TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT

DEVELOPMENT OF CONTROLLED ETOPOSIDE RELEASE MATERIALS: TESTING ON GLIOBLASTOMA CELLS

EYLEM TÜRKÜ ÖZKAN

M.Sc.

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ADANA, 2024

DECLARATION OF CONFORMITY

In this thesis study, which was prepared following the thesis writing rules of Adana Alparslan Türkeş Science and Technology University Institute of Graduate School, I declare that I provide all the information, documents, evaluations and results in accordance with scientific ethics and moral codes without resorting to any means or assistance that would be contrary to scientific ethics and traditions. I also declare that I refer to all of the articles I used in this study with appropriate references and accept all moral and legal consequences if a situation is found contrary to my statement regarding my work.

21/02/2024

[Signature]

Eylem Türkü ÖZKAN

ÖZET

KONTROLLÜ ETOPOSİD SALIM MALZEMELERİNİN GELİŞTİRİLMESİ: GLİOBLASTOMA HÜCRELERİNDE TEST EDİLMESİ

Eylem Türkü ÖZKAN
Yüksek Lisans, Biyomühendislik Anabilim Dalı
Danışman: Prof. Dr. Gözde BAYDEMİR PEŞİNT
II. danışman: Doç. Dr. Dilek GÖKTÜRK
Ocak 2024, 65 sayfa

Etoposid, kanser tedavisinde kullanılan bir kemoterapi ilacıdır. Bu ilaç, kanser hücrelerinin büyümesini ve çoğalmasını engelleyerek etki gösterir. Etoposid, genellikle solid tümörler, lenfoma ve lösemi gibi çeşitli kanser türlerinin tedavisinde kullanılır. Etoposid, DNA replikasyonu ve hücre bölünmesi süreçlerini etkileyerek çalışır. İlacın ana mekanizması, topoizomeraz II enzimini inhibe ederek DNA çiftinin çözülmesi ve tekrar birleşmesi süreçlerini etkiler. Etoposid, bu enzimin normal işlevini engelleyerek DNA'nın doğru bir şekilde çoğaltılmasını ve hücre bölünmesinin tamamlanmasını önler. Etoposid, onkologlar tarafından belirli kanser türlerinin tedavisinde kullanılır. Etoposidin etkisi, kullanıldığı kanser türüne, hastanın genel sağlık durumuna ve diğer tedavi yöntemlerine bağlı olarak değişebilir. Bu çalışmada, farklı konsantrasyonlarda etoposid yüklü HSA-nanopartiküller ile boş HSA nanopartiküller sentezlenmiş ve bu nanopartiküller kullanılarak moleküler baskılanmış HEMA/jelatin kriyojeller sentezlenmiştir. Hidroksietil metakrilat (HEMA) bazlı kriyojeller, taramalı elektron mikroskobu, zeta-sizer partikül boyutu analizi ve şişme testleri ile karakterize edilmiştir. Kriyojellerin içerisine yüklenen Etoposid'in miktarına bağlı olarak salınım kinetiği incelenmiş; Etoposid'in Glioblastoma hücreleri üzerindeki etkisini inceleyebilmek için ise sitotoksisite testi yapılmıştır. Sonucunda jele yüklenen Etoposid'in hücrelere doğrudan uygulanan Etoposid'den daha etkili olduğu ortaya çıkmıştır.

Anahtar Kelimeler: Etoposid, İnsan serum albumini, Nanopartikül, Kriyojel, İlaç Salım

ABSTRACT

DEVELOPMENT OF CONTROLLED ETOPOSIDE RELEASE MATERIALS: TESTING ON GLIOBLASTOMA CELLS

Eylem Türkü ÖZKAN

Msc. Thesis, Department of Bioengineering

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Etoposide is a chemotherapy drug used to treat cancer. This medicine works by preventing the growth and proliferation of cancer cells. Etoposide is often used to treat various types of cancer, such as solid tumors, lymphoma, and leukemia. Etoposide works by affecting the processes of DNA replication and cell division. The main mechanism of the drug is to inhibit the enzyme topoisomerase II, affecting the processes of unwinding and recombination of the DNA pair. Etoposide inhibits the normal function of this enzyme, preventing DNA from being properly replicated and cell division from completing. Etoposide is used by oncologists to treat certain types of cancer. The effect of etoposide may vary depending on the type of cancer for which it is used, the patient's general health condition, and other treatment methods. In this study, empty HSA particles were synthesized with HSA-nanoparticles loaded with etoposide at different concentrations, and molecularly imprinted HEMA/gelatin cryogels were synthesized using these particles. Hydroxyethyl methacrylate (HEMA)-based cryogels were characterized by scanning electron microscopy, zeta-sizer particle size analysis, and swelling tests. Release kinetics were examined depending on the amount of Etoposide-loaded into the cryogels; A cytotoxicity test was performed to examine the effect of Etoposide on Glioblastoma cells. As a result, it turned out that Etoposide-loaded into the gel was more effective than Etoposide applied directly to the cells.

Keywords: Etoposide, Human serum albumin, Nanoparticle, Cryogel, Drug release

Dedication

Dedication to my dear husband and my family who always believe in me.

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LIST OF ABBREVIATIONS

CNS	: Central Nervous System
GBM	: Glioblastoma
SCLC	: Small Cell Lung Cancer
WHO	: World Health Organization
MDR	: Multidrug Resistance
AIDS	: Acquired Immune Deficiency Syndrome
VP-16	: Vepesid
OVA	: Ovalbumin
BSA	: Bovine Serum Albumin
HSA	: Human Serum Albumin
pI	: Isoelectric Point
PTX	: Paclitaxel
NPs	: Nanoparticles
Dox	: Doxorubicin
HINP	: (HSA)-coated iron oxide nanoparticle
PCS	: Particle Size Analyzer
FACS	: Fluorescence-Activated Cell Sorting

PEI	: Polyethyleneimine
TENPA	: Targeting-Enhancing Nanoparticle of Paclitaxel
PLGA	: Poly (lactic-co-glycolic acid)
NNI	: National Nanotechnology Initiative
EPR	: Enhanced Permeability and Retention
VP	: Vepesid
Topo-II	: Topoisomerase II
NAB	: Nanoparticle bound
IMTb	: Imatinib base
DSC	: Differential scanning calorimetry
MIT	: Molecular imprinting technology
MIPs	: Molecularly imprinted polymers
MAA	: Methacrylic acid
AA	: Acrylic acid
2- or 4-VP	: 2- or 4-vinylpyridine
HEMA	: 2-hydroxyethyl methacrylate
Etop	: Etoposide

LIST OF SYMBOLS

nm	: Nanometer
Qt	: Cumulative amount of drug released at time t
Q0	: Cumulative amount of drug released at time t=0
C0	: Initial concentration of drug
k	: k is the first order rate constant
KH	: Release constant of Higuchi.
KHC	: Release constant of Hixson-Crowell.
Mt	: Amount of active substance
M∞	: Active substance at the equilibrium state

1. INTRODUCTION

Cancer is the uncontrollable growth and division of cells as a result of DNA damage, which allows the cells to infiltrate surrounding veins and tissues (Lammert et al, 2014). Even though our bodies go through 10,000 mutations every day, our immune systems are always on the lookout for and getting rid of any bad cells. Age, gender, genetics, race, hormonal balance, immunity, and social, biological, biological and regional factors, as well as chemical substances and malnourishment, all have an impact on the development of cancer. Malignant gliomas, or tumors of the central nervous system, account for 2.5 percent of cancer-related deaths worldwide, making it the second most deadly disease (Martin and J.D.,2021).

Of all primary malignant brain tumors, glioblastoma accounts for 57% of gliomas and 48% of primary central nervous system (CNS) tumors. It is the most common type of malignant primary brain tumor (Ostrom et al.2018). Because it is incurable, patients typically only live for three months, and even then, their survival only lasts for twelve to eighteen months (Jovčevska and I., 2019). Men are 1.6 times more likely than women to have the disease, and treatment usually entails radiation and chemotherapy in addition to surgery. Thus, creating new tactics to fight this deadly tumor is crucial. The effects of different drugs and drug delivery methods when used to treat cancer are still being studied (Brandes et al.,2008).

Etoposide is an alkaloid obtained from Podophyllum pellatum, the mandrake plant, has been utilized as an anticancer medication since 1980s. (Wise et al.,2019). The World Health Organization (WHO) lists it as an essential medication and it falls under the category of topoisomerase poisons (Davey and S.G., 2020). DNA topoisomerases are involved in fundamental biological processes and control the topological state of genetic material. Because etoposide, an epipodophyllotoxin, inhibits Topo II from reassembling broken nucleic acid molecules, it increases the rate of DNA breakage mediated by the enzyme. High amounts of DNA damage result from this, which can cause mutagenicity and cell death (Montecucco et al., 2015). Etoposide is a crucial component of many cancer treatments, especially for testicular and small cell lung cancer (SCLC) (Meresse et al.,2004). This drug is also a valuable agent in the treatment of brain tumors (Reyhanoğlu et al., 2020). It is frequently used in regimens with other antitumor medications such as cyclophosphamide, carboplatin, or cisplatin. When taken in conjunction with additional anticancer drugs, Etoposide is efficacious against small cell lung cancer. However, it has some disadvantages as well, such as low bioavailability, poor

hydrosolubility, multidrug resistance (MDR) tumor cell lines, and restricted neoplastic activity against a range of solid tumors. The primary objective of the field of cancer chemotherapy is the synthesis and production of etoposide analogs (Meresse et al.,2004).

Controlled drug release systems are biopolymer materials that deliver active ingredients to the intended organ or tissue at predetermined rates and times. By keeping the drug's plasma level at an effective dose, these systems enable frequent and repeated administration of dosage forms such as tablets or injections (Jain and K.K, 2008). They keep drug concentrations in the blood within therapeutic ranges, preventing drug efficacy from declining at lower concentrations than the threshold. Nevertheless, patients must take their medications for short periods of time using conventional drug delivery methods (Tiwari et al., 2012).

Albumin, a vital protein in the human body, plays a crucial role in various physiological processes. It is involved in maintaining oncotic pressure, transporting essential molecules, and regulating pH balance (Dobrinskikh et al., 2015). Albumin is a crucial protein with various roles in the human body, including maintaining colloidal osmotic pressure, transporting molecules, and serving as an antioxidant and signaling molecule (Belinskaia et al., 2021). It has been extensively studied in the context of liver diseases, such as cirrhosis, where impaired albumin function has been identified as a potential indicator of liver damage (Sun et al., 2019). Albumin can be employed as a diagnostic tool for illnesses like acquired immune deficiency syndrome (AIDS) and tuberculosis or as a drug delivery system (Langer et al.,2003). The therapeutic significance of the drug-albumin interaction is generally disputed, but research on it is still ongoing. Albumin accumulates in tumor cells, which is why it has been the focus of much research in cancer therapy (Kratz and F., 2010). Commercial sources of albumins include soy, milk, grains, egg white (ovalbumin), bovine serum (bovine serum albumin, BSA), and human serum (human serum albumin, HSA). Large quantities of albumins are obtained from these sources (Elzoghby et al., 2012).

Nanoparticles, derived from the roots of "nanos" and "nanoparticle," are solid-colloidal particles with distinct physicochemical properties. They range in size from 10-1000 nm (usually 50-300 nm) and can be used to transport active substances, vaccines, and biological macromolecules (Tabatabaei Mirakabad et al.,2014). Protein-based nanoparticles are promising due to their biodegradability, biocompatibility, and ease of elimination (Maham et al., 2009). They can shield drug molecules from degradation and proteolysis, improve drug bioavailability,

and reduce toxicity. Albumin nanoparticles are a common example, targeting tumors through the Enhanced Permeability and Retention (EPR) effect or receptor-mediated pathways (Meng et al.,2022). Protein-based nanoparticles are often made from BSA, gelatin, and HSA due to their nontoxicity, stability, and functional groups (Kundu et al., 2010). They offer advantages such as increased drug residence time, protection against harsh environments, and sustained and regulated drug administration. Nanoparticles have been shown to improve bioavailability and tissue localization compared to traditional methods, making them a promising platform for targeted controlled release (Vallorz et al., 2022; Chung et al., 2020). Four main factors affecting drug release from nanoparticles include particle size, particle surface feature, drug loading, and drug release. Smaller particle sizes lead to increased dissolution rates and improved bioavailability of poorly water-soluble drugs (Zhang et al., 2009). The size distribution of nanoparticles can impact drug encapsulation efficiency and release kinetics. To achieve high drug loading capacity, a nanodelivery system needs to use less matrix materials. Factors such as drug solubility, diffusion, desorption, erosion, and degradation of the particle matrix also affect the drug's release rate (Sing et al. 2009).

Different physical and chemical techniques can be used to create albumin nanoparticles. The desolvation method, emulsification, thermal gelation, nano spray dying, nab-technology, and self assembly are the primary techniques used to prepare albumin nanoparticles (Hong et al.,2020).

Molecular imprinting technology (MIT) is a versatile technique for creating molecularly imprinted polymers (MIPs) with tailored binding sites (Chen et al., 2016). It allows for the design of artificial receptors with predetermined selectivity and specificity for a given analyte, making it ideal for various application fields (Vasapollo et al., 2011). MIPs possess antibody-like recognition characteristics, providing specific identification abilities (Refaat et al., 2019). MIT has been widely used in various domains, particularly in chemical sensors (Akgönüllü et al., 2022). Its unique properties of application universality, recognition specificity, and structure predictability make it an attractive option for various fields of research and industry. MIPs have gained attention for their potential in drug delivery systems, such as controlled release of drugs like 5-fluorouracil (Puoci et al., 2007), aspirin (Kan et al., 2009), trinitroglycerin (Mohebali et al., 2016), and oxycodone (Behnia et al., 2022). Their versatility in accommodating different drug molecules for controlled release and targeted drug delivery demonstrate their potential in various fields (Cegłowski et al., 2020).

Cryogels are porous materials with unique properties like shape memory, high flexibility, and rapid size change (Çimen et al., 2021; Bölgen et al., 2010). They are preferred in molecular imprinting technology due to their wide-open pore structures, short diffusion paths, and high selectivity (Andaç et al., 2020; Bereli et al., 2020). They are suitable for tissue engineering, regenerative medicine, injectable carrier systems, drug release systems, and removal of heparin from human plasma (Çimen et al., 2021). Cryogels can be tailored to specific applications by modulating synthesis conditions, allowing for water purification, tissue regeneration, and drug delivery systems (Loo et al., 2013). Their unique properties, combined with molecular imprinting technology's selectivity and specificity, make them promising materials for various applications.

In this study, we aimed to synthesize a molecularly imprinted HEMA/gelatin cryogels material with etoposide-loaded HSA-nanoparticles and then to see the effect of this material on glioblastoma cells.

2. THEORETICAL FOUNDATIONS AND LITERATURE REVIEW

2.1. Cancer and Glioblastoma

Cancer is the unchecked division and development of cells due to damage to the cell's DNA. They invade the surrounding tissues and veins as they expand, knowing no bounds (Lammert et al, 2014). They don't react well to anything that stop them from growing. Despite the fact that our bodies undergo about 10,000 mutations per day, our immune systems are always monitoring us and eliminating any malignant cells.

The development of cancer is a very complicated process. It has been established that a wide range of internal and external variables can cause cancer. There are elements like age, gender, genetics, race, hormonal balance, immunity, and so on are examples of internal elements; social, biological, biological and regional, malnourishment, and chemical substances are examples of external influences (Lammert et al, 2014). When it comes to diseases that cause the highest death, cancer ranks second, right after cardiovascular diseases. (Jain et al.,2021).

Central nervous system tumors known as malignant gliomas account for 2.5 percent of cancer-related deaths worldwide (Martin and J.D.,2021). The most frequent malignant primary brain tumor is glioblastoma (GBM). It accounts for about 57% of all gliomas and 48% of primary malignant tumors of the central nervous system (CNS) (Ostrom et al.2018). It has an incidence of approximately 15% among brain tumors (Mirchia et al.,2020) As an aggressive neoplasm with a bad prognosis, glioblastoma, classified as stage 4 by the WHO in 2021, has an average survival duration of 3 months due to the incapacity to treat GBM in patients, and 12 to 18 months despite treatment. It is one of the neoplasms that causes the fastest death due to its poor prognosis, and while age and gender create variances in rates, the average 5-year survival time is 6.8% in patients, while the survival rate recorded in patients over 10 years is not even 1% (Jovčevska and I., 2019). GBM is primarily found in the supratentorial compartment, but it is also found in the frontal lobe in 43% of cases, the temporal lobe in 28%, the parietal lobe in 25% of cases, and the occipital lobe in 4% of cases (Figure 2.1) (Tamimi et al.,2017). GBM is an uncommon cerebellar tumor that is 1.6 times more common in men than in women, according to research. The initial course of treatment for glioblastoma should involve surgery with the goal of complete resection. However, total resection is nearly impossible and recurrence is nearly guaranteed because of the disease's infiltrative nature. Concurrent

chemotherapy and radiation after surgery is the usual course of care (Brandes et al.,2008). Therefore, creating novel strategies to combat this lethal tumor is essential. Research is still being done on the effects of various medications and drug delivery techniques when used to treat cancer.

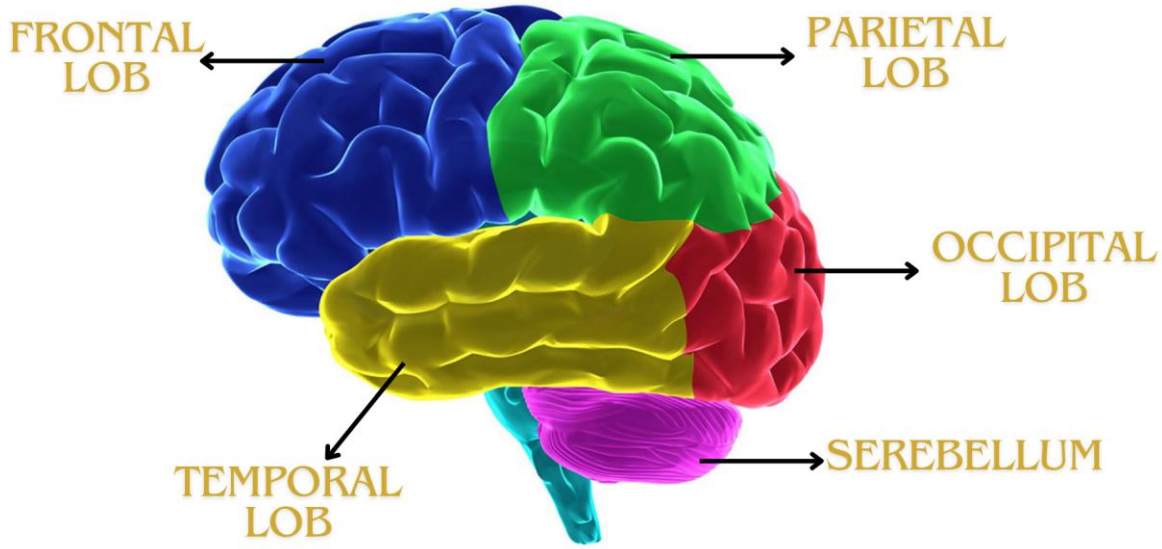

Figure 2.1. Schematic representation of the lobes of the brain.

2.2. Etoposide

Since the 1980s, Etoposide (Etop), a potent anticancer drug, has been used to treat a variety of cancers. It is an alkaloid derived from the mandrake plant, Podophyllum pellatum. Vepesid is the trade name for it (VP-16) (Wise et al.,2019). Etop is a powerful anti-tumor agent that is a member of the class of topoisomerase poisons and is listed as an essential medication by the WHO (Davey and S.G., 2020). DNA topoisomerases are vital enzymes that, by transiently transferring genetic material to DNA, regulate the topological state of genetic material. They

participate in basic biological functions like transcription, DNA repair, chromatin remodeling, and DNA replication. (Montecucco et al., 2015).

Etop is in the topoisomerase II inhibitor drug class. The late S and G2 phases of the cell cycle are when it primarily acts (Reyhanoğlu et al., 2020). The cleavable or cleavable complex, also referred to as the covalently cleaved DNA complex, is a transient phase of the reaction that the cell can withstand. It is cleaved by Topoisomerase II (Topo-II). Topo II has cytotoxic properties and becomes a toxin at high concentrations, causing a large number of transient protein-associated breaks in cell genomes. Anticancer medications can be categorized as either poisons or inhibitors according to their mode of action, depending on Topo II's capacity for cytotoxicity. The cleavable complex is stabilized by topo- II poisons, which include Etop and a number of clinically beneficial anticancer medications like doxorubicin, daunorubicin, mitoxantrone and m-AMSA. Etop is an epipodophyllotoxin that is used to treat various cancers. In vitro studies show that Etop primarily promotes Topo II-mediated DNA breakage by blocking the enzyme's ability to reassemble damaged nucleic acid molecules. The formation of the topoisomerase-drug-DNA ternary complex appears to be necessary for nucleic acid cleavage. The drug enters the complex by direct interaction with the monomer of the ATP-dependent enzyme, stabilizing only single-strand breaks per drug molecule. (Montecucco et al., 2015).

Indeed, Etop is a crucial and usual component of the management of numerous cancers, especially testicular and small cell lung cancer (SCLC). While Etop is occasionally administered as a stand-alone treatment during chemotherapy, it is typically combined with other antitumor drugs like cyclophosphamide, carboplatin, or cisplatin in regimens. It has been demonstrated that Etop is especially effective against small cell lung cancer when combined with other anticancer medications. One of the most effective treatments for lymphomas is VP-16. Etop is also used to treat non-Hodgkin's lymphoma and Hodgkin's disease, acute myeloblastic leukemia, and Kaposi's sarcoma, which is associated with the AIDS (Meresse et al.,2004). Additionally, this medication is beneficial in the treatment of brain tumors (Reyhanoğlu et al., 2020). Some of the disadvantages of Etop include limited neoplastic activity against a variety of solid tumors, including non-small cell lung cancer, low hydrosolubility, low bioavailability and cross-resistance to MDR tumor cell lines. The creation and synthesis of Etop analogs is a fundamental area of interest for the field of cancer chemotherapy (Meresse et al., 2004)

Robertson et al. (2002) demonstrated that a concentration of 50 M Etop directly induced toxicity to mitochondria and stimulated a caspase-independent release of cytochrome c. This finding highlights the importance of understanding the concentration-dependent effects of Etop on cellular processes (Robertson et al., 2002).

Ganesan et al. (2022) reported sustained release of Etop from Etop-loaded hydroxyapatite, with damage to the cell membrane of S. aureus observed, indicating the potential of this delivery system for antibacterial activity (Ganesan et al., 2022).

Mehrabi et al. (2016) evaluated the efficacy of pegylated liposomal Etop nanoparticles on breast cancer cell lines, demonstrating higher antitumor activity compared to the free drug, indicating the potential of nanoparticle formulations in enhancing the therapeutic effects of Etop (Mehrabi et al., 2016).

Smith et al. (2019) achieved prolonged in vitro release of Etop and temozolomide from a thermo-responsive biodegradable paste, highlighting the potential of this paste for sustained drug release in the treatment of malignant glioma (Smith et al., 2019).

2.2.1. Biosynthesis of Etoposide

The synthesis of Etop involves several pathways and intermediates. One approach involves the reconstitution of the biosynthetic pathway to (-)-4'-desmethylepipodophyllotoxin, the Etop aglycone, using enzymes from mayapple in tobacco (Lau & Sattely, 2015). Additionally, Etop is semi-synthetically produced from (−)-podophyllotoxin extracted from the plant Podophyllum hexandrum (Schultz et al., 2019). The American mayapple (Podophyllum peltatum L.) is a rhizomatous herbaceous perennial found in wooded areas of eastern North America (Cushman et al., 2005). It is known to contain the pharmaceutical compound podophyllotoxin (Cushman et al., 2006). Podophyllotoxin is of particular interest due to its potential use in the semi-synthesis of anticancer drugs (Howes et al., 2020). While the Himalayan mayapple (Podophyllum hexandrum Royle) contains higher levels of podophyllotoxin, the American mayapple is also a source of this compound (Howes et al., 2020; Cushman & Maqbool, 2005). Research has shown that the American mayapple contains high levels of podophyllotoxin in its leaves (Cushman & Maqbool, 2005). Furthermore, there are at least six established synthetic routes to generate Etop (Liu et al., 2016). The major biological function of Etop is to inhibit

DNA synthesis, resulting in the accumulation of double-stranded DNA breaks (Yang et al., 2012).

In summary, the biosynthesis of Etop involves complex enzymatic pathways and metabolic processes. Understanding the biosynthetic route and the factors influencing Etop resistance is crucial for advancing its production and efficacy in cancer treatment.

2.2.2. Chemical Structure of Etoposide

Podophyllum peltatum Linnaeus, commonly known as American mandrake or May apple, and Podophyllum emodi Wallich, which are native to the Himalayan regions, are sources of podophyllotoxin, a naturally occurring compound. A project began in the early 1950s and was headed by Sandoz chemists. The basic idea behind it was that glycosidic derivatives ought to possess more favorable pharmacological characteristics than their corresponding aglycones (Hande and K. R., 1998). The glycosides of podophyllotoxin, which were primarily isolated from the roots of Podophyllum, and its subsequently synthesized derivatives have shown to be more water-soluble and less toxic than their non-glycosidic counterparts (Meresse et al., 2004). A semisynthetic glucosidic derivative of podophyllotoxin is called Etop. The chemical structure of Etop and podophyllotoxin is shown in Figure 2.2. Its structure differs from that of podophyllotoxin in that it has an epimeric configuration at ring C's C-4 position, an ethylidene glucoside attached at the C-1 position, and a hydroxyl group at C-4′ rather than a methoxy group. This hydroxyl group is linked to Etop's capacity to cause single-stranded DNA breaks, and the drug's incapacity to prevent microtubule assembly is linked to its ethylidene glucoside moiety, which may lessen podophyllotoxin's harmful effects (Mukherjee and P. K., 2019).

Molecular Formula $C_{29}H_{32}O_{13}$, Molar mass: 588.557 g/mol, CAS number: 33419-42-0, Melting point: 243.5 °C, Boiling point: 798.1 °C.

Etoposide **Podophyllotoxin**

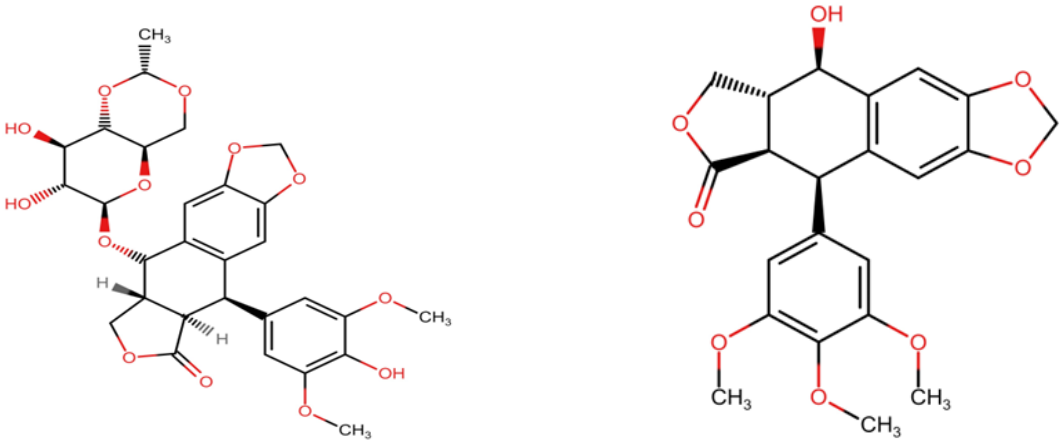

Figure 2.2. Chemical structures of Etoposide and Podophyllotoxin.

2.2.3. Clinical Trials with Etoposide

Especially in recent years, studies assessing the efficacy of Etop, a medication belonging to the topoisomerase poison class that is recognized as a potent anti tumor agent and one of the essential drugs by the WHO, on cancer cells through a variety of techniques have gained traction. Etop's low water solubility, low bioavailability, and limited neoplastic activity against different solid tumors are the main causes of this, though there are many more.

Harivardhan Reddy et al. (2005) aimed to synthesize glyceride lipid-loaded Etop nanoparticles and evaluate their drug release and in vitro steric stability properties. Homogenization, spray drying and emulsification processes were used to create nanoparticles. The results showed little increase in redispersibility and particle size. Particle size distribution was greatly influenced by experimental factors such as surfactant concentration, cycle number, and homogenization pressure. It is concluded that particle growth results from matrix-like structures surrounding the nanoparticles formed by spray drying the Poloxamer 407-stabilized nanodispersion. The in vitro steric stability testing results showed that Poloxamer 407-stabilized sodium tauroglycollate-stabilized lipid nanoparticles were more stable than the former. Spray-dried lipid nanoparticles demonstrated exceptional long-term stability at room temperature and 25°C. These stable lipid nanoparticles could be helpful as long-circulating vehicles for precise and regulated drug delivery when injected intravenously (Harivardhan Reddy et al., 2005).

Solano et al. (2013) made poly(ε-caprolactone) implants with topoisomerase II inhibitor and chemotherapeutic drug Etop by melting the material. The morphology, sterility, physical state of the drug, and content integrity of the implants were all described. When the drug release from the implants was evaluated in vivo and in vitro, it was found to have cytotoxic effects on HeLa cells. Good short-term tolerance to the implants was shown by the study's mice, suggesting that they could be used as a localized delivery system for Etop. The results of the study showed a significant relationship between the in vitro and in vivo drug release of the implants (Solano et al.,2013).

Snehalatha et al. (2008) carried out research on the Etop-loaded nanoparticle preparation process by employing emulsion solvent evaporation and nanoprecipitation methods. This study looked at how formulation factors affected drug content, zeta potential, particle size, and capture efficiency. The outcomes demonstrated that poly(ε-caprolactone) and polylactide-co-glycolic acid nanoparticles are appropriate vehicles for the controlled delivery of Etop, with high recovery rates and a good entrapment efficiency of about 80%. Up to 95% of the nanoparticles were recovered, and 1.5% of the drug was present. Nevertheless, it was also found that Etop release in vitro was decreased by raising the lactide content (Snehalatha et al., 2008).

Kovshova et al. (2023) developed co-delivery systems of Etop and paclitaxel prodrug using non-cross-linked HSA and poly (lactic-co-glycolic acid) (PLGA) nanoparticles. A wide range of techniques, such as DLS, TEM, SEM, AFM, HPLC, CZE, in vitro release, and cytotoxicity in human and mouse glioma cells, have been employed to characterize nanoformulations. Neuro2A cells seemed to be the most responsive to both PLGA- and HSA-based co-delivery systems. It is concluded that these nanodelivery systems may improve the treatment of brain tumors with combination chemotherapy (Kovshova et al., 2023).

2.3. Controlled Drug Release Systems

Biopolymer materials containing the active ingredient are systematically and regionally released into the predetermined rate and area within a specific time interval in what are known as controlled drug release systems. When an active ingredient is administered to a target organ or tissue at predetermined times and release rates, the drug release system is said to be controlled. To maintain the drug's plasma level at an effective dose, the drug must be taken often and repeatedly in the typical dosage forms—oral intake of tablets/capsules or injections—

which are commonly used in drug intake. The goal of controlled drug release systems is to deliver the medication to the intended location at the intended dose and in the intended amount of time (Jain and K.K, 2008). Therapeutic limits are maintained for the drug concentration in the blood thanks to controlled drug release systems. Drug effectiveness decreases at concentrations below the therapeutic limit value, even though the drug causes toxic side effects in the body at concentrations above the therapeutic limit value. Low dosage values can be used for treatment in controlled drug release systems, and the therapeutic limit value can be maintained for the desired amount of time (Liechty et al, 2010). Figure 2.3 is shown schematic representation of the comparison of the change in blood drug concentration over time for controlled release systems and conventional dosage forms. It has been noted that in traditional drug delivery systems, the drug first rises above the therapeutic limit value before falling below it. Because of these quick changes, patients in traditional drug delivery systems are only required to take the medication for brief periods of time (Tiwari et al., 2012). Although the controlled drug release system has many benefits, it also has drawbacks. Figure 2.4 is shown advantages of disadvantages of controlled release systems (Ummadi et al.,2013; Heng and P.W., 2018).

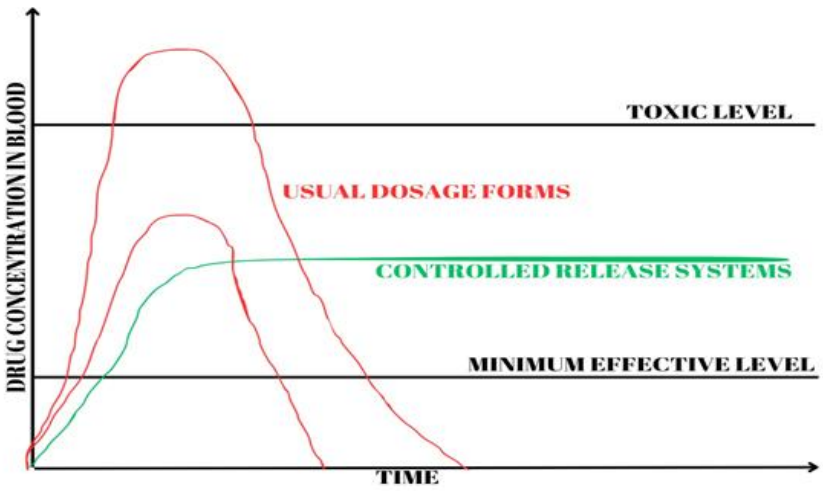

Figure 2.3. Schematic representation of the comparison of the change in blood drug concentration over time for controlled release systems and conventional dosage forms.

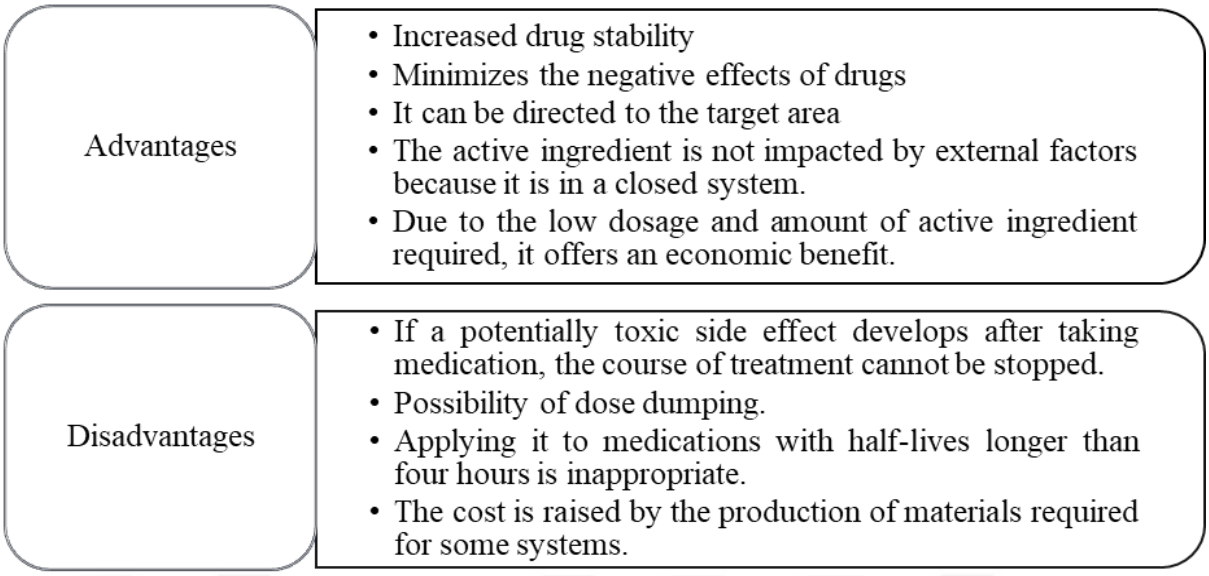

Figure 2.4. Advantages and disadvantages of controlled release systems.

2.3.1. Drug Release Kinetic

Drug release or the drug's release time can be regulated with the help of the release time design. A number of variables, including the drug's distribution within the matrix and the ratio of the drug to the carrier substance, influence the kinetics of drug release. Mathematical modeling is used to investigate drug release mechanisms and track release rates. Drug release and in vivo outcomes can be impacted by both qualitative and quantitative formulation modifications. Therefore, in order to forecast *In vivo* bioperformance, controlled-release formulations are necessary (Dash et el.,2010). To ensure optimization of drug release kinetics, to design new release systems considering general release systems, to estimate parameter effects for specific parameters in the system, to predict profiles designed for the drug and to improve the effectiveness and safety of these profiles, to determine the drug release rate profile and drug release in the polymer. Predicting diffusion behavior is among the goals of mathematical modeling (Shaikh et al.,2015).

2.3.1.1. Zero Order Model

For dosages where the drug is released gradually, the dissolution of the drug is expressed using a zero-order kinetic model. The drug release rate in the zero-order kinetic model happens

regardless of concentration (Dash et al.,2010). Zero order release kinetics can be expressed mathematically as;

$$Q_t = Q_0 + K_0 t \quad (2.1)$$

where Q_t is the cumulative amount of drug released at time t, Q_0 is the cumulative amount of drug released at time t=0 (most times, $Q_0 = 0$) and K_0 is the zero order release constant.

2.3.1.2. First-Order Model

Drugs that are water soluble and non-swellable have their release mechanism explained by the first-order kinetic model. The first-order kinetic model predicts that drug release is concentration-dependent (Azadi et al.,2017). It is expressed mathematically as;

$$\log C = \log C_0 - Kt / 2.303 \quad (2.2)$$

where C_0 is the initial concentration of drug, k is the first order rate constant, and t is the time.

2.3.1.3. Higuchi Model

Higuchi found out possibly the most used mathematical equation in 1961.

The underlying assumptions of this model are as follows:

- The systemic drug concentration at initialization is higher than the drug solubility.
- Diffusion of drugs occurs in a single dimension.
- It is possible to overlook matrix dissolution and swelling.
- The oscillatory environment achieves perfect collapse conditions (Kashani et al, 2021).

This release kinetics is expressed as;

$$Q_t = Q_0 + K_H t^{½} \quad (2.3)$$

where Q_t is the cumulative amount of drug released at time t, Q_0 is the cumulative amount of drug released at time t=0 (most times, $Q_0 = 0$) and K_H is the release constant of Higuchi.

2.3.1.4. Hixson-Crowell Model

This model is used for systems where there is a change in surface area and diameter during oscillation (Singhvi et al.,2011).

This release kinetics is expressed as;

$$Q_0^{1/3} = Q_t^{1/3} + K_{HC}t \quad (2.4)$$

where Q_t is the cumulative amount of drug released at time t, Q_0 is the cumulative amount of drug released at time t=0 (most times, $Q_0 = 0$) and K_{HC} is the release constant of Hixson-Crowell.

2.3.1.5. Korsmeyer-Peppas Model

This model depends on the drug release exponential. A graph is created against time using M_t/M_∞ values obtained from in-vitro release experiments. This equation is used to evaluate the controlled release of polymeric systems with different geometric shapes (Dash et al.,2010).

This release kinetics is expressed as;

$$\log (M_t/M_\infty) = \log k + n \log t \quad (2.5)$$

where Mt is the amount of active substance released at time t. M_∞ is the amount of active substance at the equilibrium state, k is the kinetic constant expressing the structural and geometric properties of the release system, n is the diffusional constant showing the release mechanism.

2.4. Types of Albumin

Albumin, a vital protein in the human body, plays a crucial role in various physiological processes. It is involved in maintaining oncotic pressure, transporting essential molecules, and regulating pH balance (Dobrinskikh et al., 2015). The scientific community has focused a lot of attention on albumin because of its many diverse functions. The blood contains 30–40% of the body's total albumin. They act as physiological carriers for species such as bilirubin, fatty acids, and bile salts. Maintaining the proper balance of water in tissue and blood fluids is its primary duty. Large proteins counteract the propensity of blood fluids to leak because they are

unable to flow through capillaries. Therefore, albumin is the primary protein that regulates colloid osmotic pressure, which causes water and water-soluble substances to pass through the capillaries into the tissues. Albumin is a crucial protein with various roles in the human body, including maintaining colloidal osmotic pressure, transporting molecules, and serving as an antioxidant and signaling molecule (Belinskaia et al., 2021). It has been extensively studied in the context of liver diseases, such as cirrhosis, where impaired albumin function has been identified as a potential indicator of liver damage (Sun et al., 2019). Because albumin is a component of blood, it can be used as a drug delivery system or as a stand-alone therapeutic agent (Hoogenboezem, et al.,2018). It can also be used as a diagnostic tool to help identify diseases like tuberculosis and AIDS (Langer et al.,2003). Researchers continue to extensively study the drug-albumin interaction, despite the fact that its therapeutic significance is generally debatable. A patient's clinical exposure to a drug will not be impacted by changes in plasma protein binding because individual plasma protein binding may be affected (Heuberger et al., 2013). Due to a number of factors that cause albumin to accumulate preferentially in tumor cells, albumin has been the subject of extensive research in cancer therapy (Kratz and F., 2010). There are numerous uses for albumin, particularly in basic and clinical research. Albumins are commercially obtained in large quantities from soy, milk, and grains as well as from egg white (ovalbumin), bovine serum (bovine serum albumin, BSA), and human serum (human serum albumin, HSA) (Elzoghby et al., 2012).

2.4.1. Ovalbumin (OVA)

The most prevalent protein in egg whites, ovalbumin makes up 54-69% of the total protein content. (Liu et al., 2018). Using has several advantages, such as being inexpensive, having the capacity to form gel networks, and stabilizing foams and emulsions. Its molecular weight is 47,000 Da, its isoelectric point (pI) is 4.8, and over half of its amino acid composition is hydrophobic (Tolstoguzov et al., 1997). OVA, the common form of globulin, is the only protein that has free sulfhydryl groups embedded in the hydrophobic egg white core. The presence of disulfide bonds and sulfhydryl groups in the ovalbumin structure has a significant impact on the aggregation structure of ovalbumin. OVA is widely used in research related to protein structure and properties, serpin structure and functions, molecular weight determinant for electrophoresis gel calibration, and eliciting allergic reactions in test subjects. This is because ovalbumin is present in large quantities in these application areas. The primary functional characteristics of ovalbumin include its ability to emulsify, foam, retain water, and form films

(Li et al. 2019). It can improve food taste and texture, as well as increase product stability and shelf life. (Tang et al. 2019). OVA complex nanoparticles exhibit good encapsulation efficiency (Meng et al., 2022).

2.4.2. Bovine Serum Albumin (BSA)

The molecular weight of BSA is 69,323 Da, and its isoelectric point (pI) is 4,7. (Elzoghby et al., 2012). There are several reasons to use, including its abundance, low cost, strong cell penetration ability, and possible anticancer activity. It is preferred in biochemical experiments because of its medical significance. It is widely used in drug delivery systems (Meng et al., 2022). It is well known that albumins have high concentrations of cysteine, the charged amino acids aspartic and glutamic acid, lysine, and arginine, and low concentrations of tryptophan and methionine. Glycine and isoleucine content in BSA is lower than in other proteins. BSA has been used for its stability, lack of potency, and low cost since it can be spiked in large quantities from cattle blood as a byproduct of the cattle industry. The full-length BSA protein is 607 amino acids long. The starting protein product contains 589 acids. By comparing the amount of known BSA with the amount of unknown protein, BSA is frequently used to quantify other proteins. Furthermore, during the digestion of DNA, BSA is used to stabilize certain enzymes to stop them from precipitating into the reaction tube and certain channels. Other enzymes that are not required for stabilization are unaffected by this protein (Battal Y.,2006)

2.4.3. Human Serum Albumin (HSA)

HSA constitutes half of the proteins that are synthesized by the liver in approximately 10-12 g every day and are found in high amounts in human serum (Lee and Wu, 2015). It is composed of 585 residues of amino acids and has a 66,500 Da relative molecular weight. HSA is a biocompatible substance derived from human plasma. The high biological stability and noncytotoxic properties are severel reasons of using. Also, it has more drug binding sites. Due to all these features, its use in medicine and biochemistry is very common (Meng et al., 2022). It is not a standardized protein due to its stability in the pH range between 4 and 9, its ability to be exposed to heat at 60 °C for up to 10 hours, and its ability to dissolve in organic solvents. (Elzoghby et al., 2012). While the outer part of its structure is cylinder-like and polar, the middle part is non-polar. It allows the binding of hydrophobic substrates (Wang et al., 2013) the HSA molecule has a superior ligand-binding capacity, it can bind neutral and weakly acidic

metabolites, fatty acids and steroids, etc. It is an important carrier protein for many endogenous and exogenous compounds. (Heli et al., 2007). A healthy person has between 30 and 50 g/L of HSA (Rabbani et al., 2019). Figure 2.5 shows the 3D structure of albumins (PDB ID: 1AO6, Sugio et al.,1999; PDB ID: 3V03, Majorek et al., 2012; PDB ID: 1OVA, Stein et al.,1991)

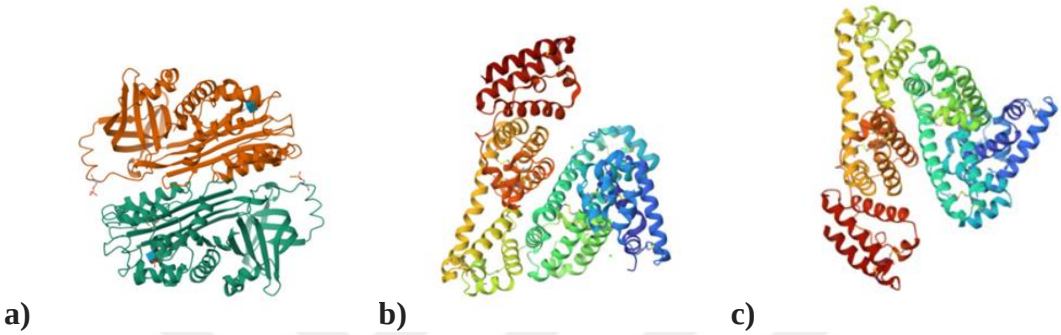

Figure 2.5. The 3D structure of albumins: (**a**) human serum albumin; (**b**) bovine serum albumin and (**c**) ovalbumin (PDB ID: 1AO6, Sugio et al.,1999; PDB ID: 3V03, Majorek et al., 2012; PDB ID: 1OVA, Stein et al.,1991).

2.5. Protein Based Nanoparticles

The words "nanos" (small) and "nanoparticle" (small particle) are the roots of the word "nano" (Kumar et al., 2014). Using natural or synthetic polymers or solid oils, nanoparticles are solid-colloidal particles with distinct physicochemical properties. They range in size from 10-1000 nm (usually 50-300 nm). The active component may be bonded to the system surface or trapped inside the nanoparticle system. Nanoparticles can be used to transport hydrophilic-hydrophobic active substances, vaccines, and biological macromolecules (Tabatabaei Mirakabad et al.,2014).

Animal proteins like albumin and gelatin, or vegetable proteins like gliadin, zein, and legumin, can be used as raw materials to create protein nanoparticles (Chakraborty et al., 2013). The majority of antitumor medications are hydrophobic and have poor targeting and side effects. Antitumor medication usage is restricted as a result of these factors. Therefore, it is imperative to create novel therapeutic strategies that enhance drug solubility, enhance targeting, and minimize side effects. Protein-based nanoparticles are a promising drug delivery system

because of their biodegradability, biocompatibility, and ease of bodily elimination. Protein-based nanoparticles have the ability to shield drug molecules from degradation and proteolysis (Maham et al., 2009). Protein-based nanoparticles can be used to deliver small molecule proteins, nucleic acids, peptides, and natural or chemical drugs. Particles of the right size can be enriched in tumor tissue, improve a drug's bioavailability, and lessen the toxicity and adverse effects of free drugs. An example of a protein-based nanoparticle that is frequently used to target tumors is albumin nanoparticles, which work by stimulating receptor-mediated pathways or EPR effect (Meng et al.,2022). In drug carrier systems, protein-based nanoparticles have so far been frequently made from bovine serum albumin, gelatin and human serum albumin due to their nontoxicity, stability and containing functional groups (Kundu et al., 2010).

2.6. Albumin Nanoparticles

A common ingredient in the creation of nanoparticles like nanospheres and nanocapsules is albumin. Albumin can be used to create nanoparticles with a size range of 50–300 nm. Natural protease digestion results in the release of drugs from albumin nanoparticles (Lohcharoenkal et al., 2014; Elzoghby et al., 2012). Because albumin nanoparticles are easily prepared, reproducible, and biodegradable, they offer numerous benefits. Because different drugs bind to proteins very well, these compounds can be effectively incorporated into the albumin nanoparticle matrix. Usually, to conjugate ligands to the surface of albumin nanoparticles, covalent bonds are formed between the ligands and functional groups on the albumin surface. (Elzoghby et al., 2012). When albumin and ligand are combined, the protein acts as a biodegradable drug delivery vehicle. Ligands are used to extend the half-life of circulation (e.g., cationic polymers), improve nanosystem stability, and alter pharmacokinetic parameters (e.g., surfactants). Based on clinical studies using exclusive HSA-based particle formulations like AbraxaneTM and AlbunexTM, they should be well tolerated (Geny et al.,1993). Furthermore, albumin nanoparticles—particularly HSA—seem like a good fit as a gene therapy agent. It can stop the unwanted serum interactions that frequently happen after transfection complexes are injected intravenously (Elzoghby et al., 2012). Anticancer medications are commonly delivered using protein nanoparticles. Albumin nanoparticles have recently been the focus of a lot of research regarding site-specific delivery of anti-tumor drugs in the treatment of cancer because albumin is one of them and tends to accumulate in solid tumors (Sebak et al., 2010).

2.6.1. Albumin Nanoparticle Preparation Methods

Different physical and chemical techniques can be used to create albumin nanoparticles. Figure 2.6 shows the albümin nanoparticle preparation methods. Each technique has its own advantages and disadvantages (Table 2.1) (Hong et al.,2020).

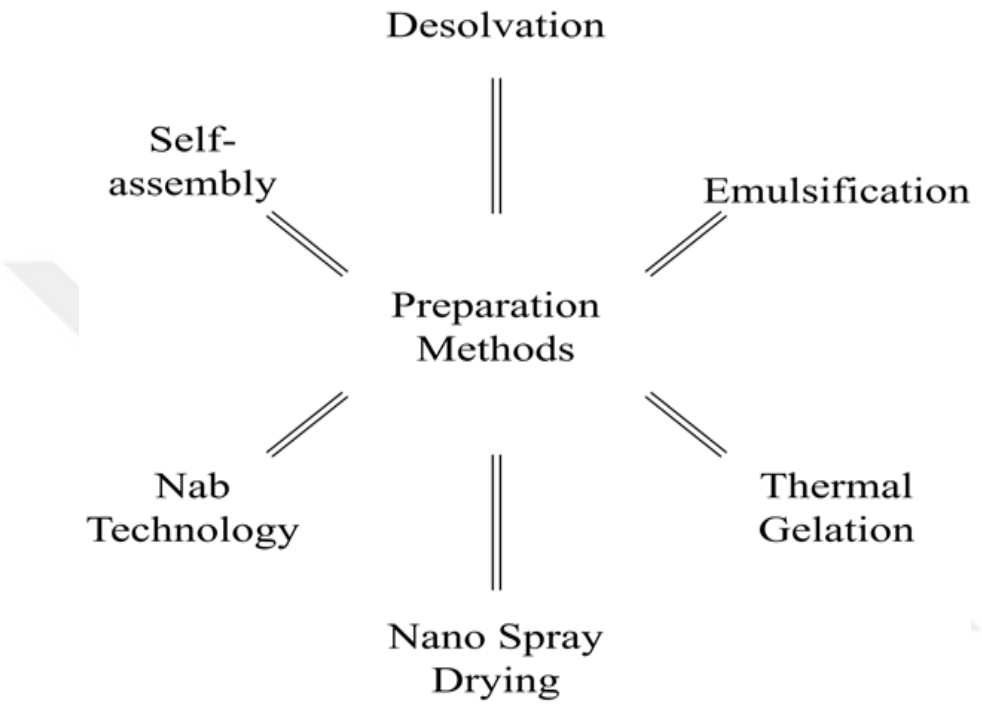

Figure 2.6. Albumin Nanoparticle Preparation Methods.

Table 2.1 The advantages and disadvantages of preparation techniques.

Advantages	**Disadvantages**
Emulsification	
A high degree of steadiness Reaction circumstances can determine the size and shape of nanoparticles. Increased encapsulation effectiveness	Producing particles that are bigger than those that come from desolvation Unstable thermodynamic states: Require stabilizers and surfactants
Desolvation	

A high degree of steadiness Easy to produce, tiny nanoparticles, increased encapsulation effectiveness Reaction circumstances can determine the size and shape of nanoparticles.	Only feasible for proteins that the transporter dilutes or that are only slightly impacted by the de-soluble process
Thermal Gelation	
Simple to use, doesn't require the addition of crosslinking agents, and produces stable goods	Unsuitable for drug proteins that are heat-sensitive
Self-assembly	
Elevated encapsulation effectiveness, tiny nanoparticles, a high degree of stability	Nanoparticles are difficult to control in terms of size and shape
Nano Spray Drying	
Regulating the morphology, size, and shape of particles Semi-continuous one-step procedure Handling heat-sensitive materials with minimal chance of deterioration Economical	Restricted to modest production A difficult task to include hydrophobic medications
NAB Technology	
Reducing the use of surfactants in a straightforward and secure manner	Needing to incorporate hazardous solvents like dichloromethane and chloroform

2.6.1.1 Desolvation

Utilizing a dehydrating agent like ethanol, the desolvation method reveals the albumin's hydrophobic region and induces phase separation as a result of the protein's decreased water solubility. Those that are not sufficiently stabilized dissolve once more when water is added to them (Figure 2.7). It is possible to create stable albumin nanoparticles by chemically cross-linking. Utilizing glutaraldehyde as a crosslinking agent is common. The desolvation method's key benefits include a straightforward preparation process, repeatability, a quick reaction, and the absence of a surfactant requirement. It is the technique most frequently employed to create albumin nanoparticles (Elzoghby et al.,2012). On the other hand, because cross-linking agents are used, some toxic side effects and a relatively long duration are among its drawbacks, as is its low efficiency (Hornok and V.,2021). Desolvation-produced nanoparticles have been extensively studied for use in antitumor applications, particularly in recent years (Meng et al, 2022).

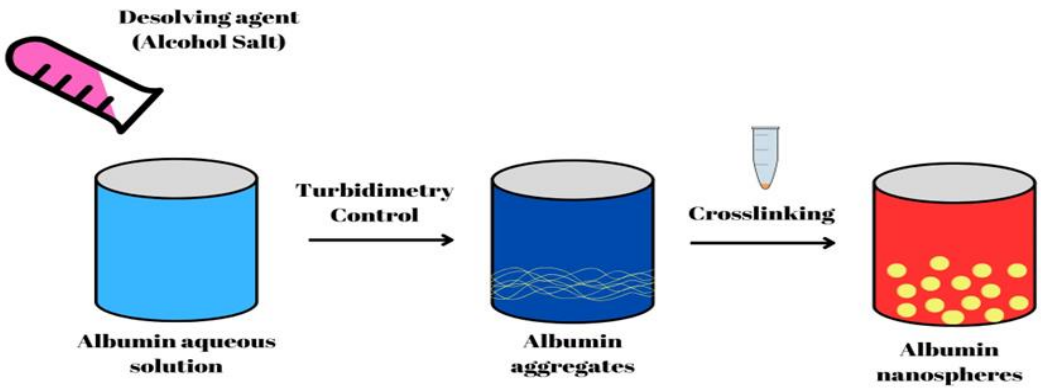

Figure 2.7. Schematic representation of desolvation method.

2.6.1.2. Emulsification

The process of emulsification is frequently employed in the creation of polymeric nanoparticles (Patil and G.V., 2003). The aqueous protein solution is emulsified in a vegetable oil (cotton, castor, etc.) using an ultrasonicator or high-pressure homogenization in the emulsification method. As stabilizers, surfactants are added to produce nanoparticles. Albumin nanospheres

produced by emulsification can be stabilized using either thermal or chemical treatment. An organic solvent is then used to extract the oil phase, resulting in the creation of nanosized proteins. The albumin is denatured and the water quickly evaporates when heated oil is added drop by drop. Finally, after the organic agents are eliminated, albumin nanoparticles are produced. Particle size in this method is influenced by the water-to-oil ratio (w/o) and protein concentration (Tarhini et al., 2017). The size of the nanoparticles increases with an increase in phase volume ratio and protein concentration. For the products to be used safely, the oil phase and organic solvent must be removed from the nanoparticles made using the desolvation and emulsion method (Lohcharoenkal et al., 2014; Elzoghby et al., 2012). The emulsification method's advantages include its relatively simple experimental procedure and conditions. Additionally, albumin nanoparticles may degrade due to high pressure and the ultrasonic effect. Furthermore, the efficiency of this method for encapsulating hydrophilic drugs is low (Quintanar-Guerrero et al.,2012; Sundar et al.,2010).

2.6.1.3. Thermal Gelation

The continuous process known as thermal gelation includes protein-protein interactions such as electrostatic, hydrophobic, hydrogen bonding, and thermally induced unfolding. The benefits of this preparation method include good product stability, ease of handling, and the absence of the need for cross-linking agents (Elzoghby et al., 2012; Meng et al, 2022). For medications that are heat-sensitive, it is not appropriate. Because of their stability, albumin nanoparticles made using the thermal gelation method typically have good mechanical properties. (Hughes et al., 2021).

2.6.1.4. Nano Spray Drying

In the pharmaceutical industry, spray drying is a popular technique for turning liquid materials into powder. This technique, in contrast to conventional spray dryers, forms tiny droplets using vibrating mesh technology (Lee et al., 2011). Additionally, electrostatic precipitation is used, which is different from common technology in that particles smaller than 2 μm are usually not captured. In the nanospray dryer, the collection mechanism is independent of particle mass. Because of the high heat during the drying process, there is a chance that the albumin used in this method will be denatured and inactivated. The ease of production, quick drying times, and even product distribution are some benefits of the Nano Spray Drying method. Dairy products,

detergents, medicine, and other industries all use this technique extensively. This method typically yields albumin nanoparticles with a high drug load (Elzoghby et al., 2012; Meng et al, 2022).

2.6.1.5. Nanoparticle Albumin Bound Technology

Utilizing a novel albumin-based nanoparticle technique called nanoparticle albumin bound (NAB) technology, American Bioscience has created nanoparticles that are capable of encasing lipophilic drugs. This technology's application procedure calls for mixing the medication with HSA in an aqueous solvent first. After that, it is subjected to high pressure to create albumin nanoparticles, which have a diameter of between 100 and 200 nm (Cortes et al., 2010). This method's use of toxic solvents like dichloromethane and chloroform is one of its drawbacks, but it also eliminates the need for the toxic glutaraldehyde material because surfactants are not needed. This approach is safe and reasonably easy to use (Elzoghby et al., 2012; Meng et al, 2022). Abraxane (NAB-PTX), prepared with this technology, is the first FDA-approved chemotherapeutic used in 130 nm diameter and metastatic breast cancer (Fu et al., 2009).

2.6.1.6. Self-Assembly

A number of techniques, including increasing the hydrophobicity of albumin molecules, lowering internal disulfide bonds, lowering primary amine groups on the protein surface, heating the process, and adding denaturants, can cause albumin to self-assembly. The hydrophobic domains of albumin are contacted by drug molecules, which facilitate albumin's self-assembly into nanoparticles. This method produces albumin nanoparticles that have active targeting properties because they can better protect the functions of the protein (Meng et al, 2022). One of the advantages of this method is that no inducer is added. However, one of its disadvantages is that chemical modification may be required for self-formation strength (Lin et al., 2016; Böker et al., 2007).

2.7. Nanoparticles in Drug Release Systems

Nanoparticles have gained significant attention in drug delivery systems due to their potential to enhance drug stability, control drug release, and improve drug adherence to target tissues (Hidayah et al., 2018). These nanoparticles offer several advantages, including increased drug residence time, protection of drugs against harsh environments, and sustained and regulated

drug administration (Al-Kassas et al., 2016; Kondiah et al., 2022; Gelperina et al., 2005; Swapna et al., 2019). They have been shown to improve the bioavailability and tissue localization of drugs compared to traditional means of administration, making them a promising platform for targeted controlled release (Vallorz et al., 2022; Chung et al., 2020). Furthermore, nanoparticles can be designed to allow controlled (sustained) drug release from the matrix, providing a versatile approach for drug delivery (Gelperina et al., 2005; Hanasaki et al., 2018).

There are four main factors affecting drug release from nanoparticles: particle size, particle surface feature, drug loading and drug release.

Studies have shown that smaller particle sizes lead to increased dissolution rates and improved bioavailability of poorly water-soluble drugs (Zhang et al., 2009). Additionally, the size distribution of nanoparticles can impact drug encapsulation efficiency and release kinetics (López-Gasco et al., 2011). For instance, a study on paclitaxel-loaded polyester nanoparticles demonstrated that size distribution affected drug encapsulation efficiency and release behavior (López-Gasco et al., 2011).

In order to achieve a high drug loading capacity, a nanodelivery system needs to use less matrix materials in its application. Drug loading can be accomplished in two ways: by incubating the nanoparticles with a therapeutic agent solution, therapeutic agents can be incorporated during the nanoparticle formation process or adsorbed after the nanoparticles are synthesized. The molecular weight and composition of the polymer, the interaction between the drug and the polymer, and the functional groups (carboxyl or ester) of the polymer all have a direct bearing on drug loading efficiency. Many factors, such as the drug's solubility, drug diffusion through the nanoparticle matrix, desorption of surface-bound or adsorbed drug, erosion or degradation of the nanoparticle matrix, and the interaction of erosion and diffusion processes, affect the drug's release rate. Therefore, the solubility, diffusion, and biodegradation of the particle matrix dictate the release process (Sing et al. 2009).

Lomis et al. (2016) focused on creating stable HSA nanoparticles (HSA-NPs) loaded with paclitaxel (PTX), an anti-cancer medication, for use in cancer treatment. Particle size, surface charge, drug loading, and in vitro release kinetics were determined to be important variables. 82% of PTX-HSA-NPs were encapsulated and 93% yield was achieved. The team also performed in vitro drug release and cytotoxicity analysis using a human breast cancer cell line,

demonstrating dose-dependent toxicity. This study showed that there were 52%, 39.3%, and 22.6% dose-related toxicity as PTX concentrations increased (Lomis et al.,2016).

Quan et al. (2011) created an iron oxide nanoparticle (HINP) formula coated in HSA and confirmed that it targets tumors. Doxorubicin (Dox) has been applied to HINPs to evaluate their potential as theranostic agents. They discovered that 10 mg of HSA matrix can hold roughly 0.5 mg of Dox and 1 mg of iron oxide nanoparticles, which can be loaded to create 50 nm hydrodynamically-sized D-HINPs (Dox-loaded HINPs). These results suggest that Dox can be continuously released by HINPs and that Dox can assist Dox in passing through cell membranes and building up in the nucleus. D-HINPs were similar to Doxil in a subsequent treatment study and concluded to have better tumor suppression efficacy than free Dox. Lastly, it has been found that this approach is applicable to other small molecules, indicating that HINP is a potentially useful theranostic nanoplatform (Quan et al.,2011).

Mehravar et al. (2009) intended to focus on two factors in order to describe the HSA desolvation process for nanoparticle preparation. These are pH value and glutaraldehyde content. The largest size reached, 388 nm, was found to be suitable for drug application, while the smallest size was found to be 91 nm. It has been observed that particle size is mostly determined by the pH value of the HSA solution and is little affected by the amount of cross-linker. The nanoparticle sample was purified after centrifugation and redispersion in 10 mM NaCl and pH values of 7.5–9. Then, it was examined using particle size analyzer (PCS) (Mehravar et al.,2009).

Sebak et al. (2010) reported on the integration and delivery of HSA nanoparticles containing the drug noscapine into tumor cells. The goal of nanoparticle design and optimization was to achieve 85%–96% drug loading efficiency at a particle size in the range of 150–300 nm. The anticancer activity and effectiveness of nanoparticles on breast cancer cells were evaluated in vitro. This study demonstrated that the coacervation process can be used to form HSA nanoparticles, which can then be used to successfully encapsulate noscapine. The pH of the preparation appeared to affect size and particle production but had no effect on drug loading capacity. According to the findings of the study, it was concluded that noscapine-loaded HSA nanoparticles can be used to provide the highest possible noscapine dosage to target areas (Sebak et eal., 2010).

Langer et al. (2003) created a desolvation process for HSA-based nanoparticles with a narrow size distribution and a controllable particle size between 100 and 300 nm. Evaluations were conducted on variables such protein content, pH level, ionic composition, solvent rate, and particle purification conditions. The pH of the HSA solution was discovered to be the main factor influencing particle size. Higher pH values resulted in the production of smaller nanoparticles. Differential centrifugation was used to reduce the size distributions. The reproducibility of the particle size and distribution was demonstrated by sedimentation rate measurement, and the cellular uptake of the nanoparticles was examined using confocal microscopy imaging and Fluorescence-Activated Cell Sorting (FACS) analysis. The stability of the nanoparticles was evaluated using Ph (Langer et al,2003).

Abbasi et al. (2012) created Dox-loaded polyethyleneimine (PEI-)-enhanced HSA nanoparticles to prevent the side effects of Doxorubicin, a drug used against breast cancer. The surface zeta potential of the nanoparticles was −+15 mV and the size was −137 nm. Blank PEI-reinforced HSA nanoparticles showed no signs of cytotoxicity, indicating their safety and biocompatibility. Under ideal transfection conditions, about 80% of the cells were transfected with HSA nanoparticles containing bovine serum albumin conjugated with tetramethylrhodamine. This study's results showed that PEI-reinforced HSA nanoparticles are effective anticancer medication carriers (Abbasi et al.,2012).

Chung et al. (2020) produced a paclitaxel targeting-enhancing nanoparticle (TENPA). Through non-covalent binding with the human serum albumin-hemin complex, they were able to encapsulate paclitaxel. The nanoparticle did not form protein coronas in the blood and retained its structural integrity and stability. Its average diameter was 140 nm, and its zeta potential was +29 mV for optimal passive targeting. Improved TENPA cancer targeting resulted in decreased systemic toxicity and total eradication of end-stage cancer in a xenografted mouse experiment (Chung et al.,2020).

Kamali et al. (2015) was aimed to create an imatinib base (IMTb) at the nanoscale and examine how well it loaded into HSA nanoparticles. IMTb-loaded HSA with an 80–90 nm average size was created using the desolvation method. IMTb's chemical structure was unaffected by non-covalent interactions with HSA, as demonstrated by Fourier transform infrared spectroscopy (FT-IR). HSA nanoparticles containing IMTb were shown to have an amorphous form by differential scanning calorimetry (DSC) analysis. It was discovered that the drug loading

capacity and encapsulation efficiency were 6.9% and 98%, respectively. In U87MG glioblastoma cells, IMTb-loaded HSA and free IMTb exhibited 90% and 55% cytotoxic effects, respectively. These results suggest that IMTb-loaded HSA nanoparticles could be a promising drug delivery option for the treatment of glioblastoma.

2.8. Molecular Imprinting Technology

Molecular imprinting technology (MIT) is a versatile and promising technique for creating molecularly imprinted polymers (MIPs) with tailor-made binding sites complementary to template molecules in shape, size, and functional groups (Chen et al., 2016). MIT allows for the design of artificial receptors with predetermined selectivity and specificity for a given analyte, making it ideal for various application fields (Vasapollo et al., 2011). These molecularly imprinted polymers possess antibody-like recognition characteristics, providing specific identification abilities (Refaat et al., 2019). MIT has been extensively practiced in various domains, producing molecular recognition materials with specific recognition capabilities (Akgönüllü et al., 2023). The technology has established itself within the broader scientific community, offering a generic, robust, and cost-effective alternative to existing techniques such as monoclonal antibodies (Whitcombe et al., 2014). Figure 2.8 shows the molecular imprinting technique.

MIT has been widely used in different areas due to its smart and unique properties of application universality, recognition specificity, and structure predictability (Akgönüllü et al., 2022). It is a versatile technology for practical applications in many areas, particularly in chemical sensors (Tang et al., 2017). Molecular imprinting technology is a versatile and promising technique with diverse applications, ranging from chemical sensors to biosensors and recognition of small molecules. Its unique properties of application universality, recognition specificity, and structure predictability make it an attractive option for various fields of research and industry.

Molecularly Imprinted Polymers (MIPs) have gained attention for their potential in drug delivery systems due to their selective binding characteristics, offering a promising approach for controlled release drug dosage forms (Puoci et al., 2007). These polymers have been designed to exhibit combined properties of molecular recognition and controlled release, making them suitable for drug delivery applications (Kan et al., 2009). The synthesis of MIPs

involves multiple-step polymerization methods, which have been explored for the remediation of pharmaceuticals from contaminated water (Zare et al., 2022).

In specific drug delivery applications, MIPs have been synthesized for the controlled release of various drugs, such as 5-fluorouracil (Puoci et al., 2007), aspirin (Kan et al., 2009), trinitroglycerin (Mohebali et al., 2016), and oxycodone (Behnia et al., 2022). These studies highlight the versatility of MIPs in accommodating different drug molecules for controlled release. Additionally, the development of reduction-responsive MIPs has been reported for the controlled release of anticancer agents, indicating the potential of MIPs in targeted drug delivery systems (Cegłowski et al., 2020).

The diverse applications of MIPs in drug delivery systems, selective separation, and targeted drug delivery demonstrate their potential in various fields, ranging from pharmaceuticals to environmental remediation and food safety.

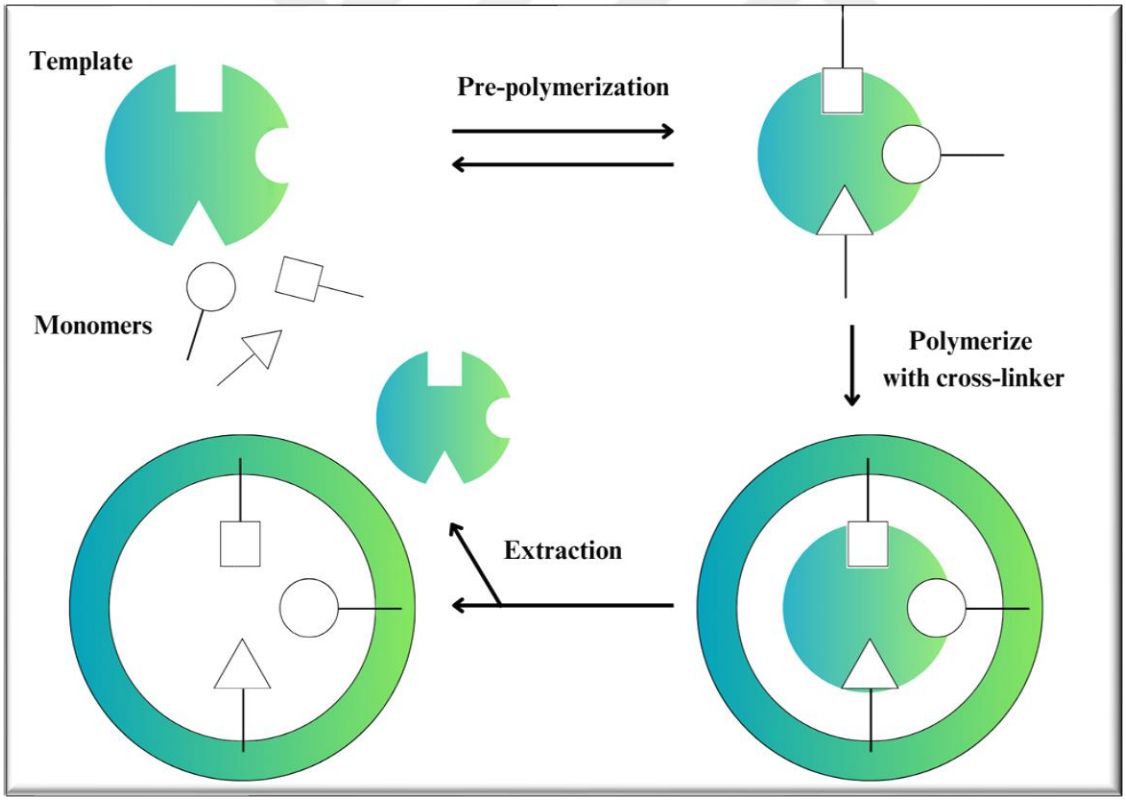

Figure 2.8. Representative illustration of molecular imprinting technique.

2.8.1. Covalent Molecular Imprinting

Covalent molecular imprinting is a technique used to create molecularly imprinted polymers (MIPs) with specific recognition sites for target molecules. This process involves the covalent binding of functional monomers to the template molecules, resulting in the formation of stable and selective binding sites within the polymer matrix (Chen et al., 2011). The covalent protein imprinting process often utilizes boronic acid derivatized functional monomers, which are limited to glycoproteins (Kalecki et al., 2020). Additionally, covalent molecular imprinting involves the covalent conjugation of functional monomers to the template molecules, such as 4-vinylphenylboronic acid and 4-vinylbenzylamine, to form stable interactions (Matsumoto et al., 2019). Furthermore, covalent molecular imprinting can be achieved through site-specific post-imprinting modification of MIP nanocavities with modifiable functional monomers, enhancing the selectivity of the imprinted polymers (Haupt & Mosbach, 2000).

In summary, Covalent molecular imprinting involves the covalent binding of functional monomers to template molecules, resulting in the formation of stable and selective binding sites within the polymer matrix. This approach offers advantages in terms of stability and selectivity, making it a valuable technique for the development of molecularly imprinted polymers.

2.8.2. Non-Covalent Molecular Imprinting

Non-covalent molecular imprinting is a technique that offers faster equilibrium kinetics compared to the covalent approach (Vasapollo et al., 2011). It relies on the ability of the template molecule to produce strong intermolecular non-covalent interactions with the functional monomers, such as H-bonding, electrostatic, or π-interactions (Lee et al., 2009). This method has been widely adopted due to its flexibility in the choice of functional monomers and template molecules (Manickam et al., 2017). However, it has been noted that non-specific binding of off-target molecules can occur in non-covalent molecular imprinting, leading to the presence of low-affinity imprinted cavities and erroneous functional monomer residues outside of the binding cavities (Matsumoto et al., 2019; Sunayama et al., 2014). Despite these challenges, non-covalent molecular imprinting has been successfully applied in various fields, such as the recognition and absorption of specific molecules like iodixanols (Liu et al., 2008), the creation of affinity sites for antibacterial drugs (Sreenivasan, 2005), and the chiral recognition and separation of β2-amino acids (Shim et al., 2004).

Both covalent and non-covalent molecular imprinting approaches have their advantages and limitations. While non-covalent imprinting offers faster equilibrium kinetics and flexibility in functional monomer and template molecule selection, it may lead to non-specific binding. On the other hand, covalent imprinting provides a more controlled and specific binding approach but may have limitations in terms of rebinding from non-aqueous media. The choice between these approaches depends on the specific requirements of the intended application. Figure 2.9 shows the non-covalent and covalent molecular imprinting procedures.

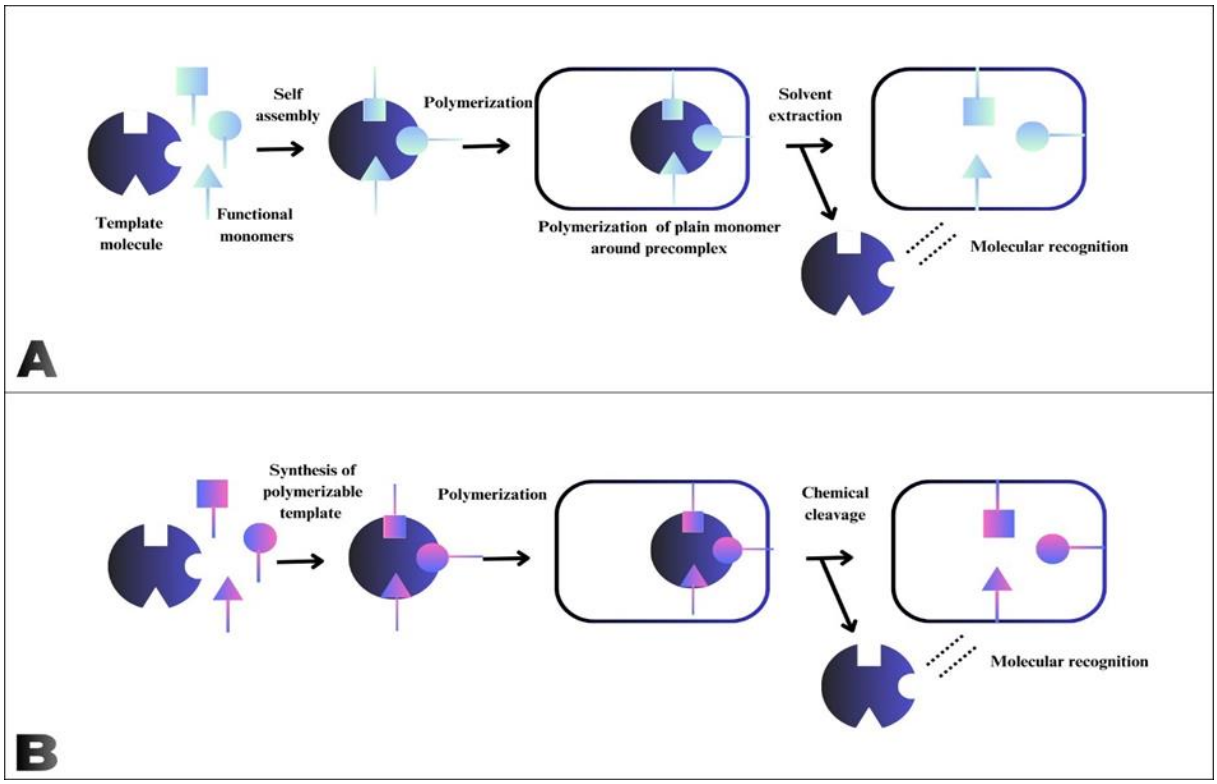

Figure 2.9. Molecular Imprinting Approaches. Schematic representation of A) non-covalent, and B) covalent molecular imprinting procedures.

2.8.3. Molecular Imprinting Synthesis Conditions

The preparation of molecularly imprinted polymers (MIPs) involves several key components, including monomers, template molecules, cross-linkers, solvents, and initiators. These components are essential for the successful synthesis of MIPs, which are synthetic polymers with specific recognition sites for target molecules. The process of molecular imprinting begins with the assembly of functional monomers around the template molecules, followed by the formation of a polymer with a composite network and specific spatial structure through

polymerization. The template molecules are then removed, leaving behind cavities with chemical properties complementary to the target molecules (Gong et al., 2004; Yoshida et al., 1999; Chronakis et al., 2005; Kempe et al., 1993).

Functional monomers play a crucial role in the molecular imprinting process by interacting with the template molecules to form specific binding sites within the polymer matrix. The choice of functional monomers is essential for achieving high binding affinity and selectivity towards the target molecules. The interaction between the functional monomers and the template molecules can be either covalent or non-covalent, and it is the basis for the subsequent recognition sites in the resultant polymers (Inan et al., 2021; Muhammad et al., 2012; Kempe et al., 1993). Methacrylic acid (MAA), acrylic acid (AA), 2- or 4-vinylpyridine (2- or 4-VP), acrylamide, trifluoromethacrylic acid and 2-hydroxyethyl methacrylate (HEMA) are most common used monomers in molecular imprinting technology.

Cross-linkers are another critical component in the synthesis of MIPs, as they contribute to the formation of the polymer network and the stability of the imprinted cavities. The role of the cross-linker is to secure the functional groups of the monomers in specific locations and directions around the template molecules, thereby preserving the structure of the binding sites or cavities within the polymer matrix (Dolai et al., 2019; Amin et al., 2018; Muhammad et al., 2012).

Various cross-linkers have been used in molecular imprinting, including ethylene glycol dimethacrylate, divinylbenzene, and epichlorohydrin, among others (Zhao et al., 2014; Wang et al., 2015; Zhao et al., 2012; Xu & Guo, 2012; Jiang et al., 2008). The selection of the cross-linker depends on the specific application and the desired properties of the MIPs. For instance, ethylene glycol dimethacrylate has been widely used as a cross-linker in molecular imprinting due to its ability to provide rigidity to the MIPs (Zhao et al., 2014; Wang et al., 2015; Li et al., 2013) On the other hand, divinylbenzene has been identified as the best cross-linking agent for the synthesis of melamine molecularly imprinted polymers (Wang et al., 2015). Additionally, epichlorohydrin has been utilized as a cross-linking agent in the preparation of ion-imprinted polymers for selective adsorption of copper ions (Xu & Guo, 2012).

Solvents and initiators are also necessary for the polymerization process. The solvent provides the medium for the assembly of functional monomers around the template molecules, while the

initiator initiates the polymerization reaction, leading to the formation of the imprinted polymer (Mayes & Mosbach, 1996; Balamurugan & Spivak, 2011; Joke Chow et al., 2016).

In summary, the successful preparation of MIPs requires careful selection and assembly of functional monomers, template molecules, cross-linkers, solvents, and initiators. Each component plays a crucial role in the molecular imprinting process, ultimately leading to the creation of synthetic polymers with specific recognition sites for target molecules.

2.9. Cryogels

Cryogels are highly porous materials with interconnected macropores and dense polymer walls, endowing them with unique structural properties such as shape memory, high flexibility, and rapid size change in response to external forces (Çimen et al., 2021; Bölgen et al., 2010). These properties make cryogels preferred in molecular imprinting technology due to their wide-open pore structures, short diffusion paths, and high selectivity, which are advantageous for various applications such as protein chromatography and biomimetic assemblies (Andaç et al., 2020; Bereli et al., 2020). The macroporous structure of cryogels also facilitates cell penetration, contact, and migration, making them suitable for tissue engineering and regenerative medicine (Tyshkunova et al., 2022; Bölgen et al., 2010; Tyshkunova et al., 2022). Furthermore, cryogels have been utilized as an injectable carrier system for in vivo cells and growth factor delivery, demonstrating their potential in biomedicine (Kim et al., 2018).

Cryogels are also preferred in drug release systems due to their biocompatibility, physical resistance, and sensitivity, making them suitable for various biomedical applications (Çimen et al., 2021). The porous structure of cryogels allows for the controlled release of drugs, providing a platform for the development of drug delivery systems with tunable properties (Zhang et al., 2022). Additionally, cryogels have been utilized in the removal of heparin from human plasma, demonstrating their potential in biotechnology, biomedicine, and pharmaceutics (Baydemir & Denizli, 2014).

The properties of cryogels can be tailored to specific applications by modulating synthesis conditions such as freezing temperature, monomer and initiator concentrations, and crosslinker ratio (Loo et al., 2013). This tunability allows for the customization of cryogels for different

purposes, including water purification, tissue regeneration, and drug delivery systems (Zhang et al., 2022; , Loo et al., 2013; , Baydemir & Denizli, 2014).

Overall, cryogels and molecular imprinting technology have been extensively studied and applied in various fields, including biomedical, environmental, and analytical sciences. The unique properties of cryogels, combined with the selectivity and specificity offered by molecular imprinting technology, make them promising materials for a wide range of applications.

Thakar et al., (2019) discussed the pH-responsive delivery of doxorubicin using a molecularly imprinted cryogel made up of HEMA and gelatin, which showed increased drug release at tumor sites (Thakar et al., 2019). This study highlights the potential of molecularly imprinted cryogels for targeted drug delivery in cancer treatment.

Bakhshpour et al., (2020) synthesized specific molecularly imprinted cryogel cartridges for the selective recognition of tyrosine, demonstrating the potential for selective drug recognition and delivery systems using molecularly imprinted cryogels (Bakhshpour et al., 2020).

Chen et al., (2015) constructed tumor-targeted nanodrugs with multimodal imaging and combination therapy capabilities using modified albumins, demonstrating the potential for advanced drug delivery systems in cancer treatment (Chen et al., 2015).

Rabieizadeh and Kashefimofrad, (2014) suggested further studies on the optimization of pH during the synthesis of lysozyme molecularly imprinted cryogel, indicating the importance of optimizing synthesis parameters for effective drug delivery systems (Rabieizadeh and Kashefimofrad, 2014).

Mofrad et al., (2015) investigated the adsorption isotherms, heterogeneity, and breakthrough curves of molecularly imprinted cryogels, providing insights into the behavior and selectivity of these materials, which are crucial for drug delivery applications (Mofrad et al., 2015).

Dolak, (2019) conducted selectivity studies using U(VI)-imprinted p-HEMA-(MAH)3 cryogel polymer, demonstrating the potential for selective adsorption of specific ions, which could be relevant for targeted drug delivery applications (Dolak, 2019).

Kumari and Kumar, (2017) studied the potential of polymeric cryogel matrices, including HEMA-gelatin cryogels, for transporting and storing mammalian cells, indicating the biocompatibility and potential biomedical applications of these materials (Kumari and Kumar, 2017).

3. METHODOLOGY

3.1. Materials

Etop, HSA, sodium hydroxide (NaOH), glutaraldehyde, gelatine, 2-hydroxyethyl methacrylate (HEMA), N,N′-methylene-bis-acrylamide (MBAA), ammonium persulfate (APS), N,N,N′,N′-tetramethylene diamine (TEMED), dimethyl sulfoxide (DMSO), ethanol, and all chemicals used in the experiments were sourced from from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade reagents and distilled water (MercAG Millipore Direct-Q 3 UV (Darmstadt, Germany)) were used to prepare the solutions.

3.2. Synthesis of Etoposide Imprinted HSA Nanoparticles

Different concentrations (5, 10, and 30 µg/mL) of Etop-loaded HSA nanoparticles (Etop-HSAnp) and non-loaded HSA nanoparticles (HSAnp) were synthesized by the desolvation (coacervation) method using ethanol as a solvent and glutaraldehyde as a cross-linker. For this purpose, 100 mg HSA was dissolved in 2 mL of distilled water. Then, Etop solutions prepared with DMSO at concentrations of 5, 10, and 30 µg/mL were added to the HSA solution and incubated at room temperature for 4 hours. Afterwards the pH of the solution was adjusted to 9 with 1 M NaOH. In order to form nanoparticles, 8 mL of ethanol was added by dropping method (at a rate of 1 mL/min) under constant stirring speed. Finally, 100 µL of 8% glutaraldehyde solution was added for stabilization of the nanoparticles. The synthesis process was carried out for 24 hours at room temperature with a stirring speed of 500 rpm. The synthesis of non-loaded HSA nanoparticles was carried out using the same method without adding Etop. The synthesized nanoparticles were separated by centrifugation and washed with distilled water to remove chemicals and impurities that did not participate in the reaction.

3.3. Preparation of Etoposide Imprinted HSA Nanoparticle Embedded HEMA/gelatin Cryogels

0.275 g MBAA was dissolved in 10 mL distilled water and 1.3 mL HEMA was added and mixed for a while (surrounded by ice). Then, different concentrations of Etop-HSAnps (5, 10 and 30 µg/mL) and HSAnps were dissolved in water and added to the mixture as a suspension (V = 1 mL). Following the addition of nanoparticles, 0.4 g of gelatin (dissolved in 4 mL of water) and 40 µL of glutaraldehyde were added, respectively. Finally, 130 µL of APS and 25

μL of TEMED were added to the mixture and mixed for a certain period of time. The mixture was divided into 24-well plates and left at -16°C for 12 hours for polymerization. The synthesized HEMA/gelatin cryogels were kept at room temperature to melt ice after polymerization. The cryogels were then washed with distilled water to remove chemicals that did not participate in polymerization.

3.4. Characterization Studies

The synthesized Etop-HSAnp and HSAnp embedded HEMA/gelatin cryogels were characterized by Swelling test, Zeta-sizer particle size analysis (Zetasizer, Nano S-90 from Malvern Instruments, London, UK), and Scanning electron microscopy (SEM) (FEI, Quanta 650 Field Emission SEM, USA).

3.4.1. Swelling Test

The swelling behaviors of the synthesized Etop-HSAnp embedded HEMA/gelatin cryogels and HSAnp embedded HEMA/gelatin cryogels were tested. For this purpose, cryogels were first swollen in distilled water for a certain period of time and then frozen. The frozen cryogels were dried with the help of a lyophilizer (UK- FD10P, Biobase, China) at -50 °C under 0.001 mbar pressure. The dried cryogels were weighed. Then, the cryogels were placed in 30 ml of distilled water and allowed to swell for 2 hours. At the end of 2 hours, the water on the swollen cryogel surface was removed using filter paper. Then, the weights of the swollen cryogels were determined. The swelling percentage was calculated using the dry weight of the cryogels after lyophilization (W_{dry}) and the received weight after swelling (W_s). The calculations were carried out with the help of the following equation:

$$\text{Swelling Ratio (\%)} = [(W_s - W_{dry}) / W_{dry}] \times 100 \quad (3.1)$$

3.4.2. Size Analysis of Nanoparticles

To determine the precise size of Etop-loaded HSA nanoparticles (Etop-HSAnp) and their non-loaded nanoparticles (HSAnp), researchers at the Nanotechnology Application and Research Laboratory of Adana Alparslan Türkeş Science and Technology University used a special instrument called the Zetasizer Nano S-90 from Malvern Instruments (London, UK). This device measures nanoparticle size using a technique called light scattering. First, the nanoparticles were completely dissolved in water to prevent any clumping or aggregation. This

was achieved using an ultrasonic water bath, which emits sound waves to gently break up any clusters. Next, a small amount (1.5 milliliters) of the nanoparticle solution was placed in a special container called a cuvette. A beam of light was then shone onto the solution at a specific angle (90°) and temperature (25°C). The instrument measured how the light scattered by the nanoparticles, which provides information about their size. To ensure accurate results, the viscosity and refractive index of water were entered into the device, along with the polymer's absorption properties. The Zetasizer calculated the number of nanoparticles per second based on the scattered light signal and confirmed that the solution had enough nanoparticles for a reliable measurement. To improve accuracy, the entire measurement process was repeated three times, and the final size reported was an average of all three measurements.

3.4.3. Scanning Electron Microscopy (SEM)

The surface morphologies of Etop-HSAnp embedded HEMA/gelatin cryogels and HSAnp embedded HEMA/gelatin cryogels were examined using SEM (FEI, Quanta 650 Field Emission SEM, USA). First, HEMA/gelatin cryogels were lyophilized for SEM analysis. The lyophilized HEMA/gelatin cryogel samples were then coated with gold under vacuum. Images of HEMA/gelatin cryogel samples coated with gold were taken with SEM at different magnifications.

3.5. *In vitro* Release Studies

This study investigated the release of Etop from HSA nanoparticles encapsulated within HEMA/gelatin cryogels at room temperature using DMSO. Five cryogels were treated with 15 mL of DMSO, and samples were collected at specific time points: 0, 30, 60, 120, and 180 minutes, as well as 24 and 48 hours. Each time a sample was collected, an equal volume of DMSO was added to maintain the total volume. The Etop concentration in the samples was determined by measuring their absorbance at 248 nm using a UV spectrophotometer (Genesys 150, Thermo Scientific, USA).

Each sample was measured at 248 nm using a spectrophotometer, and a standard graph was created. The cumulative release percentages of the samples were calculated using equation 3.4, based on the OD values obtained from UV-VIS spectrophotometer measurements.

Concentration of drug (μg/mL) = (slope × absorbance) ± intercept (3.2)

Amount of released drug (μg/mL) = [Concentration × Dissolution bath volume × dilution factor] / 1000 (3.3)

Cumulative release = R (t – 1) + Rt (3.4)

While Rt shows the cumulative release at time t, R (t – 1) represents the cumulative release amount prior to 't'.

Release (%) = (The release amount of Etop at time t, μg/mL) / (The maximum amount of Etop, μg/mL) x100 (3.5)

The release rate of Etop (%) is expressed as a percentage of the cumulative increase in the amount of Etop found in samples collected at different times. The amount of Etop in the sample denotes the Etop concentration (μg/mL) in each sample. The maximum amount of Etop in the sample represents the maximum amount of Etop that should be present in each cryogel disc (Chandrasekaran et al., 2011).

3.6. *In vitro* Cell Studies

3.6.1. Cell Culture

Glioblastoma (U-87 MG, (ATCC® HTB-14™)) (GBM) cell lines were used in this study. Cells were cultivated in a humidified incubator at 37°C with 5% carbon dioxide using Dulbecco's Modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, South America).

3.6.2. Sterilization of HEMA/gelatin Cryogels

Etop-HSAnp and HSAnp embedded cryogels used in cell studies were synthesized in 2 mL syringes. The cryogels were first cut into 0.8 cm in diameter and 0.1 cm in height, and then these pieces were divided into 4 equal parts. Cryogels were sterilized by keeping them in 70% ethanol for 2 hours. Then, it was washed twice using sterile pure water. After washing, they were immersed in sterile pure water for 2 hours.

3.6.3. Cytotoxicity Assay

$5x10^4$ cells/well were seeded on 24 well plate and cultured. Next day different numbers of Etop-loaded and empty cryogel pieces (1,2 and 3 pieces) were added to the culture except the control group. Additionally, in order to compare the effectiveness of Etop-loaded gels, different amounts of Etop were administered to the cells in another well plate. Cells were incubated for 7 days. At the end of the culture period, cell viability was evaluated by MTT cell viability assay. The culture medium and cryogels were removed and cells were washed with PBS. The MTT (0.25mg/mL) solution was added to each well and incubated for 2 hours. After the incubation period the purple precipitants were observed. The MTT solution was aspirated and DMSO was added to dissolve the precipitants. Samples were read by spectrophotometer at 570 nm.

4. ANALYSIS AND DISCUSSION

4.1. Synthesis of Etoposide Imprinted and NonImprinted HSA Nanoparticles

Etop-HSAnps containing Etop at concentrations of 5, 10, and 30 µg/mL were synthesized using the procedure given in Section 3.2 at room temperature and a stirring speed of 500 rpm. Images of the initial and final stages of nanoparticle synthesis are given below (Figure 4.1). HSAnps was also synthesized using the same procedure without the use of Etop.

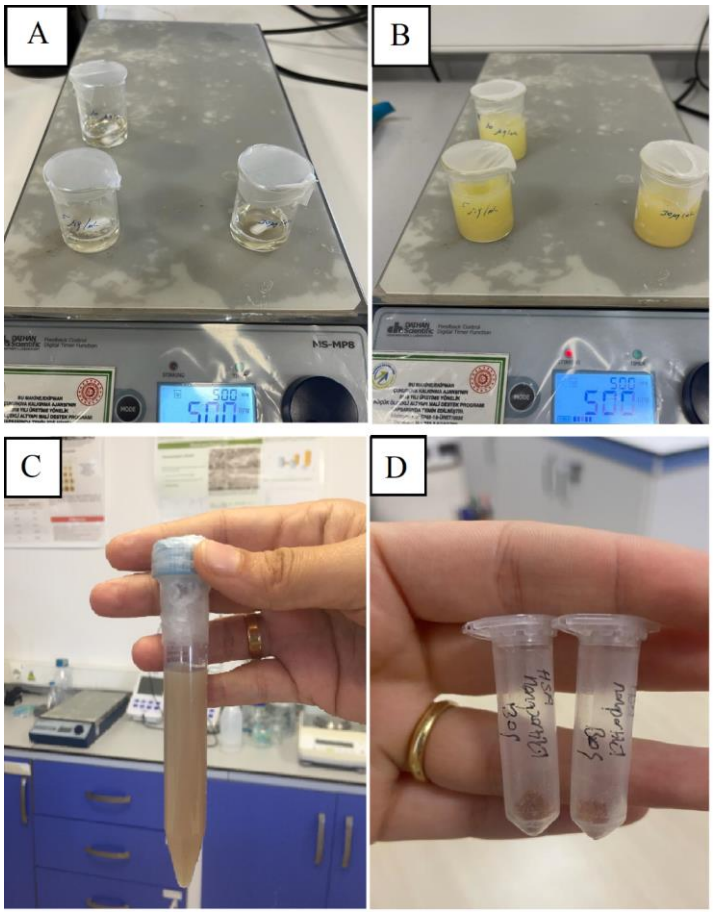

Figure 4.1. Synthesis of HSA nanoparticles A) The beginning of the synthesis, B) After the addition of glutaraldehyde, C) After 24 hours of polymerization, D) Dried appearance of the nanoparticles.

4.2. Preparation of HSA Nanoparticle Embedded HEMA/gelatin Cryogels

Nanoparticle-embedded HEMA/gelatin cryogels were prepared with HSAnps and Etop-HSAnps (5, 10 and 30 µg/mL) using the recipe given in Section 3.3. The polymerization process was carried out at -16 °C for 12 hours. At the end of polymerization, the cryogels were kept at

room temperature for the ice to melt and then washed repeatedly with distilled water. The images of the cryogels before and after polymerization were as shown in Figure 4.2.

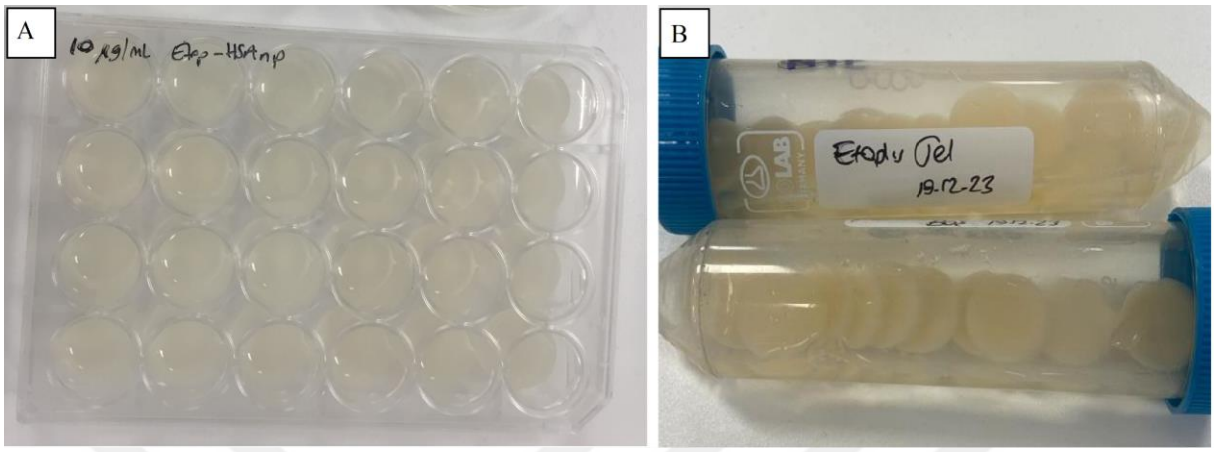

Figure 4.2. Synthesis of nanoparticle-embedded HEMA/gelatin cryogels A) Before and B) After polymerization.

4.3. Characterization Studies

4.3.1. Size Analysis of Etop-HSAnp and HSAnp Nanoparticles

Size distribution analyzes of Etop-HSAnp and HSAnp were performed using the Zetasizer device (Zetasizer Nano S-90 from Malvern Instruments, London, UK). Zetasizer analysis results of nanoparticles are given in Figure 4.3. As can be seen from the figure, thanks to the low polydispersity index values (PDI =0.108 and 0.182), it was determined that equidimensional nanoparticles with a homogeneous average size distribution were successfully produced. The presence of a single and smooth peak suggests the absence of particle aggregation and the absence of larger particles. The measurements were repeated three times and the particle sizes of Etop-HSAnps and HSAnps were determined to be 112.6 nm and 109.6 nm, respectively.

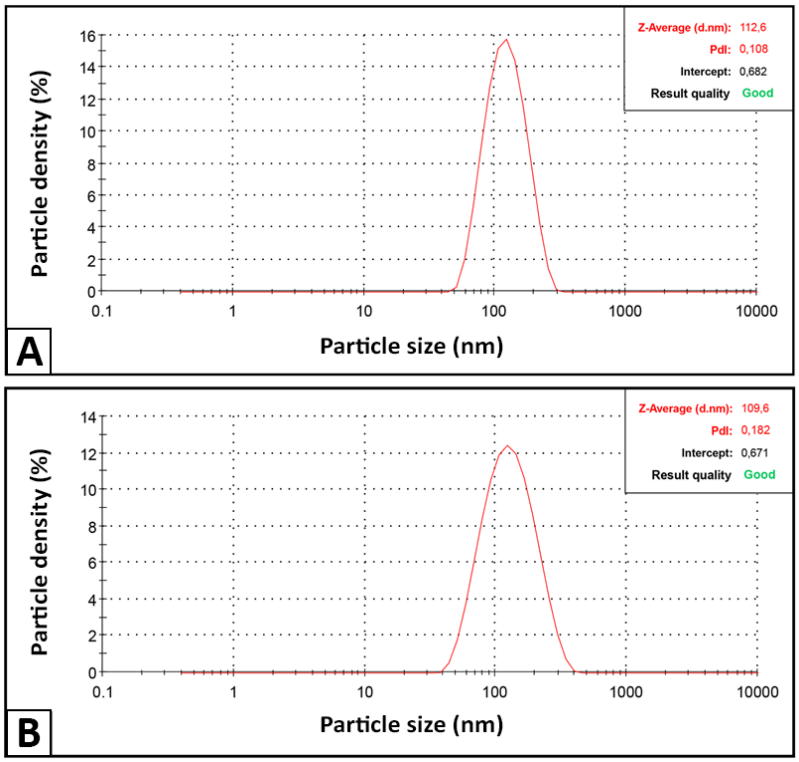

Figure 4.3. Particle sizes of A) Etop-HSAnp and B) HSAnp.

4.3.2. Swelling Test

The swelling behavior of the synthesized Etop-HSAnp and HSAnp embedded HEMA/gelatin cryogels in deionized water was examined and the swelling percentages were calculated. The findings obtained are given in Table 4.1.

Table 4.1. Swelling Ratio of HSAnp and Etop-HSAnp embedded cryogels.

Polymer	Ws (mg)	Wdry (mg)	Swelling Ratio (%)
HSAnp embedded cryogel	571.3	122.7	365.6
5 µg/mL Etop-HSAnp embedded cryogel	569.0	121.2	369.5
10 µg/mL Etop-HSAnp embedded cryogel	553.2	118.4	367.2
30 µg/mL Etop-HSAnp embedded cryogel	549.4	117.1	369.2

4.3.3. Scanning Electron Microscopy (SEM)

The surface morphologies of Etop-HSAnp, HSAnp and nanoparticle (HSAnp and Etop-HSAnp) embedded HEMA/gelatin cryogels were examined at different magnifications using SEM. The resulting SEM images are shown in Figure 4.4.

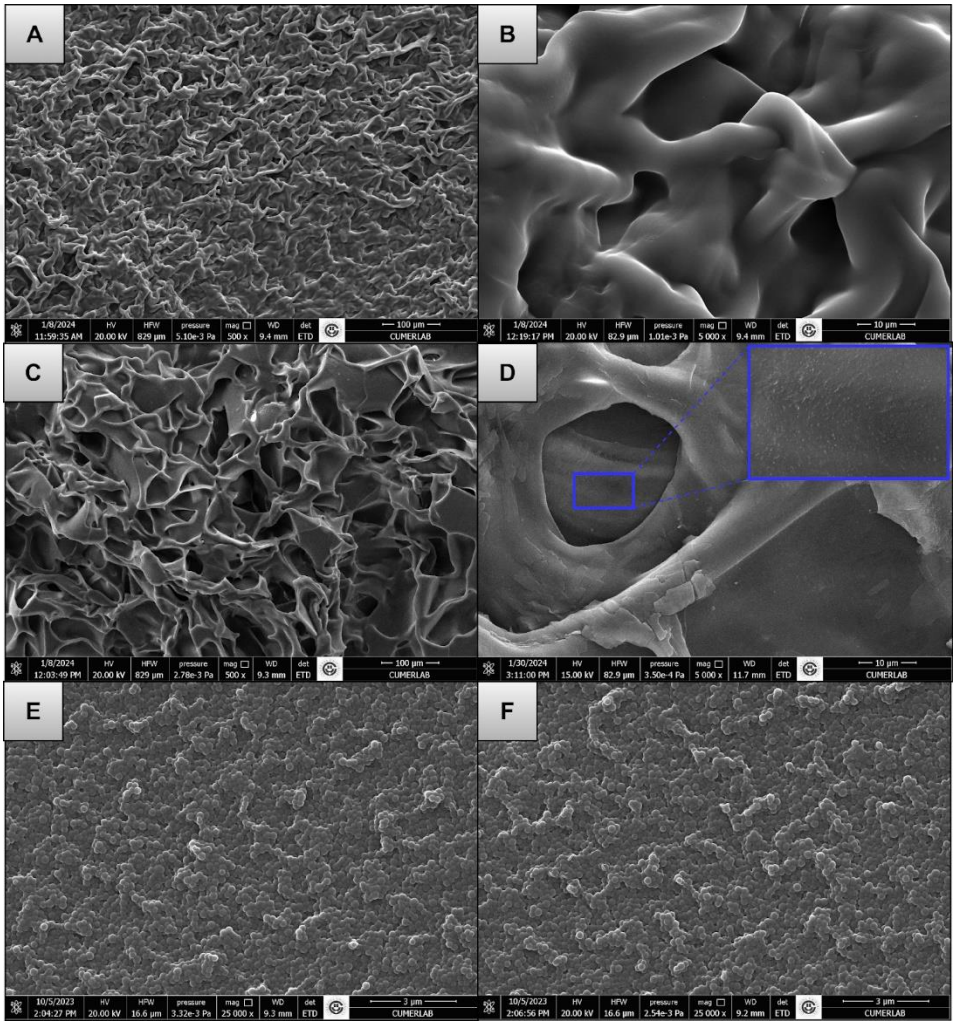

Figure 4.4. SEM images of A-B) HSAnp embedded cryogels (500x-5000x), C-D) Etop-HSAnp embedded cryogels (500x-5000x) E) HSAnp (25000 x), F) Etop-HSAnp (25000 x).

4.4. In vitro Release Studies

One of the important parameters that affect the drug release rate is the initial drug loading amount in the polymeric system. In this study, which investigated the release of Etop from cryogel disks, Etop was added to the polymerization mixture at three different concentrations

(5, 10, and 30 µg/mL). In order to determine the effect of the loading rate on the release, the same amount of DMSO (15 mL) was used and the room temperature was kept constant.

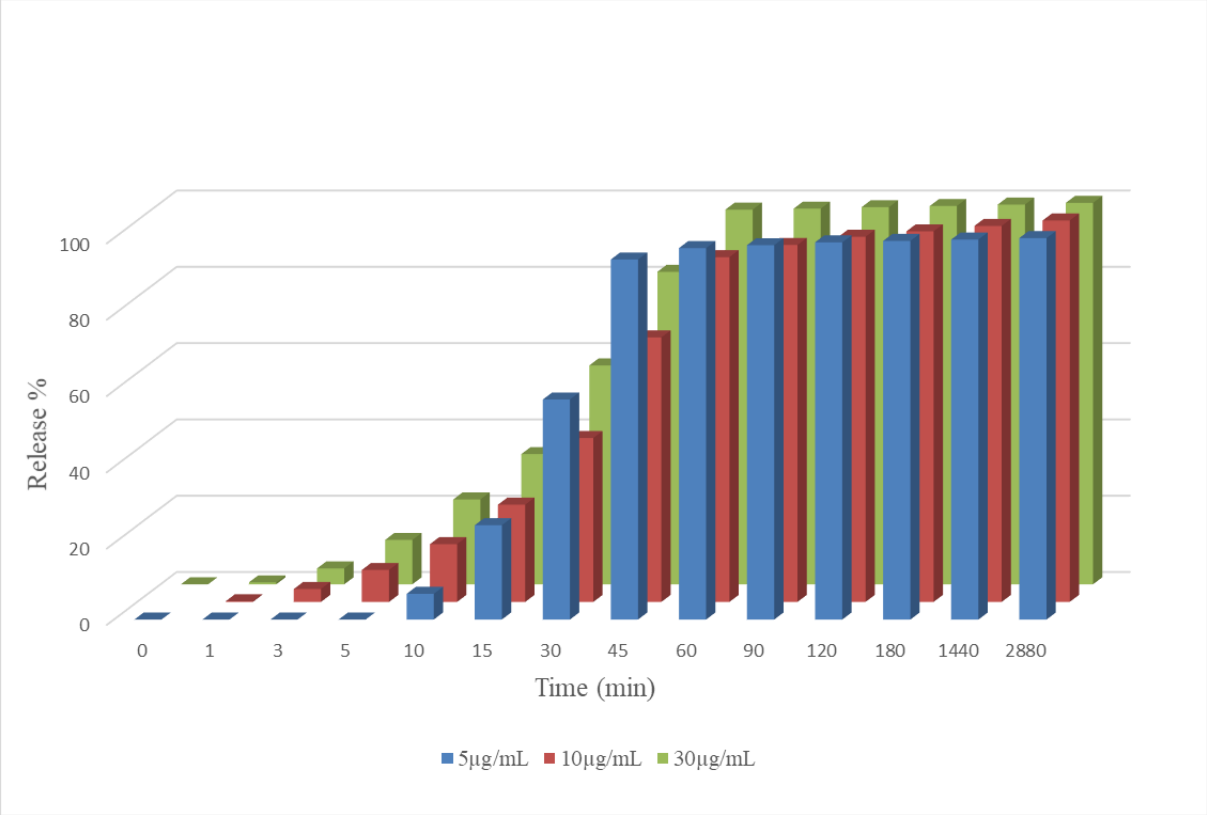

Figure 4.5. Effect of initial Etop concentration on release (%)

According to Figure 4.5, the release rate also increases as the drug loading amount increases. This is an expected result and can be explained by the increase in the driving force (concentration difference) for its diffusion from the Etop-loaded cryogel disc as the amount of drug in the polymer system increases.

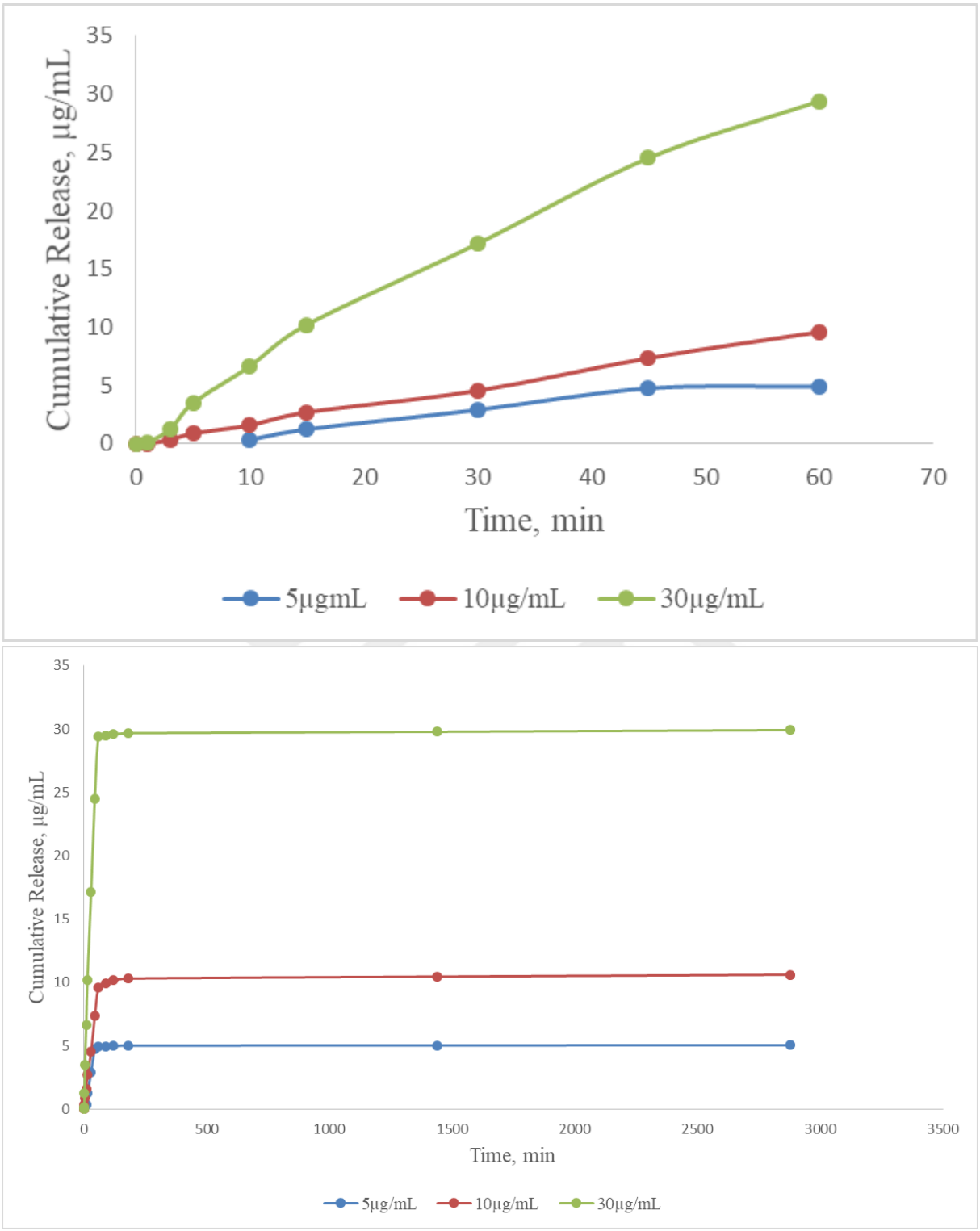

Figure 4.6. Cumulative release amount over time based on initial Etop concentration

The cumulative release graph of HSA nanoparticles containing different concentrations of Etop is given in Figure 4.6. When the graph was examined, it was seen that Etop was released at a high rate within the first hour. Later, according to the samples taken for 2 days, it was shown that the drug release continued slowly.

4.5. In vitro Cell Studies

The viability of cells exposed to different numbers of pieces of both Etop-loaded and empty cryogels was measured by the MTT assay. According to MTT assay results the effect of Etop-loaded cryogels on cell viability was calculated compared to the control, considering the effect of empty cryogels on cell viability. The amount of Etop loaded in each cryogel piece was calculated (0.03 µg) and the % cell viability was plotted against the amount of Etop (Figure 4.7.).

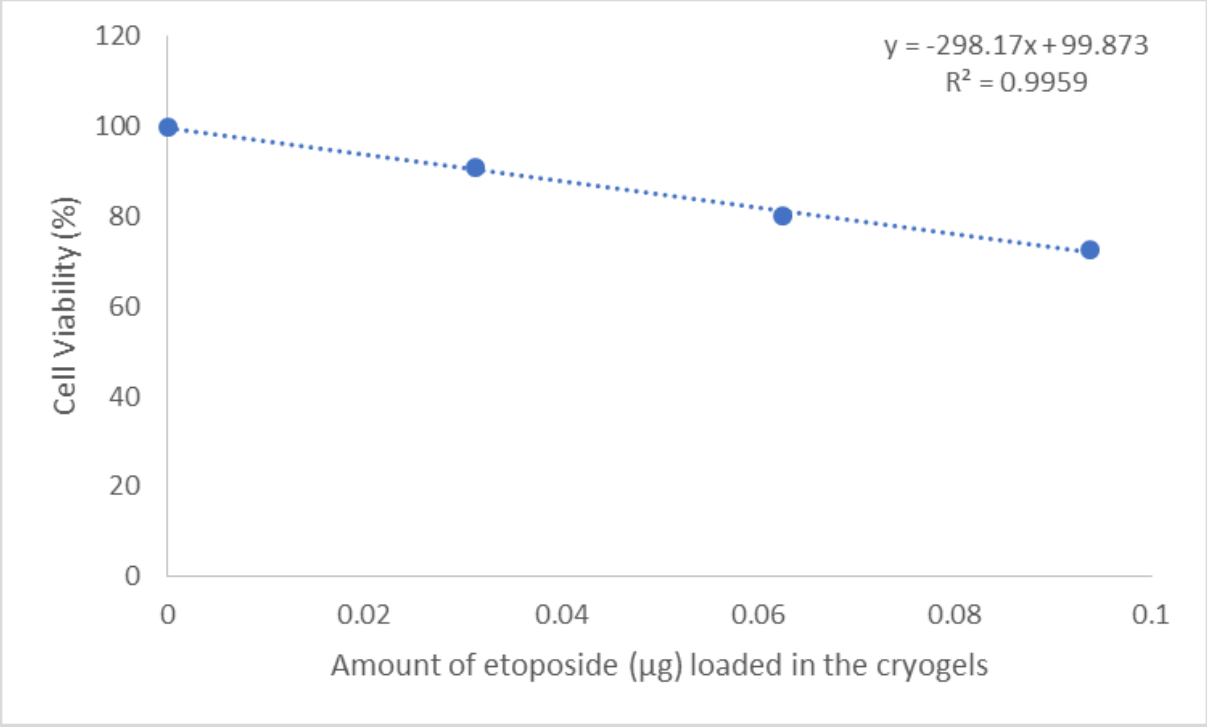

Figure 4.7. % cell viability versus amount of Etop loaded in the pieces given to the cells.

The IC25 value, the amount of Etop that kills 25% of cells, of Etop loaded cryogels was calculated as 0.08 µg from the equation of the graph in Figure 4.7. Also, the IC25 value of Etop on cells was calculated as 1.4 µg from the equation of the graph in Figure 4.8. These results revealed that Etop loaded into the gel was more effective than Etop administered directly to the cells, as the IC25 value of Etop loaded into the gel was smaller than Etop administered directly.

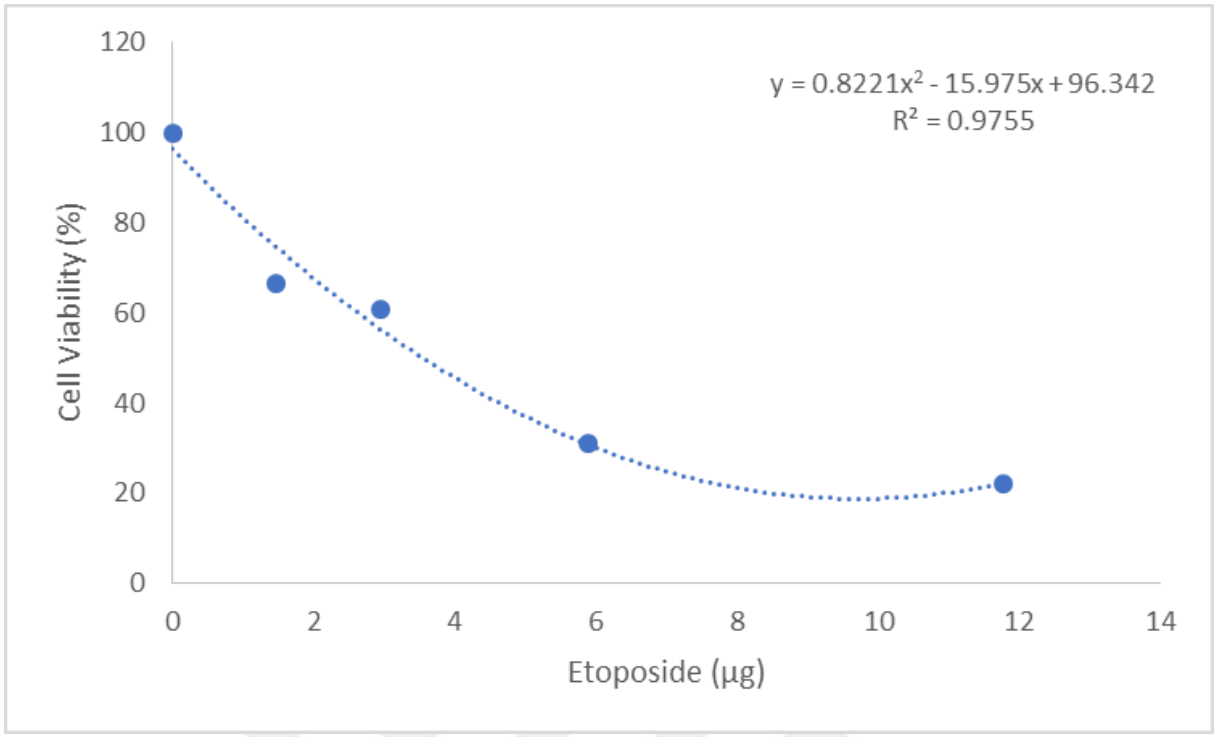

Figure 4.8. % cell viability versus amount of Etop given to the cells.

5. CONCLUSION

In this thesis, the desolvation method was used to synthesize Etop-HSAnp at 5, 10, and 30 μg/mL concentrations and non-loaded HSA nanoparticles using ethanol and glutaraldehyde. A mixture of MBAA, HEMA, Etop-HSAnps, gelatin, glutaraldehyde, APS, and TEMED was prepared. The mixture was then polymerized at -16°C for 12 hours. HEMA/gelatin cryogels were created. Zeta-sizer analysis was used to estimate the diameters of Etop-HSAnp and HSAnp nanoparticles, resulting in approximate sizes of 112.6 nm and 109.6 nm, respectively. SEM analysis supported these conclusions by confirming the precision of the size measurements. SEM was used to analyze the bulk structure and surface morphology of cryogel discs embedded with HSAnp and Etop-HSAnp nanoparticles. Examining the SEM pictures revealed that the drug was uniformly distributed throughout the polymer matrix and that there were no barriers to drug release from cryogel discs with big enough holes. Also, Etop concentration was determined by measuring absorbance at 248 nm using a UV spectrophotometer at specific time points. The study examined the release of Etop from cryogel disks at different concentrations (5, 10, and 30 μg/mL) in a polymerization mixture. The release rate increased with the drug loading amount, attributed to the increased driving force for diffusion. Increasing the concentration difference caused an increase in the release rate. The MTT assay measured cell viability in Etop-loaded and empty cryogels. The IC25 value of Etop loaded cryogels was 0.08 μg and 1.4 μg, respectively. The results showed that Etop loaded into the gel was more effective than Etop administered directly to cells, as the IC25 value was smaller.

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