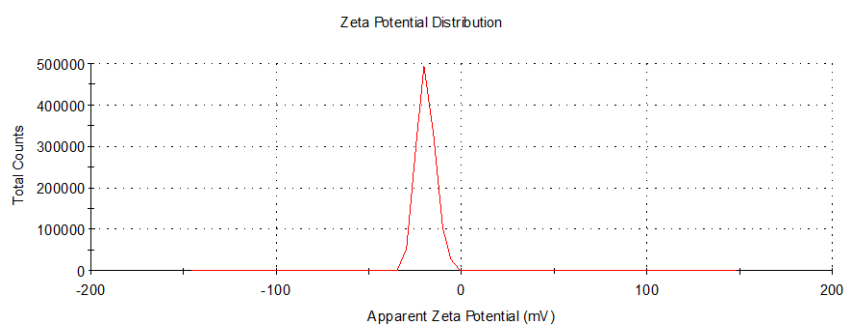


Figure 4.42. Zeta potential result of RRPFC1

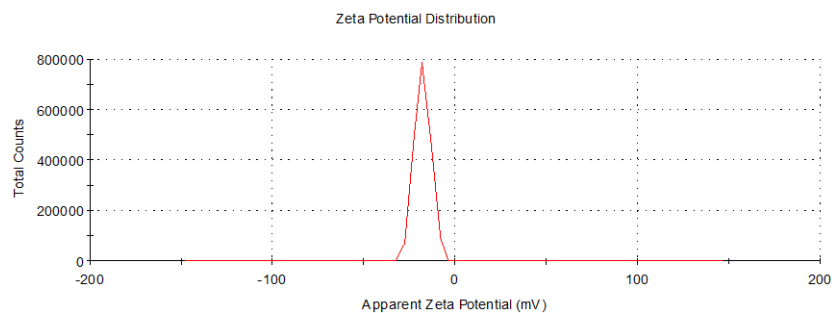


Figure 4.43. Zeta potential results of RRPFC2

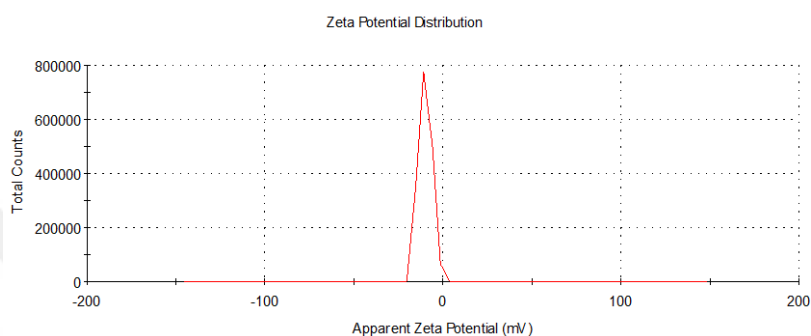


Figure 4.44. Zeta potential result of RRPFC3

The zeta size of the RRPFC1, 2, and 3 nanocarriers is presented in Figure 4.44, while the changes in zeta potential are shown in Figures 4.45, 4.46, and 4.47. The size of the nanocarriers is only between 100 and 200 nm, while SEM and TEM images indicate that they are smaller than 100 nm and are completely coated with RRPHF. Additionally, the negative surface charge percentages of the RRPFC1, RRPFC2, and RRPFC3 nanocarriers are -19.3, -17.5, and -10, respectively, which is attributed to the surface charge of the functionalized hyaluronic acid (RRPHF).

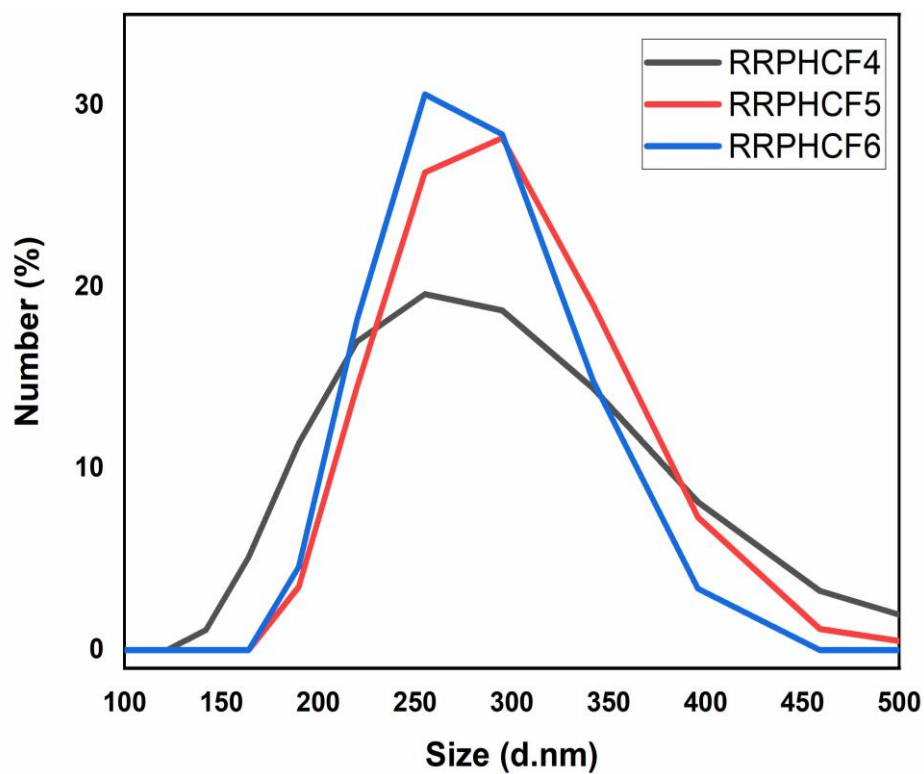


Figure 4.45. Zeta size of RRPFCF4, 5 and 6 nanocarrier

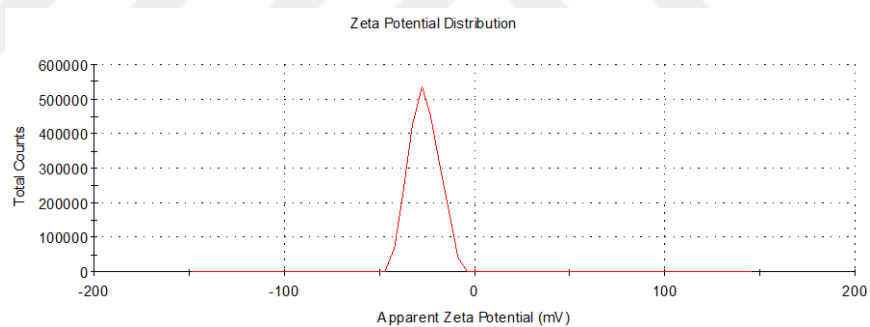


Figure 4.46. Zeta potential of RRPFCF4

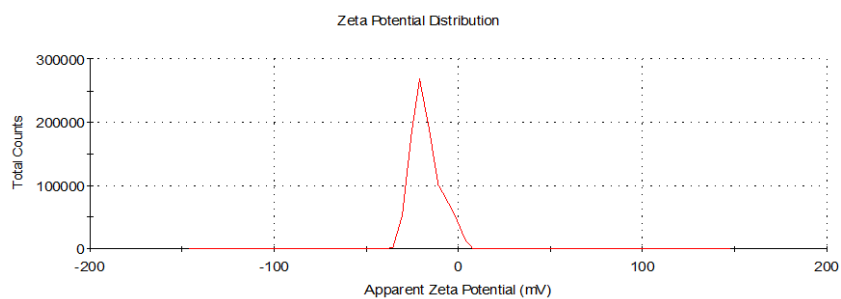


Figure 4.47. Zeta potential result of RRPFCF5

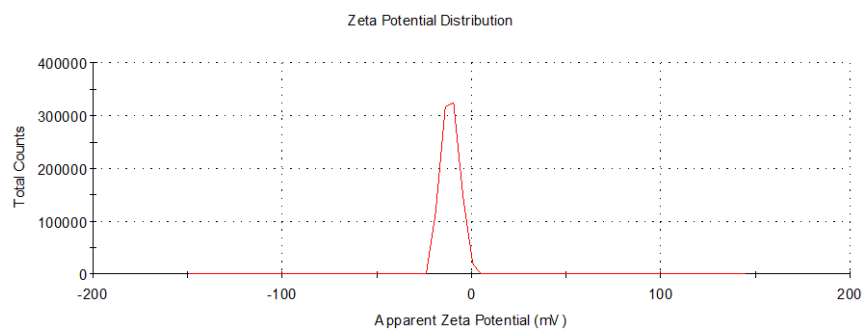


Figure 4.48. Zeta potential results of RRPFC6

The zeta size of the RRPFC4, 5, and 6 nanocarriers is presented in Figure 4.45, and their zeta potentials are shown in Figures 4.46, 4.47, and 4.48. While the size of the nanocarriers is only between 100 and 200 nm, SEM and TEM images indicate that they are smaller than 100 nm and are completely coated with RRPHF. Additionally, the negative surface charges of the RRPFC4, RRPFC5, and RRPFC6 nanocarriers are -26.3, -17.6, and -11, respectively, which is attributed to the surface charge of RRPHF.

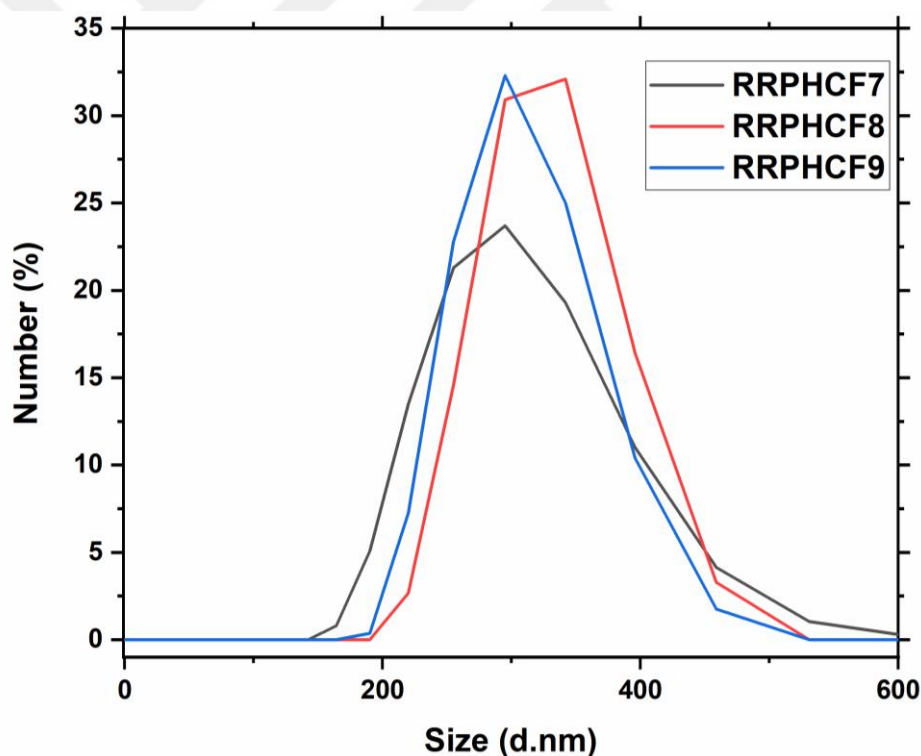


Figure 4.49. Zeta size spectrum of RRPFC7, 8 and 9 nanocarriers

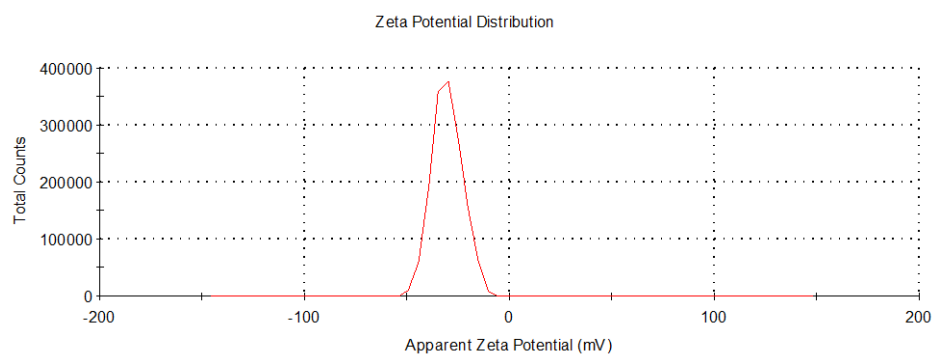


Figure 4.50. Zeta potential of RRPFC7

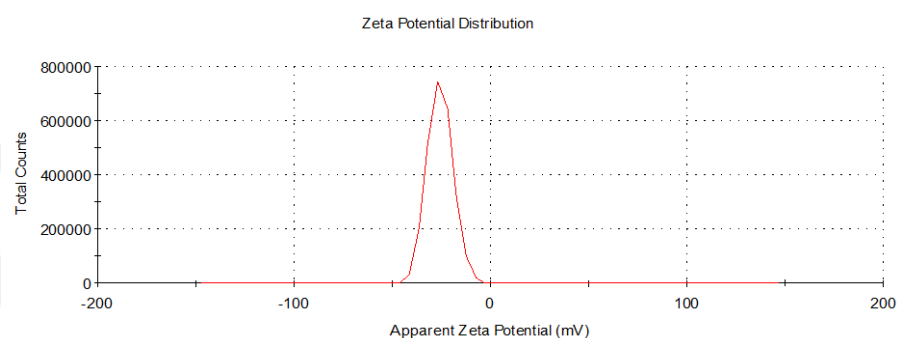


Figure 4.51. Zeta potential of RRPFC8

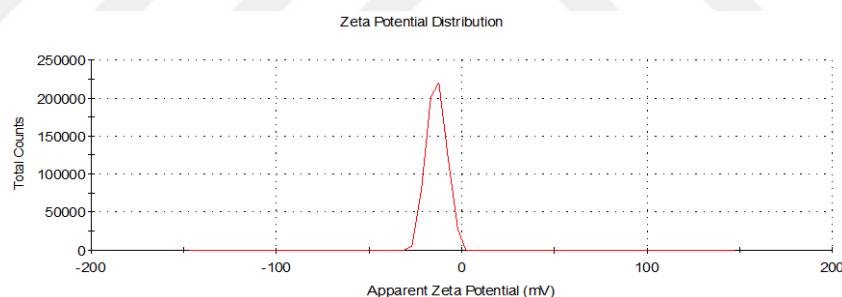


Figure 4.52. Zeta Potential of RRPFC9

The zeta size of the RRPFC 7, 8, and 9 nanocarriers is presented in Figure 4.49, and their zeta potentials are shown in Figures 4.50, 4.51, and 4.52. While the size of the nanocarriers is only between 100 and 200 nm, SEM and TEM images indicate that they are smaller than 100 nm and are completely coated with RRPHF. Additionally, the negative surface charges of the RRPFC7, RRPFC8, and RRPFC9 nanocarriers are -30, -25, and -13.5, respectively, which is attributed to the surface charge of RRPHF.

4.6. Characterization of RRPFC Nanocarriers with MALDI-TOF MS Spectrometry

MALDI-TOF mass spectrometry is an analytical technique in which samples are ionized into charged molecules, and the mass-to-charge ratio (m/z) is measured. In this soft ionization technique, Matrix-Assisted Laser Desorption/Ionization (MALDI) and Time-of-Flight (TOF) mass analyzers are used. Molecules are transferred to the gas phase by being struck by a laser on a small molecule matrix without fragmentation or decomposition. However, some biomolecules can be very large and may fragment when heated.

In our study, Boltorn H (20, 30, and 40) was functionalized with perfluorobutyl chloride and Flamma 774 fluorescent dye to obtain a dendrimer core. Folic acid, hyaluronic acid, and RGD-R8 peptide were functionalized to create a shell structure. The synthesized nanoparticles (core) were encapsulated with functionalized folic acid and peptides that form the shell structure (RRPHFC). RRPFC (1, 4, and 7: 1; BH40, 4; BH30, 7; BH20) was characterized using MALDI-TOF mass spectrometry. MALDI-TOF MS analyses were performed on Bruker Daltonic UltrafleXtreme Model MALDI-TOF mass spectrometers with positive polarization applied to obtain the spectrum. These nanocarriers were dissolved in DMSO at a concentration of 10 mg/ml. A matrix or ionization agent consisting of water containing 0.1% trifluoroacetic acid was mixed with the sample in a 10:2 ratio using 10 mg/ml 2,5-dimethylsulfoxide.

The MALDI TOF MS spectra obtained for RRPFC (1, 4, and 7) are presented in Figures 4.53, 4.57, and 4.55. Fragments for each polymeric carrier were Calculate and are listed in Tables 4.3, 4.4, and 4.5.

Table 4.3. m/z values of the fragments of the RRPFC1 synthetic nanocarrier.

Numune	Hesaplanan Fragmentlar	(m/z)
RRPFC 1	[Flamma-NH ₂ ⁺ (Na)] ⁺	680
	[Flamma-NH ₂ + 2 (Na)] ⁺	704
	[2HFB+C15H23O9] ⁺	739
	[2HFB+C15H23O10] ⁺	754
	[Flamma+N2C10H16O4] ⁺	870
	[NAGluc+Gluc-NAGluc+Folic acid+ H] ⁺	971
	[3(Gluc+NAGluc)+4H] ⁺	1087
	[4(HFB)+C10H13O4+2H] ⁺	1103
	[Flamma+FHB+C7H8O4+2H] ⁺	1141
	[Folic Asid + 2(Gluc+NAGluc)+Na+H] ⁺	1219
	[RGDFeKC4RC+H] ⁺	1319
	[RGDFeKC5R-27] ⁺	1435
	[RGD-CO-(PEG)11] ⁺	2597
	[2/6BH40] ⁺	2481
	[RGD-CO-(PEG)13] ⁺	2713
	[RGD-CO-(PEG)15+1] ⁺	2829
	[RGD-CO-(PEG)17] ⁺	2945
	[RGD-CO-(PEG)19] ⁺	3062
	[3(Gluc+NAGluc)+2Na] ⁺	3295
	[RGD-CO-(PEG)25] ⁺	3408
	[BH40] ⁺	7358
	[RGD-CO-(PEG)30] ⁺	3756
	[2/6BH40+Fn] ⁺	4106
	[4/6BH40+Fn] ⁺	6373

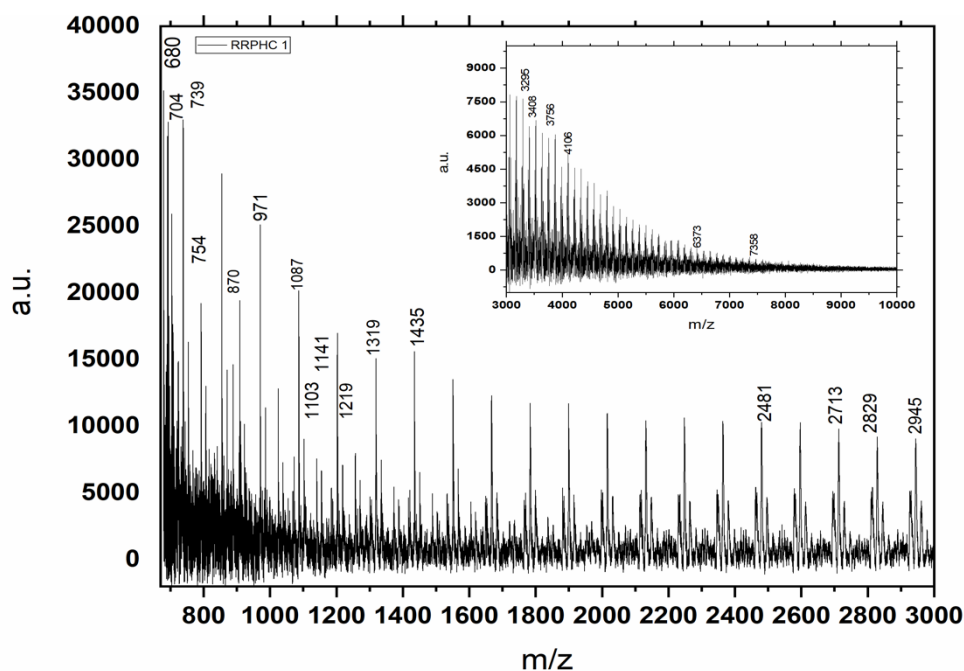


Figure 4.53. MALDI-TOF and mass spectrum of RRPFC-1

The (m/z) values observed for RRPFC-1 are shown in Table 4.3, and these values correspond to the disulfide shell made from boltorn H40 functionalized with Flamma 774 and perfluorobutyl chloride, and hyaluronic acid functionalized with folic acid and RGD-R8. The peak at 735840 m/z indicates the average size of boltorn H40, while after functionalization, the dendrimer shows peaks at 4106 and 6373 m/z due to its significantly large size in fragment form. Similarly, peaks at 971 m/z were observed for folic acid and RGD-R8 peptide, and peaks at 2928 m/z for the peptide linked to polyethylene glycol. Additionally, since the shell structure is a functionalized peptide structure, no molecular peak was detected; only fragments were identified. The MALDI-TOF MS results support the FT-IR and NMR results, indicating that the polymeric nanocarriers (RRPFC-1) structure has been successfully obtained (Cha ve Kim, 2003; Chikh et al., 2007; Knetsch et al., 2015; Sakai et al., 2007; Žagar ve Žigon, 2002).

Table 4.4. m/z values of the fragments of the RRPFC4 synthetic nanocarrier.

.Numune	Hesaplanan Fragmentler	(m/z)
RRPFC 4	[Flamma-NH+ 2 (Na)]+	704
	[2HFB+C ₁₅ H ₂₃ O ₉]+	739
	[2HFB+C ₁₅ H ₂₃ O ₁₀]+	754
	[Flamma+N ₂ C ₁₀ H ₁₆ O ₄]+	870
	[NAGluc+Gluc-NAGluc+Folik acit+ H]+	970
	[3(Gluc+NAGluc)+4H]+	1087
	[4(HFB)+C ₁₀ H ₁₃ O ₄ +2H]+	1103
	[Flamma+FHB+C ₇ H ₈ O ₄ +2H]+	1141
	[Folik Asit + 2(Gluc+NAGluc)+Na+H]+	1219
	[RGDFeKC4RC+H]+	1319
	[RGDFeKC5R-27]+	1435
	[RGD-CO-(PEG) ₁₃]+	2713
	[RGD-CO-(PEG) ₁₅₊₁]+	2829
	[RGD-CO-(PEG) ₁₇]+	2945
	[3(Gluc+NAGluc)+2Na]+	3295
	[RGD-CO-(PEG) ₂₅]+	3408
	[BH30]+	3498
	[RGD-CO-(PEG) ₃₀]+	3756
	[BH30 Max]+	6310

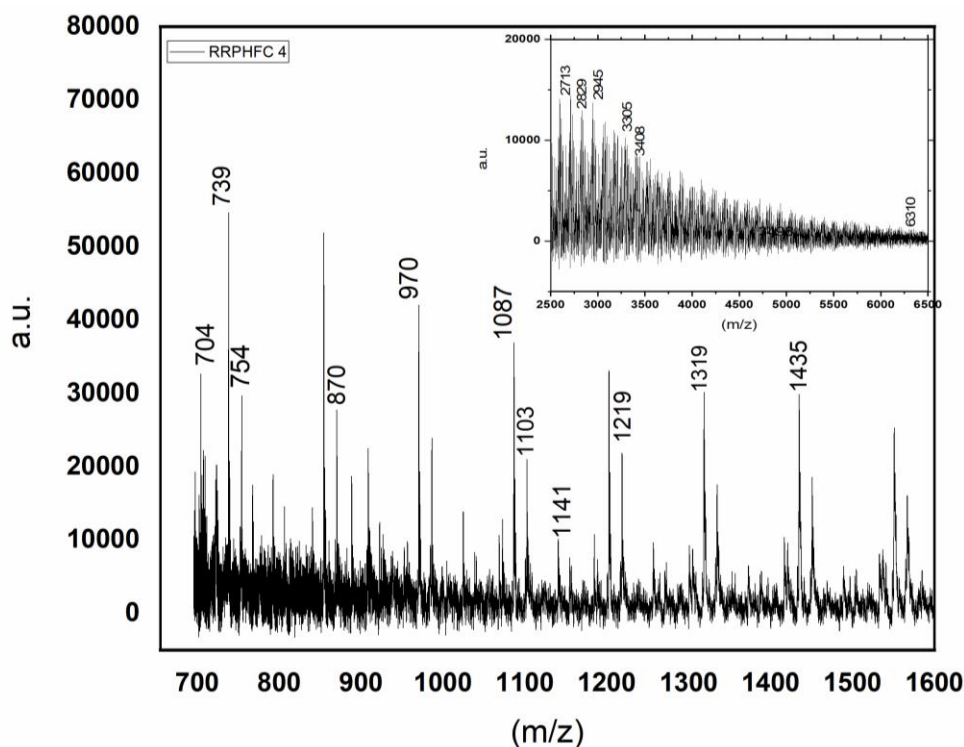


Figure 4.54. MALDI-TOF and mass spectrum of RRPHFC-4

RRPHFC-4 consists of Boltorn H30 functionalized with Flamma 774 and perfluorobutyl chloride, while its shell is made from hyaluronic acid functionalized with folic acid and RGD-R8. The observed (m/z) values show peaks at 3498 m/z , indicating the average size of Boltorn H30, and 6310 m/z after functionalization. Similarly, peaks at 970 m/z for folic acid and RGD-R8 peptide, as well as peaks at 2713, 2829, and 2945 m/z for the peptide linked to polyethylene glycol, were observed. Since the shell structure is a functionalized peptide structure, no molecular peak was detected; only fragments were identified. The MALDI TOF MS results support the FT-IR and NMR results, indicating that the polymeric nanocarriers (RRPHFC-4) structure has been successfully obtained (Cha ve Kim, 2003; Chikh et al., 2007; Knetsch et al., 2015; Sakai et al., 2007; Žagar ve Žigon, 2002).

Table 4.5. m/z values of the fragments of the RRPFC1 synthetic nanocarrier.

Numune	Hesaplanan Fragmentler	(m/z)
RRPHFC 7	[Flamma-NH ₂ + Na] ⁺	681
	[Flamma-NH ₂ + 2 (Na)] ⁺	704
	[2HFB+C ₁₅ H ₂₃ O ₉] ⁺	739
	[2HFB+C ₁₅ H ₂₃ O ₁₀] ⁺	755
	[Flamma+N ₂ C ₁₀ H ₁₆ O ₄] ⁺	871
	[NAGluc+Gluc-NAGluc+Folic acid+ H] ⁺	971
	[3(Gluc+NAGluc)+4H] ⁺	1087
	[4(HFB)+C ₁₀ H ₁₃ O ₄ +2H] ⁺	1103
	[Flamma+FHB+C ₇ H ₈ O ₄ +2H] ⁺	1141
	[Folik Asit + 2(Gluc+NAGluc)+Na+H] ⁺	1219
	[BH20] ⁺	1745
	[RGD-CO-(PEG) ₁₅ +3H] ⁺	2829
	[BH20-Fn+Na] ⁺	3328
	[3(Gluc+NAGluc)+2Na] ⁺	3294
	[RGD-CO-(PEG) ₃₀] ⁺	3756

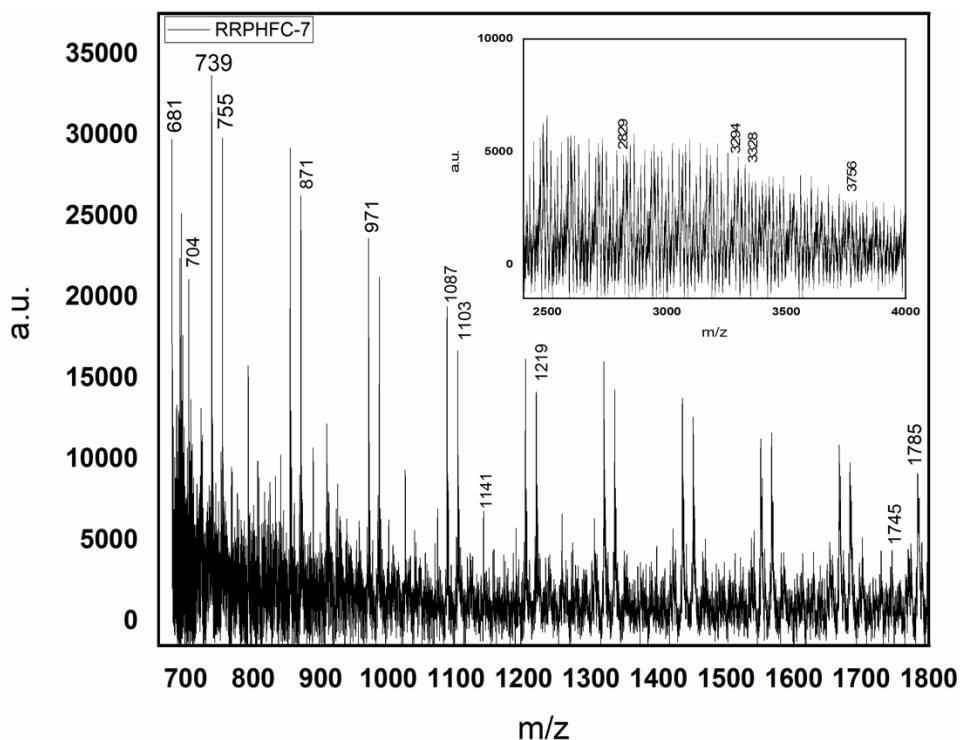


Figure 4.55. MALDI-TOF and mass spectrum of RRPFC-7

RRPHFC-7 consists of Boltorn H20 functionalized with Flamma 774 and perfluorobutyl chloride, while its shell is made from hyaluronic acid functionalized with folic acid and RGD-R8. The observed (m/z) values show peaks at 1740 m/z , indicating the average size of Boltorn H20, and 3328 m/z after functionalization. Similarly, peaks at 971 m/z for folic acid and RGD-R8 peptide, as well as 2829 m/z for the peptide linked to polyethylene glycol, were observed. Since the shell structure is a functionalized peptide structure, no molecular peak was detected; only fragments were identified. The MALDI TOF MS results support the FT-IR and NMR results, indicating that the polymeric nanocarriers (RRPHFC-7) structure has been successfully obtained (Cha ve Kim, 2003; Chikh et al., 2007; Knetsch et al., 2015; Sakai et al., 2007; Žagar ve Žigon, 2002).

4.7. Scanning Electron Microscopic (SEM) Analysis of Nanocarrier

SEM (Scanning Electron Microscopy) is a microscopy technique used to examine the surface morphology of materials. SEM analysis is a powerful characterization technique employed in various scientific fields, ranging from materials science to biology. When a focused high-energy electron beam strikes the sample surface, various signals are generated, such as secondary electrons and backscattered electrons. Different detectors are used to capture these emitted electrons, providing topographic information about the surface. The signals collected by the detectors are processed by a computer to create an image (Mohammed ve Abdullah, 2018) These images typically display the surface morphology of the sample at high resolution. Figures 4.56, 4.57, and 4.58 present the SEM micrographs of RRPHFC1, 4, and 7.

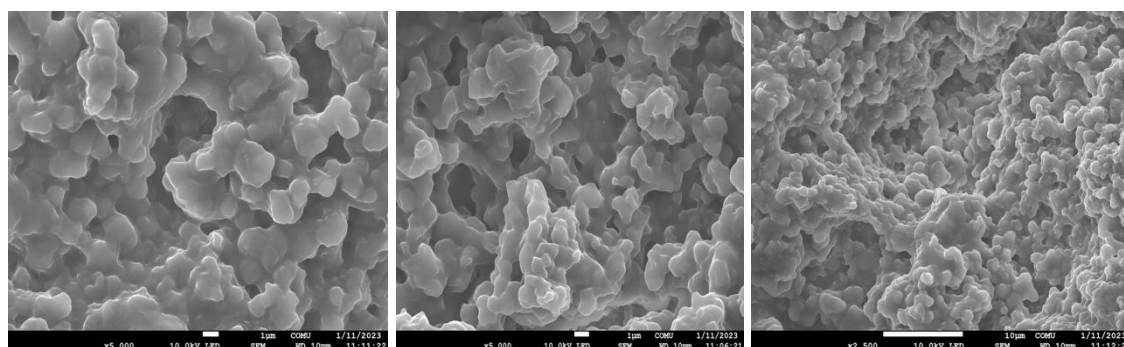


Figure 4.56. SEM micrograph of RRPHFC 1

The SEM micrographs of the Nanocarrirs RRPHFC 1 show images at different magnification levels. These images indicate that the size distribution of the particles is

homogeneous and at the nanoscale. Additionally, the TEM micrographs reveal that the particle sizes are smaller than 100 nm.

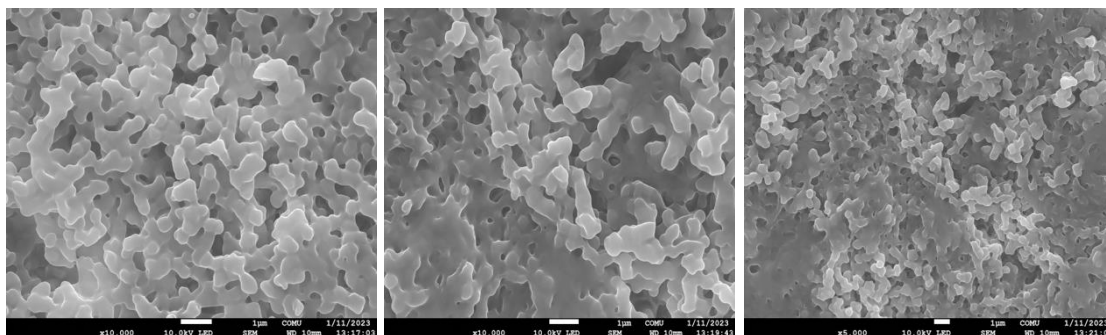


Figure 4.57. SEM micrograph of RRPFC 4

The SEM analysis results of the Nanocarriers RRPFC4 are presented with micrographs at different scales. These micrographs show that the size distributions of the particles are homogeneous and at the nanoscale. Additionally, the TEM micrographs indicate that the particle sizes are smaller than 100 nm.

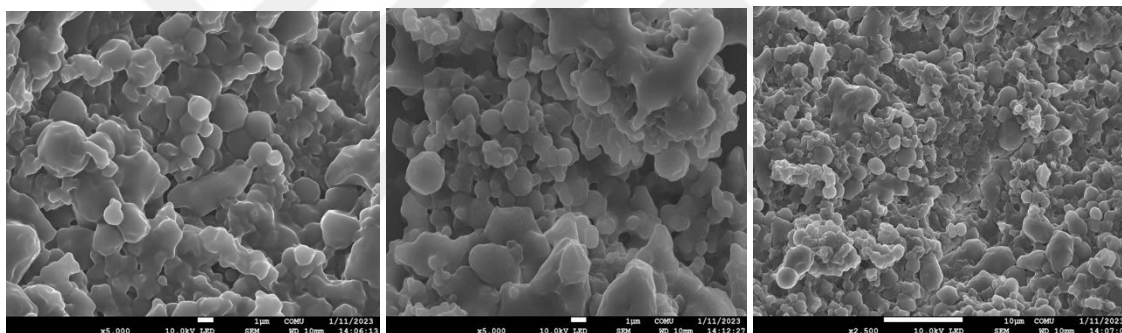


Figure 4.58. SEM micrograph of RRPFC 7 nanocarrier

The SEM analysis results of the nanocarriers RRPFC7 are presented with micrographs at different scales. These micrographs show that the size distributions of the particles are homogeneous and at the nanoscale.

4.8. Transmission Electron Microscopy (TEM) of Synthesized Nanoparticle

Transmission Electron Microscopy (TEM) is a microscopy technique used to examine the internal structure of materials and samples at the nanoscale. In TEM analyses, a focused beam of high-energy electrons passes through a thin section of the sample and operates in transmission mode. As the electrons pass through the sample, they scatter, and these scattered electrons, detected by various detectors, reveal details of the internal structure of the samples, producing high-resolution images. TEM analyses

provide detailed information about the internal structure at atomic resolution, allowing the examination of crystal structures, nanoparticle morphology, and other nanoscale details. TEM analysis has a wide range of applications in materials science, nanotechnology, biology, physics, and many other scientific fields. This technique is a critical tool for understanding nanoscale details and advanced material characterization. TEM micrographs used to examine the internal details of the nanocarriers are presented in Figures 4.59, Figure 4.60, and Figure 4.61.

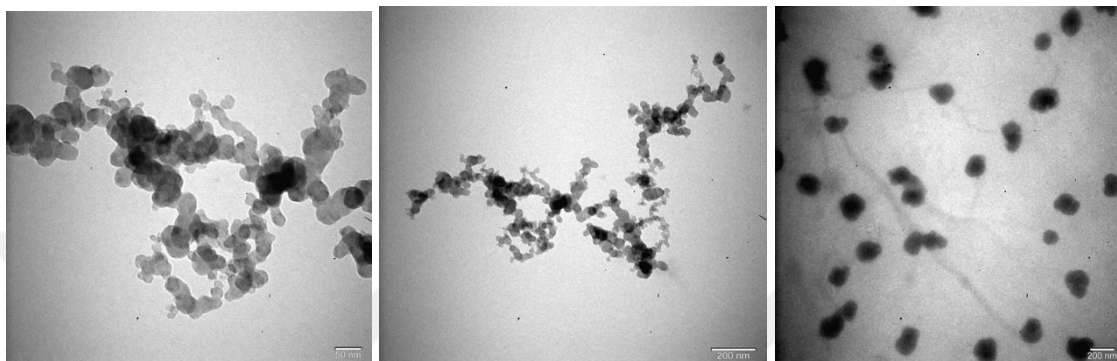


Figure 4.59. TEM micrograph of RRPFC 1 nanoparticles

The TEM results of the nanocarriers RRPFC 1 are presented with micrographs were recorded at different scales. These micrographs show that the particle size distribution is homogeneous and that the particles are coated with RRPFC. It has been determined that the particle sizes are less than 100 nm with dendrimeric morphology.

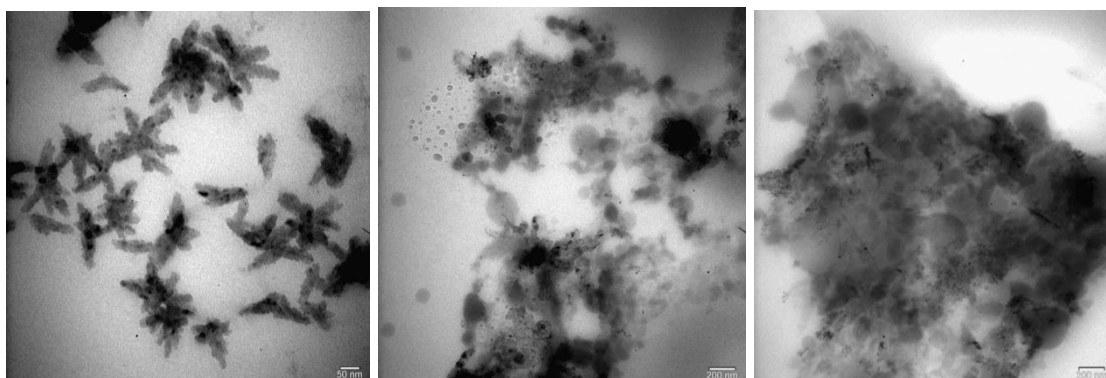


Figure 4.60. TEM micrograph of RRPFC 4

The TEM results of the nanocarriers RRPFC 1 are presented with micrographs taken at different scales. These micrographs show that the particle size distribution is homogeneous and that the particles are coated with RRPFC. It has been determined that the particles are dendrimeric in nature and less than 100 nm in sizes.

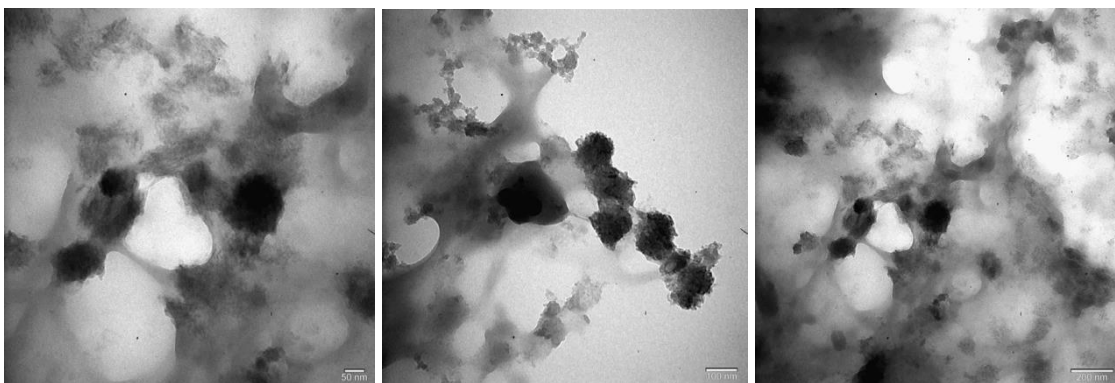


Figure 4.61. TEM micrograph of RRPFC 7

The results of the nanocarriers RRPFC 1 include micrographs taken at different scales. These micrographs indicate that the particle size distribution is homogeneous and that the particles are coated with RRPHF. It has been observed that the particle sizes are less than 100 nm.

4.9. Emission Spectra of BHX-HFBE-NH-F774

After dialysis of BHX-HFBE-NH₂-F774 (40, 30, and 20), it was freeze-dried. From the obtained solid samples, 200 μ L was taken for a multi-well plate, and the fluorescence spectrum was recorded. The emission spectrum of BHX-HFBE-NH₂-F774 in solid form is presented in Figure 4.62.

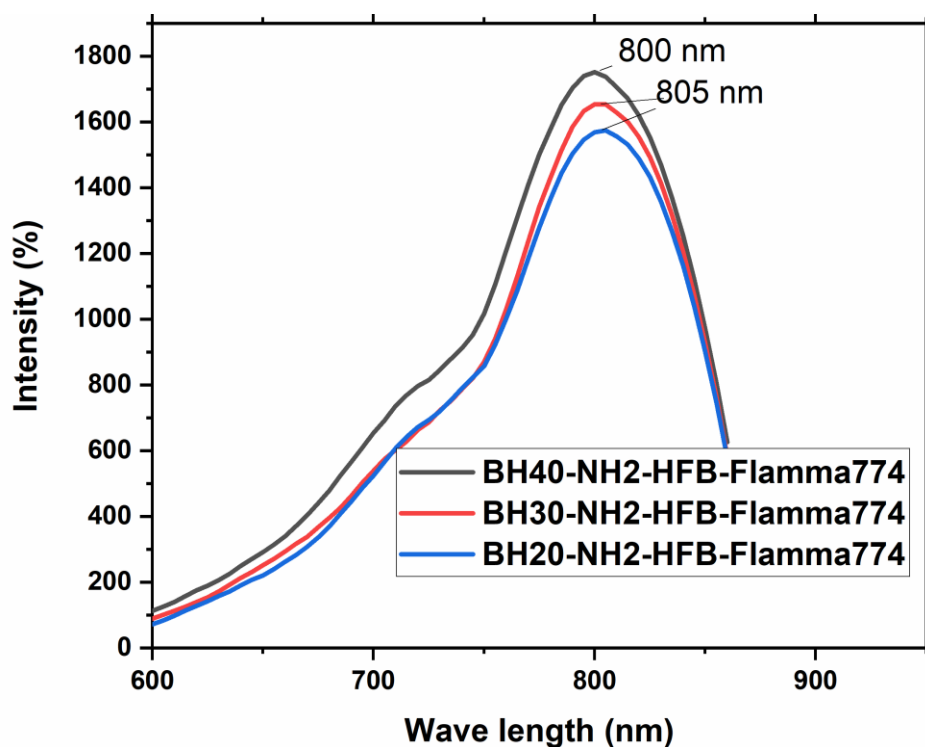


Figure 4.62. Emission spectra of BHX-HFBE-NH-F774 nanoparticles

In the emission spectrum of BHX-HFBE-NH₂-F774, a very intense emission band is observed in the wavelength range of 800-805 nm with excitation wavelength of 516 nm. This intense band indicates that a high amount of flamma-774 is bounded.

4.10. Thermogravimetric Analysis (TGA) of Ternary Complexes

Thermogravimetric Analysis (TGA) is a thermal analysis technique that examines a material's response to temperature increase. This analysis measures how the weight of a sample changes over time at a specific temperature, providing characteristic peaks or curves that determine the material's percentage weight loss (%) and its thermal properties and behavior. TGA is a powerful characterization technique with a wide range of applications in materials science, chemistry, polymer science, and many other fields.

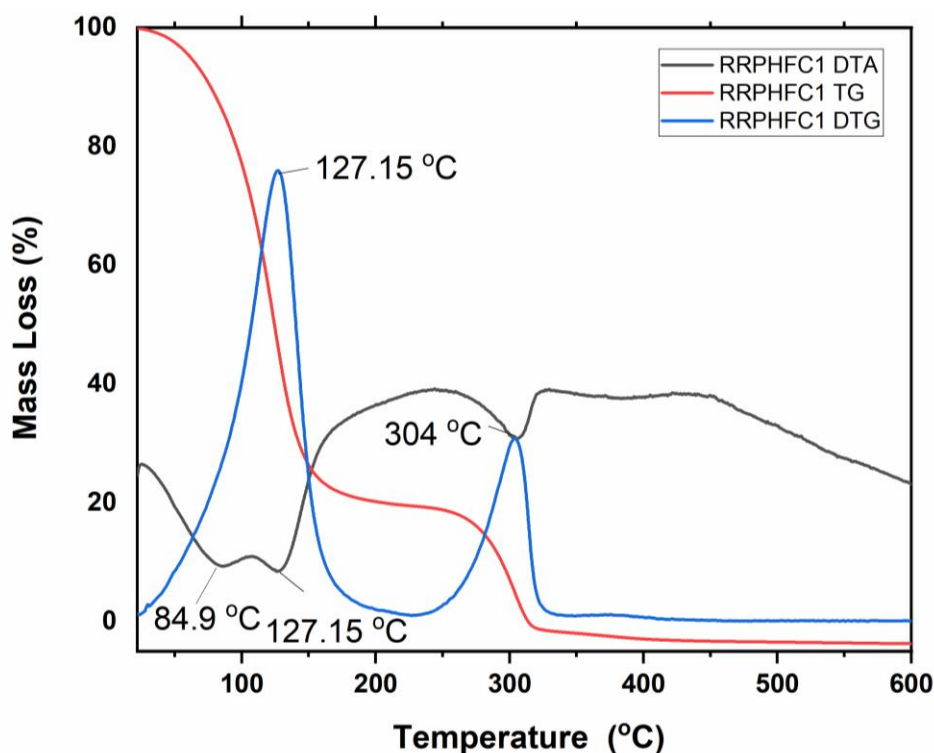


Figure 4.63. DTA/TG/DTG curves of the RRPFC 1 synthetic nanocarriers

The DTA/TG/DTG curves of the RRPFC1 nanocarriers as given in Figure 4.63, measured between 20-600 °C, are presented in Figure 25. A mass loss is observed in the TG curves between 22 °C and 317 °C. The TG and DTG curves indicate that degradation occurs in two stages: 1. The first degradation stage occurs between 22-148.8 °C with a mass loss of 73.4%, and 2. The second degradation stage occurs between 148.8-314 °C with a total mass loss of 100%. In the DTA curve, a weak endothermic peak is observed at 84.9 °C, and a sharp peak at 127.15 °C for the first degradation stage in the range of

22-160 °C. The endothermic peak at 304 °C corresponds to the second degradation stage in the range of 250-350 °C.

When examining the TG and DTG curves of RRPHFC1, it is understood that the material remains thermally stable up to 304 °C.

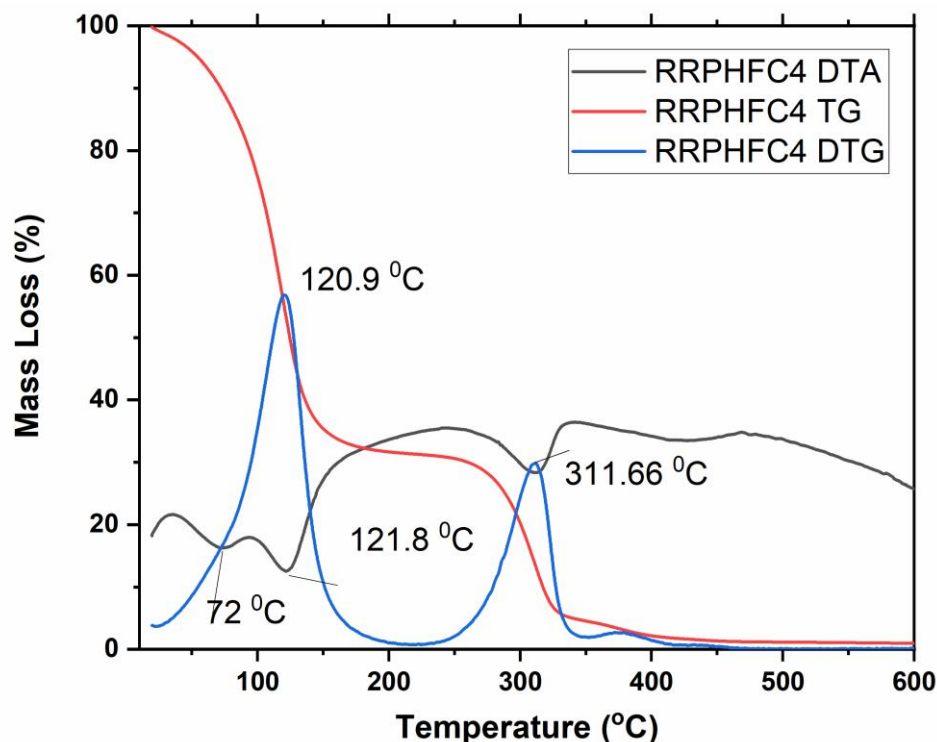


Figure 4.64. DTA/TG/DTG curve of the RRPHFC4 nanocarrier

The DTA/TG/DTG curves of the RRPHFC4 nanocarrier as given in Figure 4.64, measured between 20-600 °C. A mass loss is observed in the TG curves between 19 °C and 450 °C. The TG and DTG curves indicate that degradation occurs in two stages: 1. The first degradation stage occurs between 19.4-148.8 °C with a mass loss of 64.4%, and 2. The second degradation stage occurs between 148.8-337.7 °C with a mass loss of 94.5%. In the DTA curve, a weak endothermic peak is observed at 72 °C, and a sharp peak at 121.8 °C for the first degradation stage in the range of 22-160 °C. The endothermic peak at 311.66 °C corresponds to the second degradation stage in the range of 250-350 °C. When examining the TG and DTG curves of RRPHFC4, it is understood that the material remains thermally stable up to 391 °C.

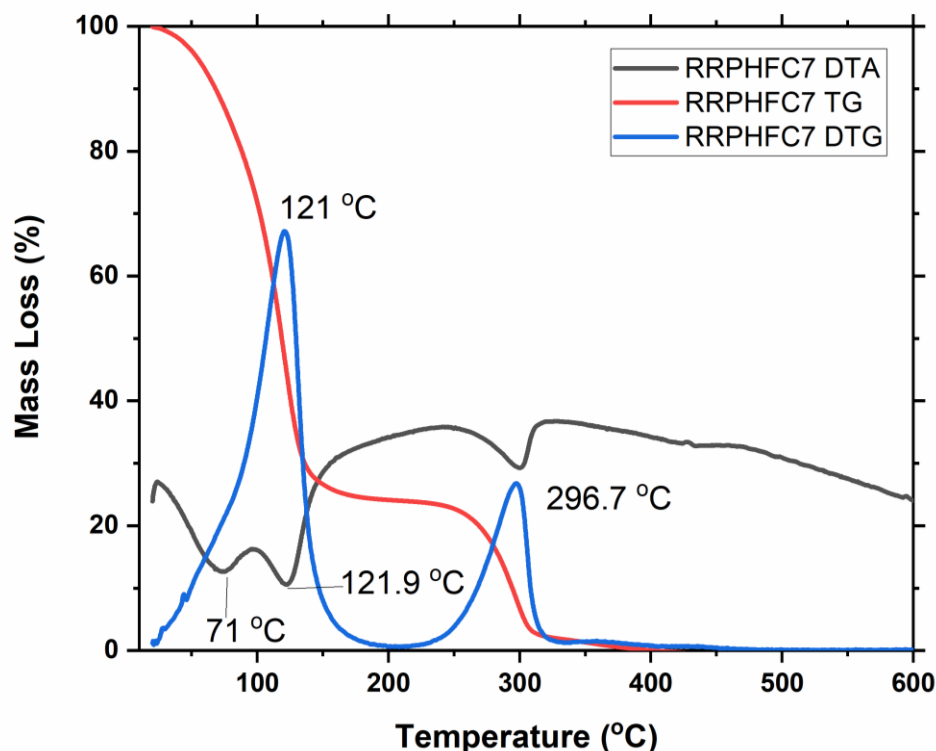


Figure 4.65. DTA/TG/DTG curve of the RRPFC7 nanoparticle

In the DTA curve, a weak endothermic peak was noted at 79 °C and a sharp peak at 127 °C during the first degradation stage between 22-160 °C. The second degradation stage showed endothermic peaks at 321 °C and 326 °C in the 250-350 °C range. For the unloaded RRPFC1, a weak endothermic peak was observed at 84.9 °C and a sharp peak at 127.15 °C during the first degradation stage, with an endothermic peak at 304 °C in the second degradation stage as given in Figure 4.65.

The increase in the DTA/TG/DTG curves of the sgRNA and afatinib-loaded RRPFC1 nanocarriers indicates that the plasmid containing afatinib and sgRNA is successfully incorporated into the RRPFC1 nanocarrier. The shift from 124.9 °C to 127.15 °C in the loaded RRPFC1 compared to the unloaded RRPFC1 suggests the breaking of hydrogen bonds between the circular plasmid and indicates that afatinib requires more energy to disrupt the aromatic ring.

4.11. Biodegradability Study of Polymeric Nanocarriers

The *in vitro* degradation studies of the polymer were conducted at 37°C in PBS buffer solutions with pH 5.3 and 7.4. A quantity of 5 mg of nanocarrier was added to 5 mL of the buffer solutions (media). The supernatant of each sample was periodically removed every 12 hours over a period of two weeks, and fresh media was added in its

place. At the end of the duration, the samples were collected from the media and freeze-dried. The mass loss in the samples was analyzed gravimetrically. The results obtained are presented in Table 4.6.

Table 4.6. Biodegradability results of synthesized nanocarrier

No.	Samples	Mass Retain (%)		Mass Retain (%)	
		pH = 7.4		pH= 5.3	
	Days	7 Day	14 Day	7 Day	14 Day
1	RRPHFC1	95.81	81.31	91.60	77.36
2	RRPHFC2	95.60	80.24	90.87	77.13
3	RRPHFC3	95.59	80.20	90.19	76.18
4	RRPHFC4	91.25	77.91	84.85	76.08
5	RRPHFC5	90.91	77.22	84.70	76.56
6	RRPHFC6	90.56	76.85	83.80	76.14
7	RRPHFC7	91.42	78.24	85.90	74.12
8	RRPHFC8	91.41	77.61	85.50	74.11
9	RRPHFC9	91.11	76.69	84.92	73.91

According to the results obtained from the biodegradation study, at pH 7.5, a degradation of 5% was observed in RRPFC1, 2, and 3 by the end of the first week, while by the end of the second week, the degradation increased to 20%. For RRPFC4, 5, 6, 7, 8, and 9, the average loss/degradation was found to be 10% by the end of the first week and 22% by the end of the second week. In the biodegradation studies of RRPFC1, 2, and 3 ternary complexes at pH 5.3, a degradation of 10% was noted by the end of the first week and 27% by the end of the second week. For RRPFC4, 5, 6, 7, 8, and 9, the degradation rates were determined to be 15% and 25% in the first and second weeks, respectively.

4.12. Gel Retardation Assay

The gel retardation test was performed to determine the condensation ability of the nanocarrier (Chuang et al., 2025). It was carried out on 1% agarose gel using plasmid DNA containing 0.2 ng sgRNA. For this purpose, 0.2 ng sgRNA-containing plasmid DNA and varying amounts (1, 2, 4, 8, 16 μ g) of BHx-NH-HFBC-Flamma774 were loaded onto 1% agarose gel. In the first well, a DNA ladder was loaded. In the second well, 0.2

ng sgRNA-containing plasmid DNA was loaded. In wells 3 to 7, 0.2 ng sgRNA-containing plasmid DNA was incubated with different amounts (1, 2, 4, 8, 16 mg) of BHX-HFBC-NH-Flamma774 for 30 minutes. The 8th well was prepared as a control group and contained only 16 µg of BHX-HFBC-NH-Flamma774 without plasmid DNA. The gel retardation assay was performed by running the samples in 1% agarose gel prepared with TAE buffer (10 mmol·L⁻¹ Tris/HCl, 1% acetic acid, 1 mmol·L⁻¹ EDTA) at 100 V for 30 minutes. The gel was visualized using a UV lamp-equipped imaging system. The results are presented in Figure 4.66.

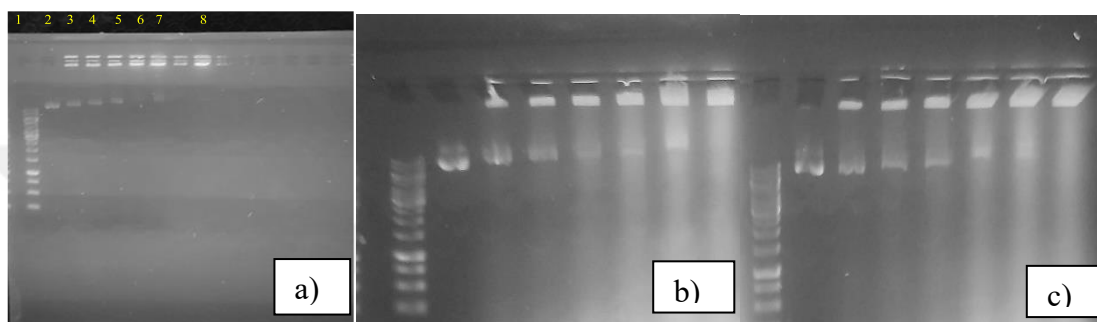


Figure 4.66. Jel retardation test of Plasmid DNA loaded BHX-HFBE-NH-F774
(a)RRPHFC1 (b) RRPFC4 ve (b) RRPFC7

It was observed that as the amount of BHX-HFBE-NH-F774 increased, the plasmid bound more effectively to the polymer. Under the condition where 8 mg of BHX-HFBC-NH-Flamma774 was used, a very high level of retardation of plasmid DNA containing 2 ng of sgRNA was observed in the agarose gel. Under the condition where 16 mg of RRPFC4 and RRPFC7 were used, a very high level of retardation of the plasmid DNA containing 0.5 µg of sgRNA was observed in the agarose gel. This indicates that the plasmid containing sgRNA binds strongly to the RRPFC polymer and that its movement through the gel is significantly slowed.

4.13. Drug Release Study

First, different concentration of cisplatin was studied under UV-vis spectrometer and their absorption value at 207 nm was taken. The data obtained from UV-vis spectrometric study is given in Table 4.7 and a calibration curve as given in Figure 4.67 was drawn. The R² value from linear equation was 0.9843 while Y=0.1541x+0.1076 was obtained.

Table 4.7. Absorption value of cisplatin at different concentration

Cisplatin Concentration (µg)	Absorbance (207)
125	1,961
62,5	1,114
31,25	0,628
15,6	0,315
7,8	0,151

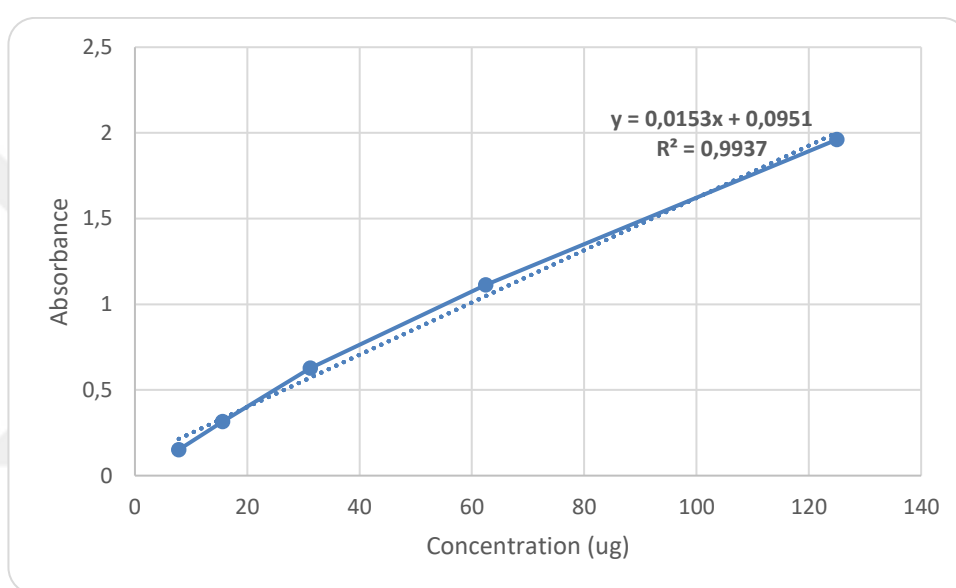


Figure 4.67. Calibration curve of cisplatin at different concentration against absorbance

To calculate the presence of free cisplatin, supernatant was studied under UV-vis spectrometer and pellet containing RRPFC (1,4 and 7) loaded with cisplatin was used for drug release study. Free cisplatin concentration in supernatant for RRPFC (1, 4 and 7) was calculated and the remaining drug was considered loaded into nanocarriers. The drug encapsulation efficiency (EE) of RRPFC1, 4 and 7 were found to be 83.8%, 85.4% and 94.5% respectively. The drug loading capacity (DLE) of RRPFC1, 4 and 7 were found to be 5.29%, 5.38% and 5.08% respectively as given in Table 4.8.

Table 4.8. Drug encapsulation efficiency (EE) and drug loading efficiency (DLE) by percentage values

Sample	EE%	DLE%
RRPHFC1	83.8	5.29
RRPHFC4	85.4	5.38
RRPHFC7	80.4	5.08

Furthermore, *in vitro*, drug release study was performed against different pH (7.4 and 6.5) in PBS environments. During the study of drug release, 30 mg of nanoparticles were taken into MW: 1KDa dialysis membrane and put into 25 mL of PBS buffer at different pH. After a specific interval of time (15-minute, 30 minute, 1 h, 2 h, 4 h, 8 h, 16 h and 32 h) sample was taken and studied under UV-vis spectrometer.

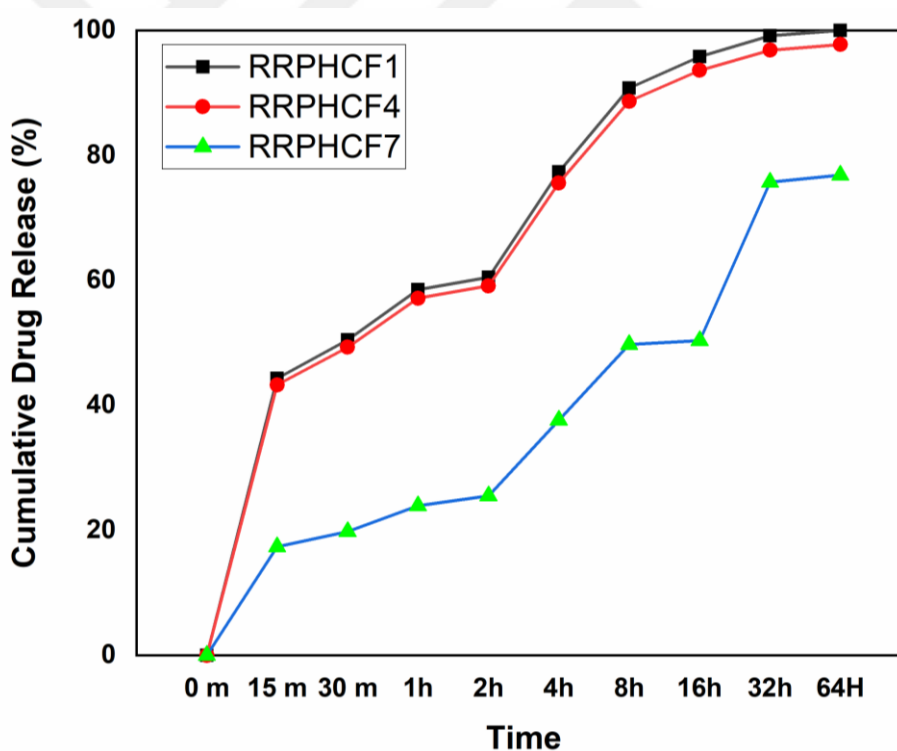


Figure 4.68. Cisplatin release study at 7.4 pH in PBS buffer environment

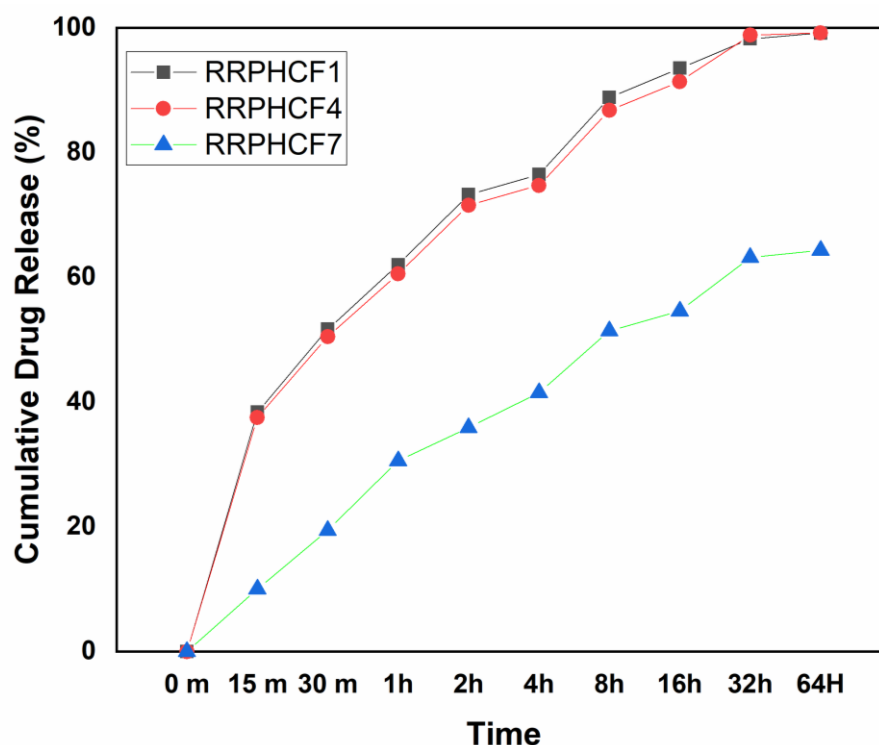


Figure 4.69. Cisplatin release study at 6.5 pH in PBS buffer environment

In vitro cisplatin release study of cisplatin in PBS environment is given in Figure 4.69 for pH 7.4 and Figure 4.68 for pH 6.5 for RRPFC 1, 4 and 7. The percentage of the drug release was found to be maximum at acidic pH compared to basic one. In addition, the RRPFC7 have shown slower release compared to RRPFC1 and 4 nanocarriers.

4.14. Determination of IC₅₀ Value in CAL51 Cell Lines

Foremost CAL51 and MCF10A cell line grown in optimum conditions were treated with afatinib to determine the natural resistance against afatinib without prior treatment. Table 4.9 shows the presence of drug resistance against afatinib in CAL51 cell line as 2.4 after 5 days of treatment.

Table 4.9. Time dependent IC₅₀ value in CAL51 and MCF10A cell lines

Cell Line	IC ₅₀ (Afatinib (μM))				
CAL-51	4.3±1.2	4.0±0.9	3.1±0.7	3.0±1.1	2.4±0.2
MCF10A	0.3±0.05	0.28±0.04	0.13±0.06	0.1±0.01	0.04±0.02

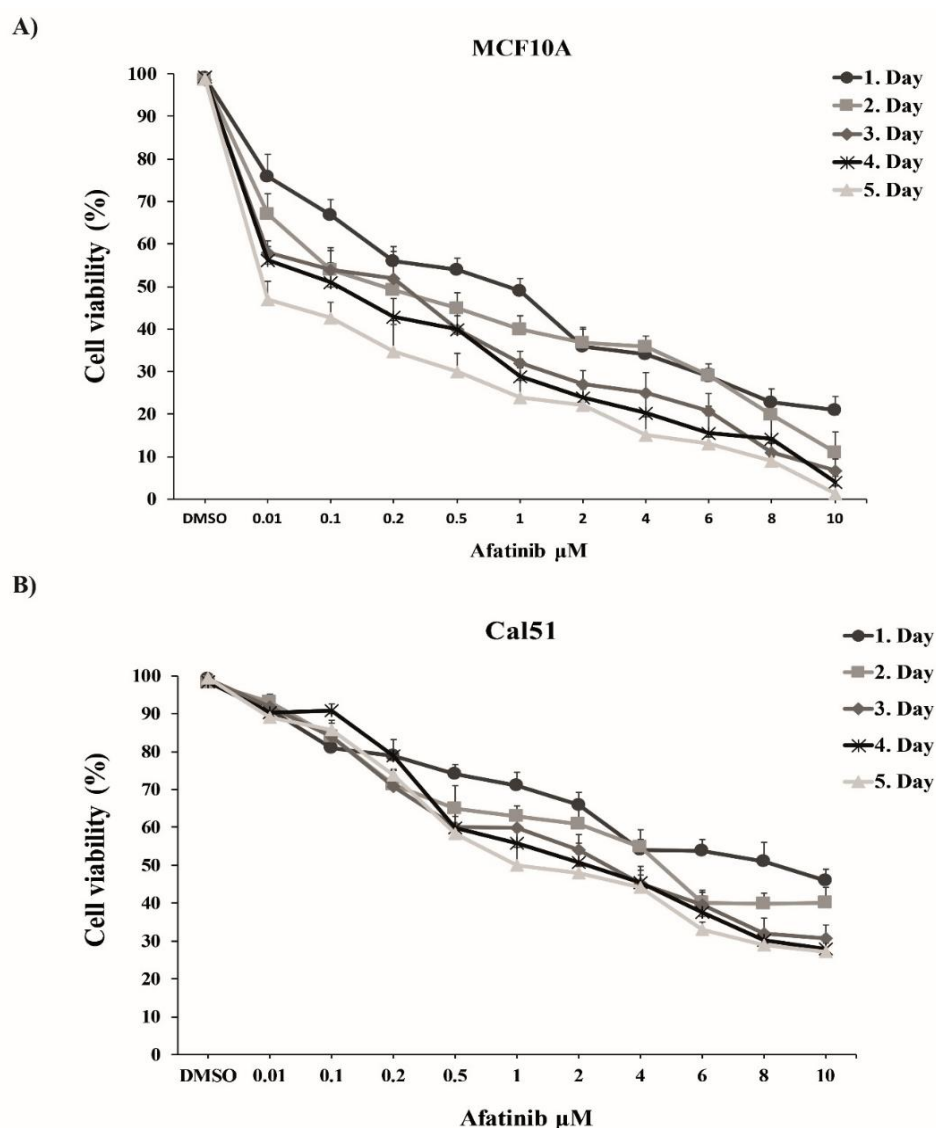


Figure 4.70. Graphical representation of percentage of alive a) MCF10A and b) CAL 51 cell line after treatment with afatinib to determine IC₅₀ value

Figure 4.70 represents the percentage of alive cell in CAL51 and MCFA10 Cell line after being treated with afatinib to determine the IC₅₀ values. It is observed that CAL 51 cell line already had developed resistance compared to MCF10A, a normal cell line. Moreover, the percentage of dead cell are more in MCF10 Cell line with IC₅₀ value of 0.04 ± 0.02 after five day of treatment with Afatinib compared to CAL51.

4.15. Afatinib Application and Determination of Afatinib Resistance

For the Afatinib application, one normal CAL51 cell line and one resistance to afatinib CAL51 cell line cultured long-term in Afatinib-containing medium were used for imaging. All cell lines were cultured in 100 mm plates under sterile conditions in an

environment with 5% CO₂ at 37°C. When the cell density reached 70–80%, the cells were passaged using 0.25% trypsin/1 mM EDTA. 5 µM Afatinib was dissolved in DMSO and applied to the cells such that the final DMSO concentration was 0.1%. Once both the resistant and non-resistant CAL51 cell lines reached sufficient confluency, they were counted and seeded into 100 mm plates at a density of 30,000 cells/cm². Afatinib was applied at a concentration of 5 µM for 1–5 days. The results obtained were imaged and are presented below In Figure 4. 71-4.75.

CAL 51 Cells

Afatinib Resistance CAL51 Cells

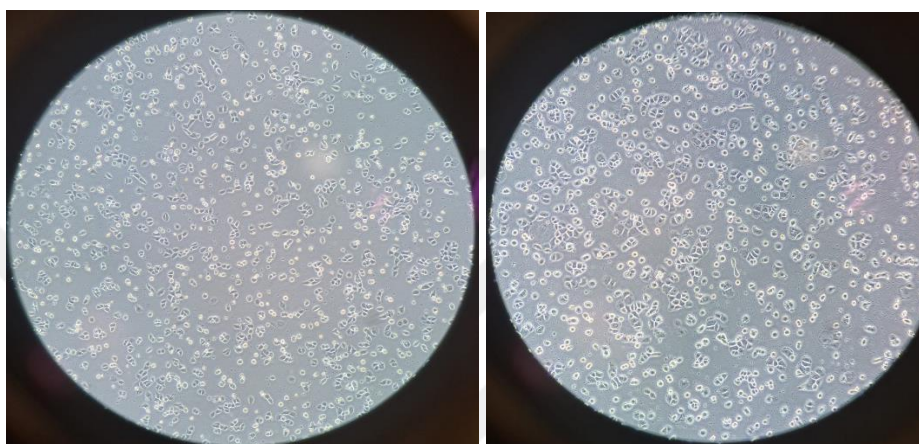


Figure 4.71. Imaging on Day 1 After Afatinib Treatment in Normal and Resistant CAL51 Cells

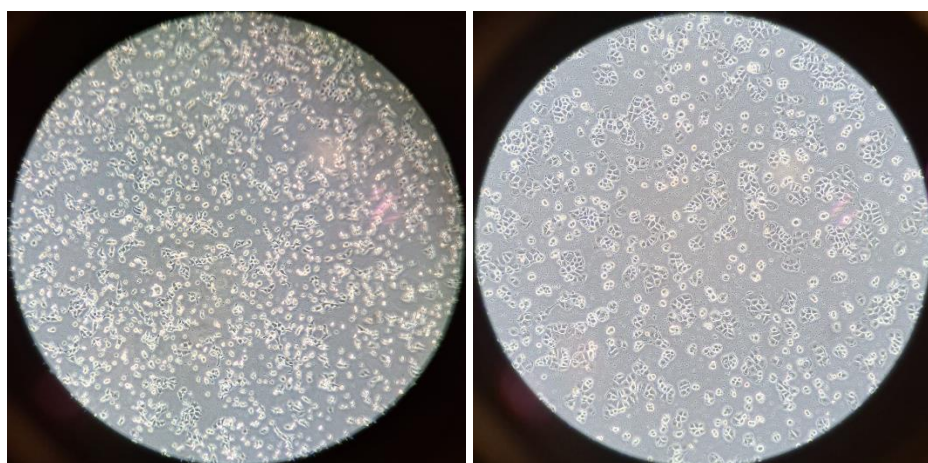


Figure 4.72. Imaging on Day 2 After Afatinib Treatment in Normal and Resistant CAL51 Cells

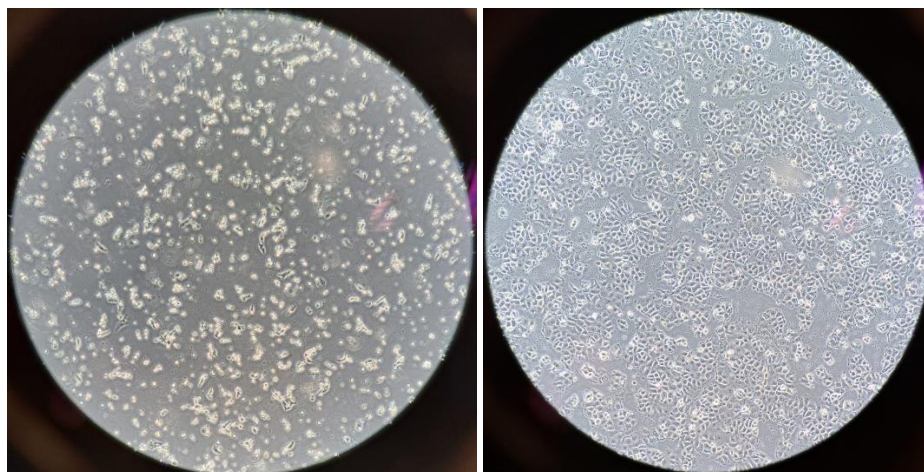


Figure 4.73. Imaging on Day 3 After Afatinib Treatment in Normal and Resistant CAL51 Cells

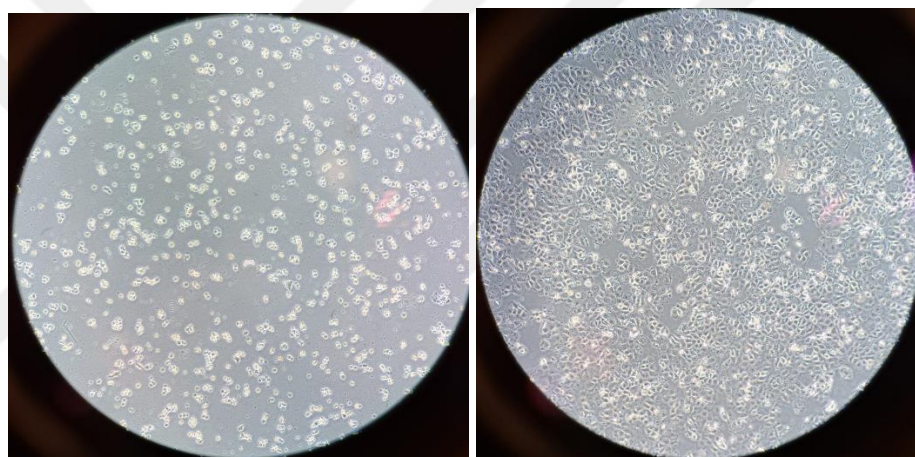


Figure 4.74. Imaging on Day 4 After Afatinib Treatment in Normal and Resistant CAL51 Cells

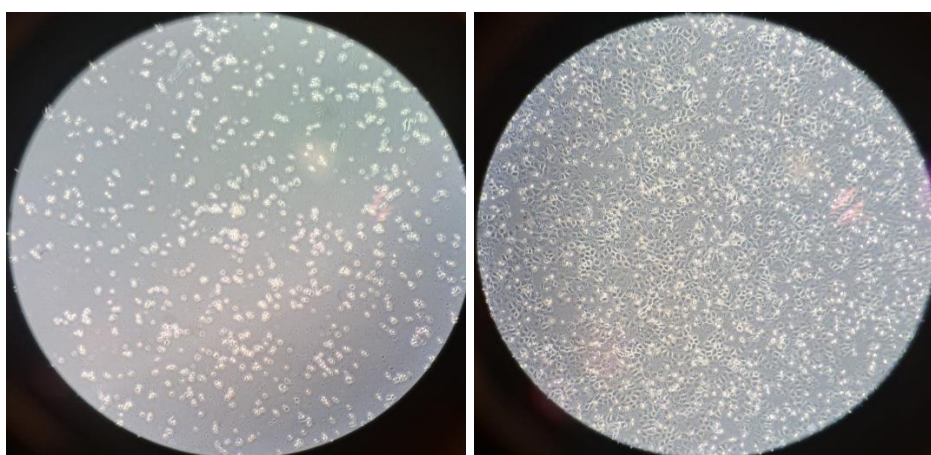


Figure 4.75. Imaging on Day 5 After Afatinib Treatment in Normal and Resistant CAL51 Cells

The death of normal CAL51 cells and the survival of resistant CAL51 cells on the 5th day after Afatinib treatment indicate the development of resistance to the drug Afatinib. The development of afatinib resistance in CAL51 Cell line with its IC 50 value is given in Table 4.10.

Table 4.10. Time dependent IC50 value in CAL51 after treatment with afatinib

Cell Line	IC50				
	Afatinib ($\mu\text{mol.L}^{-1}$)				
	1. Day	2. Day	3. Day	4. Day	5. Day
CAL-51	5.1 $\pm$ 0.6	4.7 $\pm$ 1.3	4.4 $\pm$ 0.3	4.1 $\pm$ 0.5	3.9 $\pm$ 0.9

The development of resistance in CAL 51 cell line was further represented by comparing the number of alive cells in Figure 4.76. It is observed that at 5 μM of afatinib drug concentration, CAL 51 cells have developed resistance almost 40 percent compare to afatinib non treated CAL 51 cells.

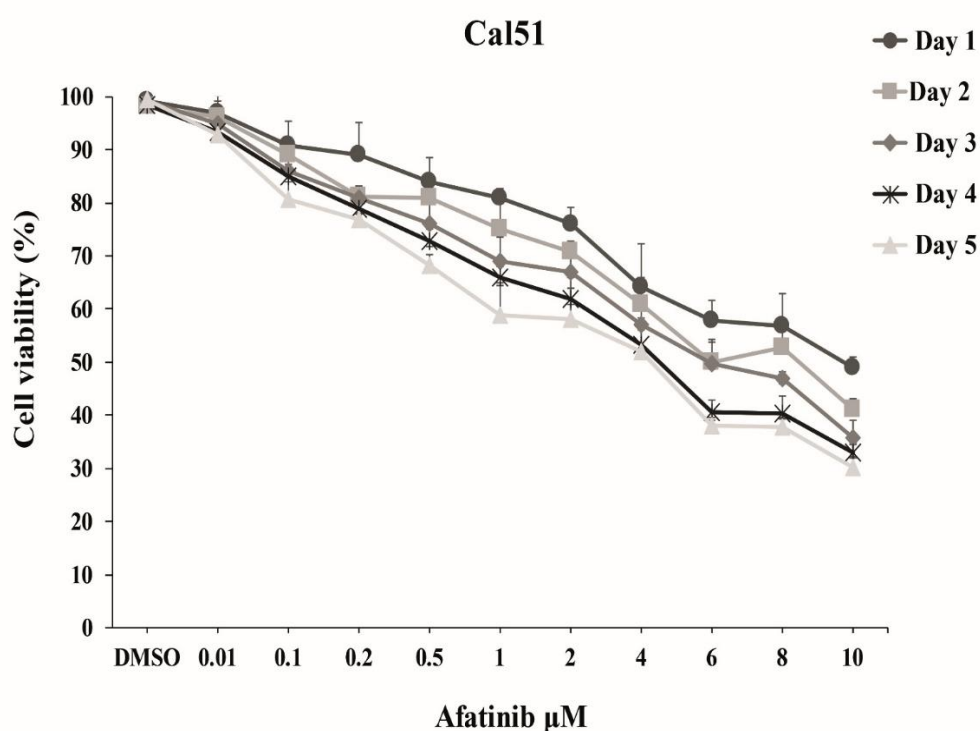


Figure 4.76. Graphical comparison of non-resistant and resistant cell death in Cal 51 cells

After five days of treatment with afatinib, cells have developed resistance, the percentage of alive cells is represented in Figure 4.76. Which indicate Cal 51 cells grown in afatinib environment have shown resistance after passage of time. The group which was grown in afatinib environment have shown more resistance compared to the group which was never grown in afatinib environment.

4.16. . Cytotoxicity Test of RRPFC (1, 4 and 7)

The cytotoxicity test is a colorimetric method used to assess cell viability in CCD18CO and Afatinib-resistant CAL 51 cells. In this study, CCD18CO and Afatinib-resistant CAL 51 cells were seeded into 96-well plates at a density of 10,000 cells per well and incubated for 24 hours to allow cell attachment. After incubation, RRPFC (1, 4, and 7) nanocarriers were added at concentrations ranging from 1 to 500 μ M, and the cells were incubated for 24, 48, and 72 hours. At the end of the incubation period, resazurin solution was added to each well, and the cells were incubated for another 2 hours. The 96-well plate was then measured using a UV spectrophotometer (EPOCH) ELISA reader at a wavelength of 570 nm to obtain optical density values. Cell viability was calculated by comparing the optical density values of the experimental groups to those of the control group, thereby determining the effect of the tested substances on the cells.

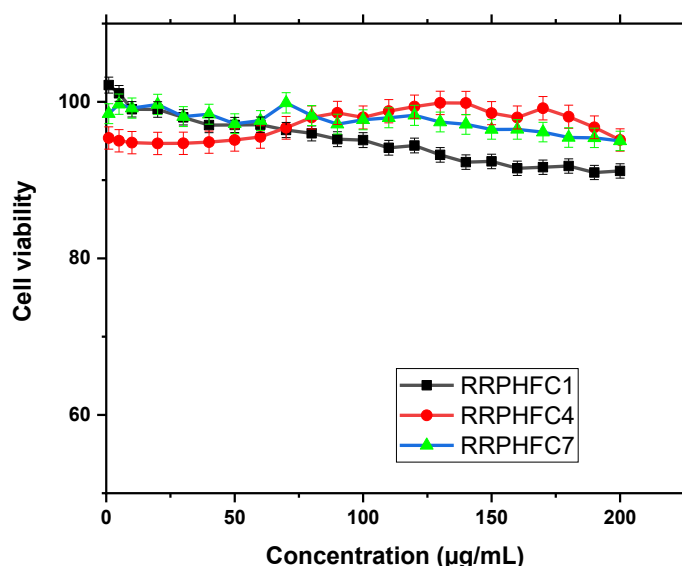


Figure 4.77. The effect of RRPFC (1, 4, and 7) on cell viability in CCD18CO cells at 24 hours in the study.

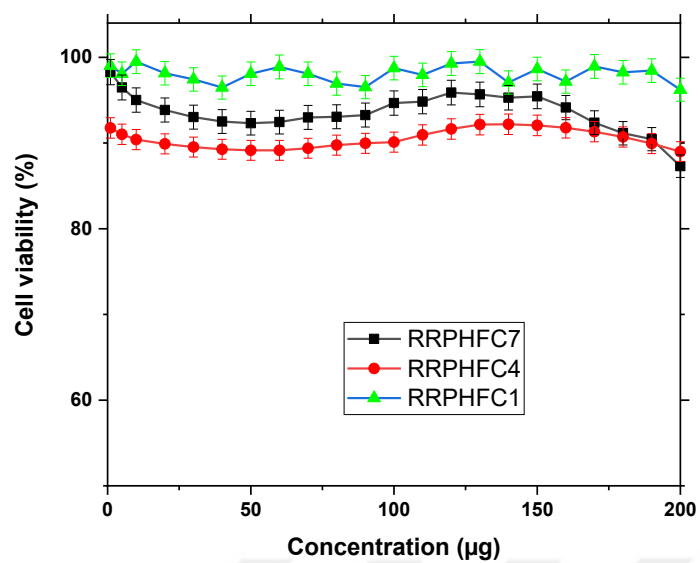


Figure 4.78. The effect of RRPHFC (1, 4, and 7) on cell viability in CCD18CO cells at 48 hours in the study.

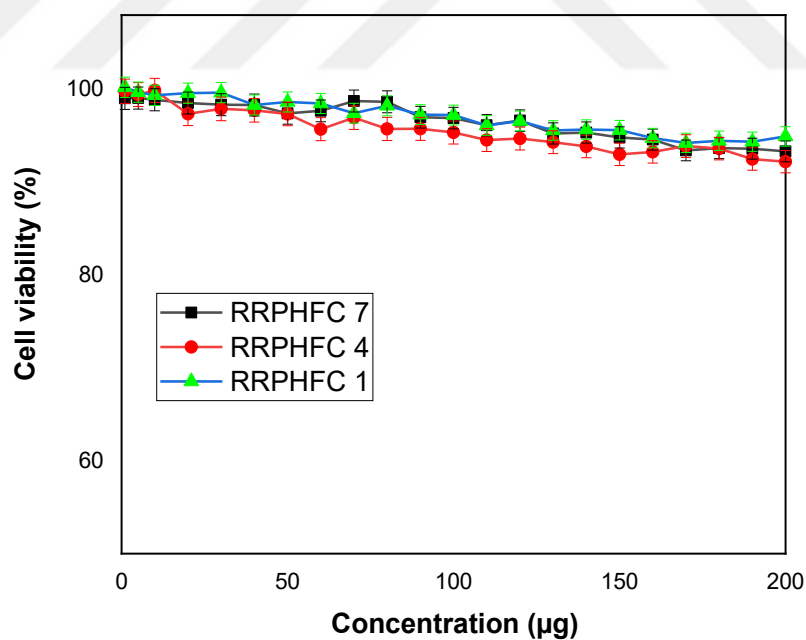


Figure 4.79. The effect of RRPHFC (1, 4, and 7) on cell viability in Afatinib-resistant CAL51 cells at 24 hours in the study.

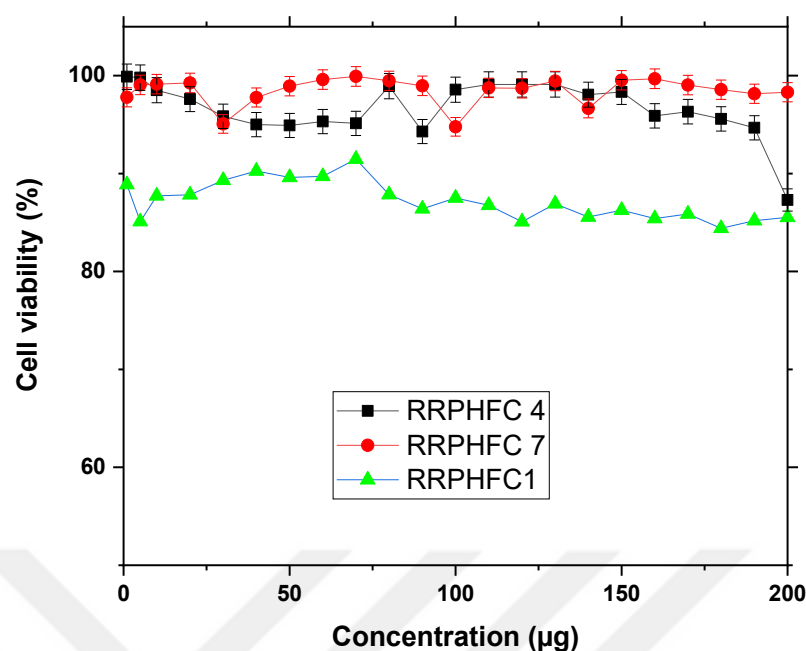


Figure 4.80. The effect of RRP HFC (1, 4, and 7) on cell viability in Afatinib-resistant CAL51 cells at 48 hours in the study.

The cytotoxicity of the RRP HFC (1, 4, and 7) nanomaterials is presented in Figures 4.77-4.80. The toxic effects of the nanomaterials, within the concentration range of 1–200 µg/mL, were tested on the normal CCD18CO cell line and the Afatinib-resistant CAL51 breast cancer cell line. The results revealed that the functional nanomaterials RRP HFC (1, 4, and 7) did not exhibit any toxic effects on either the normal or cancer cell lines till 200 µg/mL. All the data presented in Figures 4.81 showed almost identical results, indicating that the functional nanomaterials are safe for further experimental studies. This study is also supported by previously published data (Shao et al., 2017).

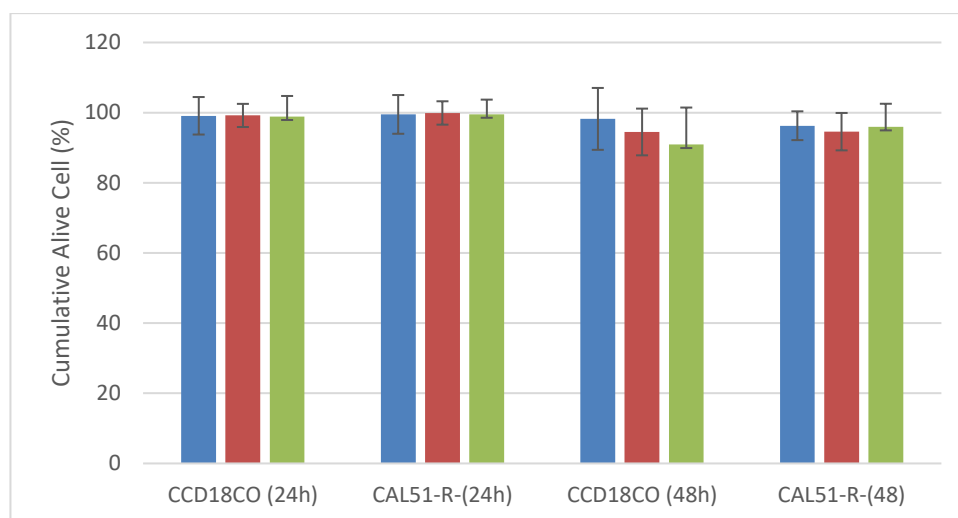


Figure 4.81. The cytotoxicity effect of RRPHFC (1, 4, and 7) on CCD18CO and afatinib resistant CAL51 cell lines in 24 and 48 hours of duration

4.17. Cytotoxicity Effect of Cisplatin Free and Loaded in RRPHFC Nanocarriers

The cytotoxicity of afatinib alone and afatinib loaded in RRPHFC nanocarriers was evaluated on CCD18CO (a normal cell line) and afatinib-resistant CAL51 cells at different time intervals. The IC₅₀ value of free cisplatin is 19.28 and 10.3 µg/mL for CCD18CO, while it is 33.7 and 15.5 µg/mL for afatinib-resistant CAL51 cells at 24 and 48 hours of incubation, respectively. According to the literature the IC₅₀ value of cisplatin in normal cell line should be higher compared to cancer cell line. Because normal cells activate their repairing mechanism to show some resistance. However, the elevated IC₅₀ value in the CAL51 cell line indicates that cells have developed resistance which activate the pathways to resist free cisplatin compared to normal cell line. However, in another case, when cisplatin loaded into RRPHFC (1,4 and 7) nanocarrier is studied on both afatinib resistance and CCD18CO cell line. The nanocarrier loaded with cisplatin has shown greater activity against the afatinib-resistant cell line, with an IC₅₀ value of 15.2, 9.6 and 14.9 for CCD18CO cell line and 9.3, 13.2 and 8.4 µg/mL against afatinib resistance CAL51 cell line in 24 hours of treatment. This activity enhanced when the time duration of incubation lead to 48 hours with IC₅₀ value of CCD18CO is 37.2, 20.1, and 36.5, while for CAL51 is 19.9, 35.37 and 19.5. Similarly, after 48 hours of incubation, cisplatin exhibited increased toxicity, and the activity of the RRPHFC nanocarrier was further enhanced.

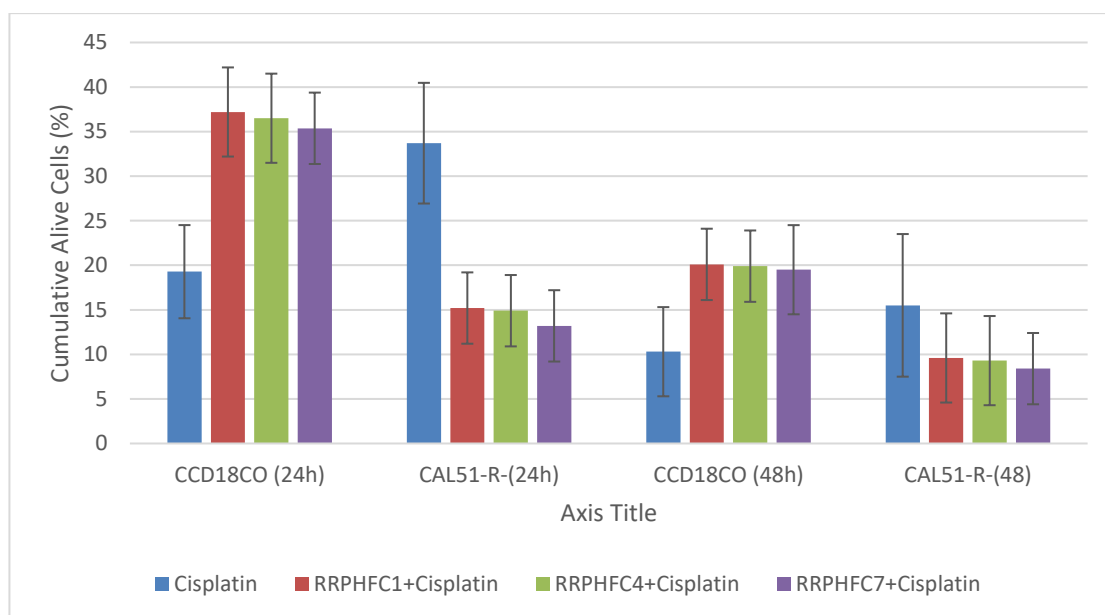


Figure 4.82. The cytotoxicity effect of cisplatin alone and loaded into RRPHFC (1, 4, and 7) on CCD18CO and afatinib resistant CAL51 cell lines in 24 and 48 hours of duration

The results presented in Figure 4.83 show that the cytotoxicity of free cisplatin was higher in the normal cell line compared to the afatinib-resistant cancer cell line. According to previous studies, cisplatin typically exhibits greater toxicity in cancer cells than in normal cells. This is because normal cells possess efficient DNA repair mechanisms that can prevent apoptosis when treated with cisplatin. However, in our case, the afatinib-resistant cancer cells have already developed resistance mechanisms, likely involving specific pathways that allow the cells to survive under stress conditions. As a result, the IC₅₀ value of free cisplatin was lower in normal cells compared to the afatinib-resistant cells. In contrast, the nanocarriers loaded with cisplatin were able to overcome the resistance mechanisms of the CAL51 cell line. This led to increased cytotoxicity in cells treated with RRPHFC loaded with cisplatin at both 24 and 48 hours.

5. CONCLUSION AND DISCUSSION

In this study, multifunctional nanocarrier has been synthesized with the core form of Boltorn HX (X=40, 30 and 20), functionalized with heptafluorobutanoyl chloride and Flamma 774 isothiocyanate molecule. Moreover, the shell of the nanocarrier is synthesized by functionalizing hyaluronic acid with folic acid and RGD-R8 peptide as a biomarker for targeting therapy. During the synthesis process, each step is characterized by FT-IR, DSC and NMR spectrometry. After the functionalization of both core and shell part of the nanocarrier, turnery complex is formed and further characterized with SEM, TEM and Zeta sizer to measure the particle size, its morphology and charge on the surface to find out the stability of nanocarriers. Later on, nanocarrier loaded with cisplatin and their loading capacity is calculated. For the release study of the nanocarrier loaded with cisplatin, PBS buffer solution of different pH (7.4 and 6.5) is used, data is taken after specific time interval and measured under UV-vis spectrometer. The cytotoxicity study of the nanocarrier with and without cisplatin is calculated through MTT assay on afatinib resistant CAL51 and CCD18CO cell lines.

Foremost, terminal hydroxyl groups of Boltorn BH (40, 30 and 20) have been functionalized with NH_2 group according to the method reported in literature (Rajesh et al., 2005). Boltorn H (40,30 and 20) has reacted with sodium azide in the presence of triphenylphosphine and di-isopropyl azodicarboxylate as catalysts as represented in Figure 3.1. Functionalized BH40- NH_2 is characterized with FT-IR spectroscopy as given in Figure 4.1, Figure 4.2 and Figure 4.3 respectively. The N-H bending vibration belong to NH_2 groups is observed at 1653 cm^{-1} which indicates the successful synthesis of BHX- NH_2 (X= 40,30 and 20). In addition, DSC study about BHX- NH_2 (X= 40,30 and 20) is given in Figure 4.5, Figure 4.6 and Figure 4.7 respectively. In BH40- NH_2 , there is an endothermic peak observed at $200\text{ }^\circ\text{C}$, while this peak is not seen in the BH40 and BH40- NH_2 polymers. Additionally, the endothermic peak for BH40 is observed at $322\text{ }^\circ\text{C}$, shifting to $311\text{ }^\circ\text{C}$ for BH40- NH_2 . The endothermic peaks observed at $54\text{ }^\circ\text{C}$ and $55\text{ }^\circ\text{C}$ for the BH-30 and BH30- NH_2 polymers can be attributed to the breaking of hydrogen bonds and the formation of new ones. Furthermore, the endothermic peak for BH30 observed at $315\text{ }^\circ\text{C}$ is seen as a more intense peak for BH30- NH_2 with the peak shifting to $323\text{ }^\circ\text{C}$. In the BH20 polymer, an endothermic peak is observed at $72.7\text{ }^\circ\text{C}$ while an extra endothermic peak is observed at $130\text{ }^\circ\text{C}$. Moreover, some peaks are noted at $60\text{ }^\circ\text{C}$ for the BH20- NH_2 . Additionally, the endothermic peak observed for BH20 at $324\text{--}329\text{ }^\circ\text{C}$

is seen as more intense for BH20-NH₂ at 317-321 °C. These results indicate that the polymer has been modified. Functionalization of amine group with BHX is further confirmed by ¹H-NMR spectroscopy is given in Figure 4.8. The peak is observed at 1.04 ppm belongs to -NH₂ functionalized onto BHX (X=40, 30 and 20) polymers. Moreover, the functionalization of amine group is also measured by ninhydrin test as given in Figure 4.28 showing the successful modification of polymer.

For further modification of BHX-NH₂ (X=40,30 and 20) is reacted with heptafluorobutanoyl chloride molecules at 4 °C condition in methanol as solvent as given in Figure 3.2. In the FT-IR spectra of BH40-NH₂-HFBE, strong C-F peaks associated with heptafluorobutanoyl chloride, which are expected to be observed in the range of 1400-1000 cm⁻¹, overlap with the peaks of BHX (*Heptafluorobutyryl Chloride*, n.d.-a). The C=O peaks in the HFBC molecule also overlap with the peaks at 1732 cm⁻¹. The peak observed at 1222 cm⁻¹ in BH40-NH₂-HFBE1, 1234 cm⁻¹ in BH30-NH₂-HFBE1 and 1232 cm⁻¹ in BH30-NH₂-HFBE1 can be attributed to the C-F stretching vibration, overlapping with the peaks of the unmodified BHX (X= 40, 30 and 20) and BHX-NH₂ polymers. These findings are consistent with the literature (Su et al., 2018). Functionalization of HFBC on BHX-NH₂ terminal hydroxyl group is further characterized with DSC thermogravimetry analysis as given in Figure 4.5, Figure 4.6 and Figure 4.7. In the BH40-NH₂-HFBE1, BH40-NH₂-HFBE2, and BH40-NH₂-HFBE3 polymers, an endothermic peak is observed at 200 °C, while this peak is not seen in the BH40 and BH40-NH₂ polymers. However, an endothermic peak at 311 °C for BH40-NH₂ shifts to 226 °C, 324 °C, and 322 °C, respectively, and is observed as a more intense endothermic peak. These results indicate that the BH40 polymer has been successfully modified. In addition, the endothermic peaks observed at 65 °C, 76 °C, and 78 °C for the BH30-NH₂-HFBE1, BH30-NH₂-HFBE2, and BH30-NH₂-HFBE3 polymers can be attributed to the hydrogen bonds breaking and rebounding as new ones. The additional endothermic peak at 100 °C in the BH30-NH₂-HFBE1, BH30-NH₂-HFBE2, and BH30-NH₂-HFBE3 polymers are not observed in the BH30 and BH30-NH₂ polymers. Furthermore, an intense endothermic peak is observed at 323 °C and 328 °C for BH30-NH₂-HFBE2 and BH30-NH₂-HFBE3, respectively, indicating that the polymer has been modified. While in the functionalized BH20, peaks are noted at 60 °C, 56 °C, and 52 °C for the BH20-NH₂-HFBE1, BH20-NH₂-HFBE2, and BH20-NH₂-HFBE3 polymers, respectively. Moreover, an intense peak for BH20-NH₂-HFBE1, BH20-NH₂-HFBE2, and BH20-NH₂-HFBC3 at 317-321 °C, 312 °C, and 316 °C, respectively is observed.

The ^{19}F -NMR spectra of functionalized hyperbranched BHX-HFBE-NH-F774 ($x=40, 30, 20$) dendrimers are given in Figures 4.15, 4.16, and 4.17. The ^{19}F -NMR spectrum obtained after the polymer is functionalized shows three peaks at -126.29, -116.36, and -80.13 ppm. A strong coupling is observed between heptafluorobutyryl-BH40 and its nearest neighbors. The peak at -126.29 ppm is attributed to the fluorine adjacent to the -COO group, while the peaks at -116.2 and -80.13 ppm correspond to the typical CF_2 and CF_3 groups. The ^{19}F -NMR spectra of the BH30-HFBE and BH20-HFBE polymers show peaks at the same positions, but the intensity of the peaks is lower in the hyperbranched BH20-HFBE polymer.

To evaluate the distribution of drug loaded functionalized nanocarrier in the targeted cells, BHX-HFBE-NH₂ is further functionalized with Flamma™-774 ($\lambda_{\text{abs}}=778$ nm, $\lambda_{\text{em}}=808$ nm) as presented in Figure 3.3. Since the BHX-NH₂-HFBE compound was functionalized with 9.9×10^{-7} mol of Flamma 774 to obtain -BHX-NH₂-HFBE-Flamma 774, the peaks corresponding to functional groups such as Ar-H, S-O, and C=N in the Flamma 774 structure were observed at very low intensity. The vibrational peaks observed between $2310\text{-}1944\text{ cm}^{-1}$ are attributed to the benzene ring as given in Figure 4.1, figure 4.2 and Figure 4.3. Therefore, the peaks expected for Flamma 774 overlapping with those of BHX-NH₂-HFBE and BHX-NH₂-HFBE-Flamma 774 compounds, and these peaks were not separately observed in the FT-IR spectrum. The binding of Flamma 774 to the structure has been confirmed by fluorescence and ^1H -NMR analyses. In boltorn HX ($X= 40, 30$ and 20) ^1H -NMR (DMSO-d_6) δ ppm spectra peaks observed at (7.175 -NH), (7.482, 7.76-7.99 -benzene ring) are attributed to Flamma 774. The peaks observed between 0.5-1.17 ppm originate from the -H of the -CH₃ groups in linear, terminal, and dendritic positions. Moreover, ^{13}C -NMR spectrum of the hyperbranched BHX-HFBE-NH₂-F774 dendrimer is given in Figure 4.18, 4.19 and 4.20. In ^{13}C -NMR spectrum (16.76 and 16.84 -CH₃), (38.93, 39.13, 39.34, 39.55, 39.76, 39.97, 40.18, 40.46, 40.47 DMSO, -NH₂), (46.23, 48.25, 50.23 NH₂, -C-, C-O-C), (63.74, 63.89, 65.05, 65.52 CH₂-OH, CH₂-OR), (173.04, 173.17, 174.31 C=O) peaks are observed. However, peaks related to Flamma 774 overlap with the peaks of BHX ($X= 40, 30$ and 20) in the ^{13}C -NMR spectrum. An absorption spectrum of Flamma 774 functionalized on terminal ending of hyperbranched BHX-HFBE-NH-Flamma 774 is given in Figure 4.62. the absorbance band at 571nm showing the successful functionalization of polymer.

The outer shell of nanocarrier is made up of hyaluronic acid functionalized with folic acid and R8-RGD. Synthesis of HA-FA is done by the method described in the

literature (Tong et al., 2020). On the other subunit of hyaluronic acid, poly(ethylene glycol) 2-aminoethyl ether acetic acid ($\text{NH}_2\text{-PEG-COOH}$) is added and then further functionalized with [c(RGDfK)-RRRRRRRR-Cys] RGD-R8 in the presence of Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) as catalyzers as given in Figure 3.4, 3.5 and 3.6. In the FT-IR spectra of hyaluronic acid functionalized with FA and RGD-R8 peptide is given in Figure 4.4 shows the N-H stretching vibrations at 3427 cm^{-1} , 3336 cm^{-1} , and in the range of $3420\text{-}3330\text{ cm}^{-1}$ overlap with a strong broad peak from the -OH group. The spectral peaks at 2925 , 2846 , and 2849 cm^{-1} correspond to the symmetric and asymmetric stretching vibrations of aliphatic CH groups. A strong peak at 1682 cm^{-1} is attributed to the symmetric stretching vibration of the C=O group. Additionally, the peaks at 1682 , 1627 , 1624 , and 1570 cm^{-1} can be assigned to the bending vibration of -CO-NH- from primary amines. The peaks at 1382 and 1371 cm^{-1} arise from the bending vibrations of the OH in the COOH group. The strong peak at 1109 cm^{-1} is attributed to the C-O stretching vibrations in the C-OH group, while the strong peaks at 1096 and 1081 cm^{-1} correspond to C-O-C stretching vibrations. The FT-IR spectra of HA-FA and HA-RGD are similar due to the presence of the same functional groups in both structures. Furthermore, In the FT-IR spectra, the N-H stretching vibrations observed at 3427 cm^{-1} , 3336 cm^{-1} , and in the range of $3420\text{-}3330\text{ cm}^{-1}$ overlap with a strong broad peak from the -OH group. The spectral peaks at 2925 , 2846 , and 2849 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of aliphatic CH groups. A strong peak at 1682 cm^{-1} is attributed to the symmetric stretching vibration of the C=O group. Additionally, the peaks at 1682 , 1627 , 1624 , and 1570 cm^{-1} can be assigned to the bending vibration of -CO-NH- from primary amines. The peaks at 1382 and 1371 cm^{-1} arise from the bending vibrations of the OH in the COOH group. The strong peak at 1109 cm^{-1} is attributed to the C-O stretching vibrations in the C-OH group, while the strong peaks at 1096 and 1081 cm^{-1} correspond to C-O-C stretching vibrations. The FT-IR spectra of HA-FA and HA-RGD are similar due to the presence of the same functional groups in both structures. The $^1\text{H-NMR}$ spectrum of hyaluronic acid functionalized with folic acid and conjugated with RGD-R8 peptide dissolved in D_2O is presented in Figure 4.12, Figure 4.13 and Figure 4.14. The different positions of the -H atoms in the HA conjugate can be observed. The peaks in the range of $1.12\text{-}1.29\text{ ppm}$ correspond to the - CH_3 protons of hyaluronic acid. A peak at 1.97 ppm , attributed to the - COCH_3 protons in hyaluronic acid, overlaps with the peak from the -NH group proton attached to the benzene ring in folic acid, appearing at 1.99 ppm . The protons of the - CH_2 groups in hyaluronic acid are

observed at 2.592-2.73 ppm. Peaks observed at 2.934-4.050 ppm correspond to the H(CH₂), CH) protons. However, after conjugation with polyethylene glycol 2-aminoethyl ether acetic acid, the peak at 3.660 ppm appears as a more intense peak. The peaks between 4.39-4.902 ppm belong to D₂O. The peak observed at 6.84 ppm corresponds to the protons of the heterocyclic rings present in folic acid and RGD. The peaks at 7.33 and 7.71 ppm are associated with the aromatic rings in folic acid and the protons of the -NH₂ group linked to these rings. The peak at 8.01 ppm corresponds to the -NH proton of the polyethylene glycol 2-aminoethyl ether acetic acid attached via C=O. The results indicate that folic acid and RGD-R8 peptide have been successfully conjugated with the polyethylene glycol 2-aminoethyl ether acetic acid chain and hyaluronic acid.

Both the core molecule BHX-HFBE-NH-Flamma 774 and shell part of HA-FA-RGD was combined to form ternary complex RRPFC. The ternary complex was further characterized by a zeta sizer to calculate the size and potential on surface. Moreover, SEM and TEM results also show the particles are nanosized and good in morphology. The MALDI-TOF analysis as given in Figure 4.53, Figure 4.54 and Figure 4.55 and their interpretation are given in Table 4.3, Table 4.4 and Table 4.5. which indicate the successful functionalization of both Boltorn HX and HA. According to the results obtained from MALDI-TOF spectra of molecular size, the RRPFC 1, RRPFC4 and RRPFC 7 is around 8000, 6000 and 4000 m/z respectively. The SEM and TEM images given in Figure 4.56-4.61 show the size is less than 100 nm and particles are good in morphology. The TEM images also show the formation of ternary complex and core structure covered with shell structure of modified hyaluronic acid.

RRPFC (1, 4 and 7) nanocarriers were loaded with cisplatin, their drug loading capacity and release study was calculated by uv-vis spectrometer. The drug loading efficiency was found 83.8, 85.4 and 90.4 % in RRPFC1, RRPFC4 and RRPFC 7 respectively. Cumulative drug release study in PBS buffer solution at different pH was calculated. Release of Cisplatin in RRPFC7 is steadier compared to RRPFC 1 and RRPFC 4. In early 15 minutes the first burst is calculated. Later, cisplatin release is increasing with the passage of time as shown in Figure 4.67. Moreover, the release in acidic environment was found faster compared to basic environment as shown in Figure 4.68.

In vitro cytotoxicity study on afatinib resistant cells (CAL51) and a normal cell line (CCD18CO) is tested. Foremost, CAL51 was cultured in afatinib containing media to develop resistance in TNBCs. With the passage of time, drug resistance against afatinib

is developed as shown in Figure 4.69-4.73. After 5 days of incubation, afatinib resistant CAL51 cells stay alive in 6 μ M afatinib containing media while nonresistance CAL51 cells die at same concentration of afatinib. Cytotoxicity test of RRPHFC1, RRPHFC4 and RRPHFC 7 is done on CAL51 and CCD18CO cell line at 24 and 48 hours of time as given in Figure 4.77-4.81. It is found that RRPHFC1, RRPHFC4 and RRPHFC 7 have no toxicity to CAL51 and CCD18CO cell line till 200 μ g/mL. The cytotoxicity of both free afatinib and afatinib loaded in RRPHFC1, RRPHFC4 and RRPHFC 7 nanocarriers was investigated using the CCD18CO normal cell line and the afatinib-resistant CAL51 breast cancer cell line over 24 and 48-hour incubation periods. Free cisplatin exhibited an IC₅₀ value of 19.28 μ g/mL in CCD18CO and 33.772 μ g/mL in CAL51, indicating higher toxicity in normal cells. This contrasts with previous research, where cisplatin generally showed greater toxicity toward cancer cells due to their deficient DNA repair mechanisms(Jie et al., 2025a; O’Grady et al., 2014; Yusoh et al., 2025). In our case, the reduced sensitivity of the CAL51 cell line is likely due to the development of resistance mechanisms that enable survival under stress conditions. Notably, RRPHFC nanocarriers loaded with cisplatin were able to overcome this resistance, showing significantly enhanced cytotoxicity in CAL51 cells. This effect was more pronounced after 48 hours, indicating the sustained and improved efficacy of the nanocarrier system in delivering cisplatin to resistant cancer cells.

We can conclude that hyperbranched polymer is successfully functionalized with HFBC and Flamma 774, as well as hyaluronic acid with folic acid and RGD-R8 peptide. The ternary complex is good in morphology and nanometer in size with high loading efficiency, no cytotoxicity and high biocompatibility. Moreover, RRPHFC hyperbranched nanocarriers have potential to overcome the developed resistance in cancer cell lines. This study was aimed to design a multifunctional nanocarrier against afatinib drug resistance TNBC cell lines. As the development of drug resistance in cancer patients after chemotherapy is the most common problem. After specific time of treatment cells started to show no response against therapeutic agent. This led to a more severe form of cancer leading to death in most cases(Rao et al., 2023). Therefore, doctors use to treat with combination therapy or enhance the dose of the therapeutic agent which left major side effects in the body of the patient. Hence there was an urgent need to overcome this problem and here nanotechnology plays its role. Nanocarrier has been attracting the attention of scientists and researchers now a days. There are so many other nanocarriers used to overcome drug resistance, however, they face some hurdles in delivering of

therapeutic agent such as target specificity. In this study Boltorn HX of different molecular weight was used and functionalized with heptafluorobutyl chloride to develop stability and hydrophobicity in the structure as well as Flamma 774, a near infrared dye was bounded to help in diagnosis purposes. To make it more target specific nanocarriers were covered with envelop of hyaluronic acid functionalized with folic acid and RGD peptide which are key targeting molecules for cancer cells. The nanocarrier was successfully synthesized and characterized with FT-IR, NMR, MADI-TOF MS, Zeta sizer, thermogravimeter, SEM and TEM analyzers. The results obtained from characterization analysis show the successful synthesis of nanocarrier. The synthesized nanocarrier was further tested on normal CCD18CO and afatinib resistant CAL51 cancerous cell lines to check its cytotoxicity. 200 μ g of these nanocarriers have shown no cytotoxicity to both normal and cancerous cell lines. Furthermore, these nanocarriers were loaded with cisplatin a model drug has shown good encapsulation efficiency. Their release study tested at various pH conditions as well as on invitro culture have shown time dependent slow and pH dependent more release in acidic environment. Moreover, these nanocarriers were found effective in afatinib resistant TNBCs compared to normal cell lines. Hence, our results indicated the successful synthesis of hyperbranched nanocarriers with high loading capacity and targeted therapy of TNBCs.

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