

DEVELOPMENT OF A HYDROGEL SYSTEM TO SUSTAIN LOCAL
MICROSPHERE RELEASE FOR POST-OPERATIVE TREATMENT OF
BREAST CANCER

A THESIS SUBMITTED TO
THE INSTITUTE OF GRADUATE PROGRAMS
OF
İSTANBUL BİLGİ UNIVERSITY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOENGINEERING

**DEVELOPMENT OF A HYDROGEL SYSTEM TO SUSTAIN LOCAL
MICROSPHERE RELEASE FOR POST-OPERATIVE TREATMENT OF
BREAST CANCER**

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Date of Approval: 6/26/2025



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ABSTRACT

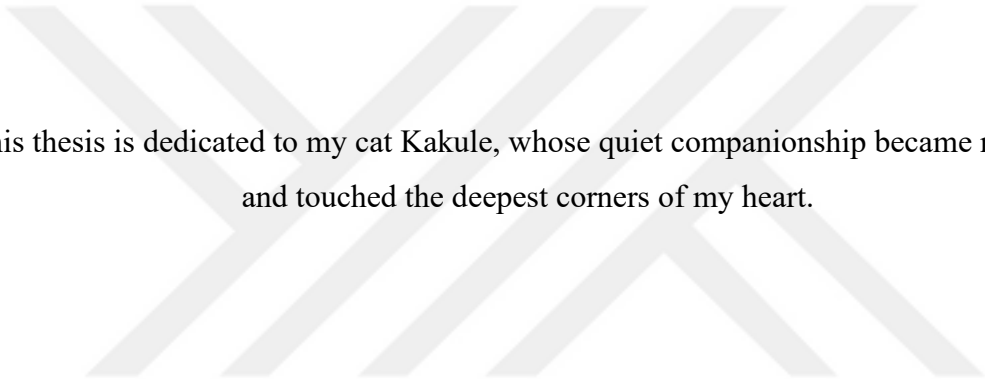
Early intervention through post-operative treatment for breast cancer patients is essential to prevent locoregional recurrence or the metastasis of residual microtumor cells thus the clinical demand for site-specific treatments is highly significant, as conventional drug delivery systems often fall short of fulfilling these requirements. Accordingly, this study presents a novel poly(vinyl alcohol)/pullulan (PVA/PUL) hydrogel platform designed for post-surgical breast cancer treatment, facilitating the localized delivery of doxorubicin (DOX). The primary carrier platform, the hydrogel, was fabricated using two combined methods: lyophilization and ice embossing techniques, which created interconnected micro and macropores that serve as reservoirs for DOX- encapsulated polycaprolactone microspheres (DOX- PCL- MSs), enabling controlled and sustained drug release. The glutaraldehyde (GA) crosslinked, structurally stabilized PVA/PUL hybrid hydrogel system demonstrated enhanced water retention and a slow degradation profile. Furthermore, when examining DOX release from different platforms, including direct release from the hydrogel and the hydrogel incorporating DOX- PCL- MSs, it was observed that the hydrogel incorporating DOX- PCL- MSs exhibited a delayed release, with no burst release, reaching up to $56.6 \pm 4.5\%$, whereas direct DOX release from the hydrogel resulted in a $76.79 \pm 19.72\%$. Additionally, in vitro studies were conducted using the human breast cancer cell line MCF- 7, in both monolayer and spheroid forms, and the results indicated that both cell morphologies were disrupted at concentrations as low as $12.5 \mu\text{g/mL}$ of DOX- PCL- MSs. Additionally, the MCF- 7 cell viability after 7 days of incubation was significantly reduced, conversely, free DOX- loaded hydrogel exhibited a loss of cytotoxic efficacy by day 4. The cytocompatibility of the hybrid hydrogel systems was also investigated with L929 fibroblast cells; the hydrogel supported the adhesion of these cells. These findings regarding the PVA/PUL hydrogel incorporating DOX- PCL- MSs highlight the system's potential as a localized and sustained drug delivery system for the post-operative treatment of breast cancer.

Keywords: Breast Cancer; Locaregional Delivery; Hydrogel; Microsphere; Spheroid

ÖZ

Meme kanseri hastalarında cerrahi müdahale sonrası tedavi yoluyla erken müdahale, lokal bölgesel tekrarı veya kalan mikrotümör hücrelerinin metastazını önlemek için önemlidir. Geleneksel ilaç verme sistemleri genellikle bu gereklilikleri karşılamada yetersiz kaldığından, bölgeye özgü tedavilere yönelik klinik talep oldukça önem kazanmıştır. Bu doğrultuda, bu çalışma, cerrahi müdahale sonrası meme kanseri tedavisi için tasarlanmış, doksorubisinin (DOX) lokalize iletimini kolaylaştıran yeni bir poli (vinil alkol)/pullulan (PVA/PUL) hidrojel platformu sunmaktadır. Birincil taşıyıcı platform olan hidrojel, DOX kapsüllenmiş polikaprolakton mikroküreleri (DOX- PCL- MS'ler) için rezervuar görevi gören birbirine bağlı mikro ve makro gözenekler oluşturan liyofilizasyon ve buz kabartma teknikleri olmak üzere iki birleşik yöntem kullanılarak üretilmiştir ve kontrollü ve uzun süreli ilaç salımını mümkün kılmıştır. Glutaraldehit (GA) çapraz bağlı, stabilize edilmiş PVA/PUL hibrit hidrojel sistemi, su tutma kapasitesi ve yavaş bir degradasyon profili göstermiştir. Hidrojelden doğrudan salım ve DOX-PCL-MS'leri içeren hidrojel olarak farklı platformlardan DOX salımı incelendiğinde, DOX-PCL-MS'leri içeren hidrojelin gecikmeli bir salım sergilediği, ani bir salım olmadan $56.6 \pm 4.5\%$ 'e ulaştığı, buna karşın hidrojelden doğrudan DOX salımının $76.79 \pm 19.72\%$ ile sonuçlandığı gözlemlenmiştir. Ek olarak, hem tek katmanlı hem de sferoid formlarda insan meme kanseri hücre hattı MCF-7 kullanılarak in vitro çalışmalar yürütülmüş ve sonuçlar her iki hücre morfolojisinin de $12,5 \mu\text{g/mL}$ kadar düşük DOX-PCL-MS konsantrasyonlarında dahi bozulduğunu göstermiştir. Yedi günlük inkübasyon sürecinden sonra ise MCF-7 hücre canlılığı önemli ölçüde azalmıştır, serbest DOX yüklü hidrojelin ise 4. günde sitotoksik etkinliğinde azalma gözlemlenmiştir. Hibrit hidrojel sistemlerinin sito-uyumluluğu L929 fibroblast hücreleriyle de araştırılmıştır; hidrojelin hücrelerin yapışmasını desteklediği gözlemlenmiştir. DOX-PCL-MS'leri içeren PVA/PUL hidrojeline ilişkin bu bulgular, sistemin meme kanserinin cerrahi müdahale sonrası tedavisi için hedefe yönelik ve kontrollü salım sağlayan ilaç taşıma sistemi olarak potansiyelini vurgulamıştır.

Anahtar Kelimeler: Meme Kanseri; Bölgesel İlaç Salımı; Hidrojel; Mikroküre; Sferoid



This thesis is dedicated to my cat Kakule, whose quiet companionship became my light
and touched the deepest corners of my heart.

“My dream life and my waking life were lives apart, with not one thing in common save
myself.” — Jack London, *Before Adam*

ACKNOWLEDGMENTS

Pursuing this master's degree has been both a challenging and rewarding experience. I would like to take this opportunity to express my sincere gratitude to my advisor, Assist. Prof. Dr. Ali Deniz Dalgıç, who has supported me throughout this journey, beginning from my undergraduate studies. I am profoundly thankful not only for his supportive and kind supervision but also for his genuine interest and encouragement. His patience towards me during my learning process and my mistakes has held both personal and academic significance. Additionally, the scientific perspective he instilled in me has been the most valuable aspect of this journey. I am deeply honored to be his student. Furthermore, I would like to extend my sincere gratitude to Assist. Prof. Dr. Yuk Yin Ng, who has guided me and broadened my knowledge, as well as to Assist. Prof. Dr. Sesil Çınar, under whom I had the opportunity to work as a teaching assistant during her course. Her cheerful and positive demeanor consistently provided a supportive and encouraging environment throughout my studies. My heartfelt gratitude is expressed to my mama, Neslihan Serap Barer, and to my father, Cenk Barer, who has never let go of my hand since the day I was born. I feel the courage and support you gave me in each breath. I will always be grateful to feel protected and supported, and I thank you for understanding my needs and emotions without me ever having to say a word. I would also like to express my gratitude to all individuals I have encountered at Istanbul Bilgi University. Over the years, I have had the opportunity to meet numerous people, each of whom has contributed to the unique experiences that have influenced my development in various ways—not only pertaining to my academic journey but also in terms of my personal and emotional growth. These experiences will always hold a special place in my heart, serving as a sanctuary to which I will always return.

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LIST OF SYMBOLS

W_0 :	initial dry weight
W_t :	dry weight of the sample at time t



LIST OF ABBREVIATIONS

1-h-GA:	1 hour of GA
30 min-GA:	30 minutes of GA
ATR-FTIR:	Fourier Transform Infrared Spectroscopy
BC:	Breast Cancer
CAR:	Chimeric Antigen Receptor
CED:	Convection-Enhanced Delivery
CTLA-4:	Cytotoxic T-Lymphocyte–Associated Protein 4
CYP:	Cytochrome P450
DCM:	Dichloromethane
DNA:	Deoxyribonucleic Acid
DOX:	Doxorubicin
DR5:	Death Receptor 5
DOX-PCL-MSs:	Doxorubicin Loaded Polycaprolactone Microspheres
EMT:	Epithelial-Mesenchymal Transition
ER+:	Estrogen Receptor-Positive
FADD:	Fas-Associated Death Domain
FBS:	Fetal Bovine Serum
FE-SEM:	Field Emission Scanning Electron Microscopy
GA:	Glutaraldehyde
HCL:	Hydrochloric Acid
HDMS:	Hexamethyldisilazane
HER+:	Human Epidermal Growth Factor Receptor-Positive
HR+:	Hormone Receptor-Positive
IA:	Intra-arterial

IARC:	International Agency for Research on Cancer
IT:	Intratumoral
LUT:	Look Up Table
LYS:	Lysozyme
MRI:	Magnetic Resonance Imaging
mRNA:	Mitochondrial Ribonucleic Acid
MTX:	Methotrexate
OCT3/4:	Octamer-Binding Transcription Factor 3/4
PBS:	Phosphate Buffer Saline
PCL:	Poly ϵ -Caprolactone
PD1:	Programmed Cell Death Protein
PD-L1:	Programmed Death-Ligand 1
PR+:	Progesterone Receptor-Positive
PUL:	Pullulan
PVA:	Polyvinyl Alcohol
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
SOX 2:	SRY-Box Transcription Factor
TNBC:	Triple-Negative Breast Cancer
TOP II:	Topoisomerase II
TOP II α :	Topoisomerase II α
TOP II β :	Topoisomerase II β
W/O:	Water in oil
ZEB 1:	Zinc Finger E-box Binding Homeobox 1

INTRODUCTION

1.1. Overview of Breast Cancer and Post-Operative Therapeutic Strategies

The global incidence of breast cancer (BC) is expected to reach approximately 2.4 million cases by 2025, according to the International Agency for Research on Cancer (IARC), estimated using the GLOBOCAN 'Cancer Tomorrow' tool [1]. This data reflects the yearly steady increase and its growing prevalence worldwide, emphasizing BC's status as one of the major health challenges. This most common type of cancer among women is classified as a heterogeneous neoplasm (an abnormal growth of cells) that develops in the breast lobules, tubes, or connective tissue [2]. This diverse disease includes various entities with unique pathogenic characteristics and biological behaviors. The occurrence of cancer in specific areas of the breast and the type of cells offer insights for classifying it as sarcomas or carcinomas [3]. The neoplasms that occur in the non-epithelial part of the gland are called sarcomas, whereas carcinomas occur in the epithelial component of the cells in lobules and ducts [4]. The invasive BC is further classified based on their intrinsic subtypes related to their hormone receptors, including estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), human epidermal growth factor receptor-positive (HER+), and triple-negative (TNBC), which lacks any of the receptor expressions mentioned [5]. According to the subtypes, they exhibit different histopathological features, risk factors, and responses to therapies [6]. Over the years the treatment of BC has changed in terms of medically and surgically. There are several post-operative therapeutics for BC, such as chemotherapy, radiotherapy, endocrine therapy, immunotherapy, gene therapy, and surgery. However, the therapy decision will be influenced by the type of BC because of the heterogeneity at the molecular and histological levels [7]. Chemotherapy is a drug-based treatment that uses powerful medications to kill and disrupt rapidly dividing cells in the body, primarily used for breast carcinomas [8].

It compromises several cytotoxic drugs with distinct mechanisms of action, such as antimetabolites and alkylating agents. Where they act primarily as enzyme suppressors/blockers and hinder cell division [9]. Chemotherapy could be utilized in the neoadjuvant (before surgery treatment) or adjuvant (after surgery treatment) setting for BC treatment. For unreachable tumors that were being explained as non-metastatic but inoperable BC, neoadjuvant chemotherapy was administered. Thereafter, the therapy was started being used before surgery for operable tumors [10]. Adjuvant chemotherapy is administered after primary treatment when there is a risk of recurrence and to patients with lymph node metastasis [11]. Radiotherapy became one of the fundamental approaches in cancer treatments with the discovery of X-rays. It is utilized with high-energy radiation for specific anatomical regions. This treatment can be applied to the entire breast or other localized areas after a mastectomy (a surgical procedure for the removal of the breast) [12]. Immunotherapy is an individualized technique that utilizes biological substances to enhance the immune system's ability to attack cancer cells [13]. Although therapy began around 20 years ago, the incorporation of patient care has progressed more slowly compared to other tumor types [14]. There are three different types: passive, active, and combinatorial immunotherapy, which depend on the therapeutic agents and the patient's immune system [15]. In active therapy, the immune system is stimulated through vaccination or by targeting specific antigen receptors. Passive therapy is generally applied to patients with weak or dysfunctional immune systems, where lab-made therapeutics enhance the anti-tumor response [16]. Combinatorial therapy combines different anticancer agents for treatment [17]. Gene therapy is a transformative application where genetic material is sent with a vector to the target cells [18]. It includes targeting transcription factors, DNA or RNA vaccination and gene editing. The mechanism works with the presence of foreign genetic material such as DNA or RNA by entering the target cell with a carrier/vector and as a result of this incidence gene editing, expression or silencing occurs [19].

Endocrine therapy is classified as adjuvant therapy for metastatic breast cancer and can not be applied as a treatment to all subtypes of breast cancer. It could be administered to cancers that are hormone receptor-positive (HR+) by blocking or modifying the effect of estrogen and progesterone. The most frequent type of BC is HR+ (~ 70%); hence, endocrine therapy offers an effective approach for HR+ patients [20]. Promising agents and new drug combinations are in the pipeline for endocrine therapy to increase survival rates [21]. Surgery's intention for the treatment of BC is to attain local control and prevent local recurrence. There are severely different approaches for surgical treatment, such as masectomy with or without reconstruction and primary, delayed, or breast-conserving therapy [22]. Removal of the breast is performed via mastectomy and requires reconstruction after the procedure [23]. On the other hand, breast-conserving therapy preserves the natural appearance of the breast while excising the tumor. In this method, it is crucial for patients to be well-informed, as multiple surgical options often more than one surgical solution with the same oncological and surgical outcomes; therefore, fully informed consent must be obtained [22]. As mentioned earlier, there are several post-operative treatments to be applied after surgery for BC. Alongside the benefits of those treatments, there are some persistent concerns regarding certain limitations presented in Table 1.1

Table 1.1. Current Breast Cancer Therapeutic Approaches, Advantages, Disadvantages/Limitations

Therapeutic Modality	Mode of Action	Advantages	Limitations/Disadvantages
Chemotherapy	Application of the anticancer agents to the vigorously growing cancer cells [24].	Improved likelihood of targeting metastatic cancer cells/Control cancer cells [25].	Development of the chemoresistance. (e.g., drug inactivation, apoptotic dysregulation, epithelial to mesenchymal transmission) [26].
Radiotherapy	Application of high-energy radiation [27].	Local inference over tumor [28].	Initial positioning discrepancy of the patient during scanning [29].
Immunotherapy	Cancer suppression via targeting checkpoint inhibitors [30].	Enhanced overall survival rate with immune checkpoint inhibitors [31].	Difficulty in manipulating the immune system due to various cellular and molecular interactions [32].
Gene Therapy	Silencing the endogenous genes and regulating the gene expressions in cancer cells [33].	Suppressing the associated pathogenic genes and regulation of the apoptosis of cancer cells [34].	Vector-associated toxicity occurrence [35].
Endocrine Therapy	Blocking/modification of the estrogen and progesterone [36].	Application of more targeted agents, thus decreased mortality [37].	Several side effects due to blockage/reduction (e.g., infertility, menapausal symptoms, osteoporosis) [38].
Surgery (Mastectomy)	Physical removal of the cancerous tissue [39].	Local disease control and perturbation of metastatic homeostasis [40].	Complications that may occur after surgery (e.g., skin or nipple necrosis, wound dehiscence, infection) [41].

Numerous studies indicate that surgical treatment, whether partial or total mastectomy, particularly in end-stage (IV) breast cancer, decreases mortality and provides prolonged survival [42], [43], [44]. It could be inferred that surgery is the preferred means to treat breast cancer; however, the success rate hinges on the cleaning of residual cancer cells and the microtumors which more than 50% of the patients hold [45]. Therefore, this challenge remains the primary reason for the failure of surgery [46], [47]. During or post-mastectomy, problems that might arise include skin-flap necrosis, lack of magnetic resonance imaging (MRI) surveillance on residual glandular breast tissue, where it is being seen as the risk of recurrence [48]. There are various reasons why some surgeons may intentionally leave some of the glandular tissue during mastectomy, in a case where avoiding harming the blood supply to the nipple or lowering the risk of ischaemic necrosis or aesthetic purposes, and those purposes might lead to locoregional relapse [49]. Additionally, tumor tissues are exposed to surgical intervention and face hypoxia and mechanical stress, leading to rapid gene expression in the tumor microenvironment [50]. Although various factors such as nodal status, tumor size, grade, margin status, and those mentioned above are known to be involved in recurrence, prior studies have documented that careful consideration needs to be given to the site where the primary tumor is formed due to the fact that it is also the location of recurrence [51]. Even though there are many treatment options for cancer therapeutics, and in general, mortality has decreased over the last several decades due to advancements in technology and improved early detection still, there are fundamental shortcomings, such as treatment-associated morbidity and recurrence rates. To overcoming those obstacles, the local delivery approach is crucial in cancer treatment, and there is a clinical need for it this. This can be attributed to various factors. During the surgical resection, some undetected microscopic diseases may remain at the site, which emphasizes the need for adjuvant therapies to control surgical excitation. It is hypothesized that these therapies must eliminate all residual malignant tumor cells because a single cancer cell has the potential to grow into a tumor or metastasize through circulation and a local delivery approach could be administered to avoid those main drawbacks [52].

1.2. Local Drug Delivery Strategies: Advantages and Limitations

Local drug delivery approaches are primarily employed after surgeries to resection cavities in order to eliminate hidden residual cancerous tissues near the margins. There are two major factors that contribute to the lack of treatment for solid tumors: 1) Imbalance between the drug concentration for the tumor mass in an adequate period and 2) Inadequate specificity in drug targeting for tumors [53]. These barriers can be mitigated by implementing a high dose regimen, however, resulting in toxicity. At this stage, the system and method of drug administration, which are crucial for effective treatment, play a pivotal role in the tumor microenvironment via determining the biological pathway within the patient's body and eventually affecting the bioavailability of the drug [53]. Drug administration affects not only bioavailability but also healthy tissue preservation. If the drug is delivered without consideration, it might elicit serious off-target effects. Thus local/site-specific drug delivery is thoroughly examined since it aims to minimize the drug toxicity [54]. The concept of local or site-specific drug delivery is broadly examined within two main categories: systemic delivery and locoregional delivery, along with their respective subtopics, systemic nontargeted delivery, systemic targeted delivery, locoregional infusion-based delivery, and locoregional depot-based delivery [53]. Regarding the toxic distribution of the drug within the tumor and body circulation, targeted drug deliveries from systemic delivery showed significantly high off-target and toxic results, while locoregional deliveries demonstrated much lower toxicity in the same context [55], [56], [57]. In comparison with systemic delivery, locoregional delivery aims to lower multiple biological barriers by selectively penetrating the tumor site or bypassing the blood circulation since it is administered in proximity. The mechanism works via the delivery of the drug either by direct infusion or implantation of depots that release it [53], [58], [59].

Bastiancich et al. previously presented a study of systemic delivery based on 15 publications and 5 meta-analyses selected for efficiency comparison between systemic and locoregional delivery. However, drug type, delivery system, and duration of the studies were excluded. Statistical results indicated that locoregional delivery is more effective than systemic delivery [60]. Consequently, innovative strategies are being demonstrated for this promising delivery system. Locoregional delivery is primarily classified into two major categories: infusion-based and drug-loaded depot-based delivery. The infusion-based approach for local drug delivery to the tumor site does not consider drug retention as opposed to the depot-based method [53]. An infusion-based approach can be performed with a syringe(s) that contains the therapeutic agent(s). This approach's critical consideration is the injectability of the agent; it is applied via the standard gauge needle in a syringe [61]. This approach includes subtypes such as direct intratumoral (IT) injection, convection-enhanced delivery (CED), and intra-arterial (IA) delivery [53]. IT injection mainly consists of immunotherapeutic agents that can be administered intravenously, such as monoclonal antibodies targeting immune checkpoint proteins (PD-1, PD-L1, CTLA-4) and chimeric antigen receptor (CAR) T cells [57], [62], [63]. This approach provides several benefits, while maximizing the therapeutic index of various immunomodulatory therapies and minimizing systemic toxic exposure [64]. CED is an infusion based application technique where high-concentration therapeutic agents are delivered via cannula attached to a microfusion pump to generate pressure gradient at the site. This method is mostly preferred for nervous system related diseases such as glioblastoma [65], [66]. IA delivery relies on catheter-based treatments. In this treatment, therapeutic agents are injected through the arteries to the tumor site to higher tumor response [67]. However, the response of this approach according to drug distribution is highly reliant on the vascularization level of the tumor [53]. Although infusion-based techniques are advantageous in many aspects and able to reduce off target toxicity, this approach is yet accompanied by certain challenges.

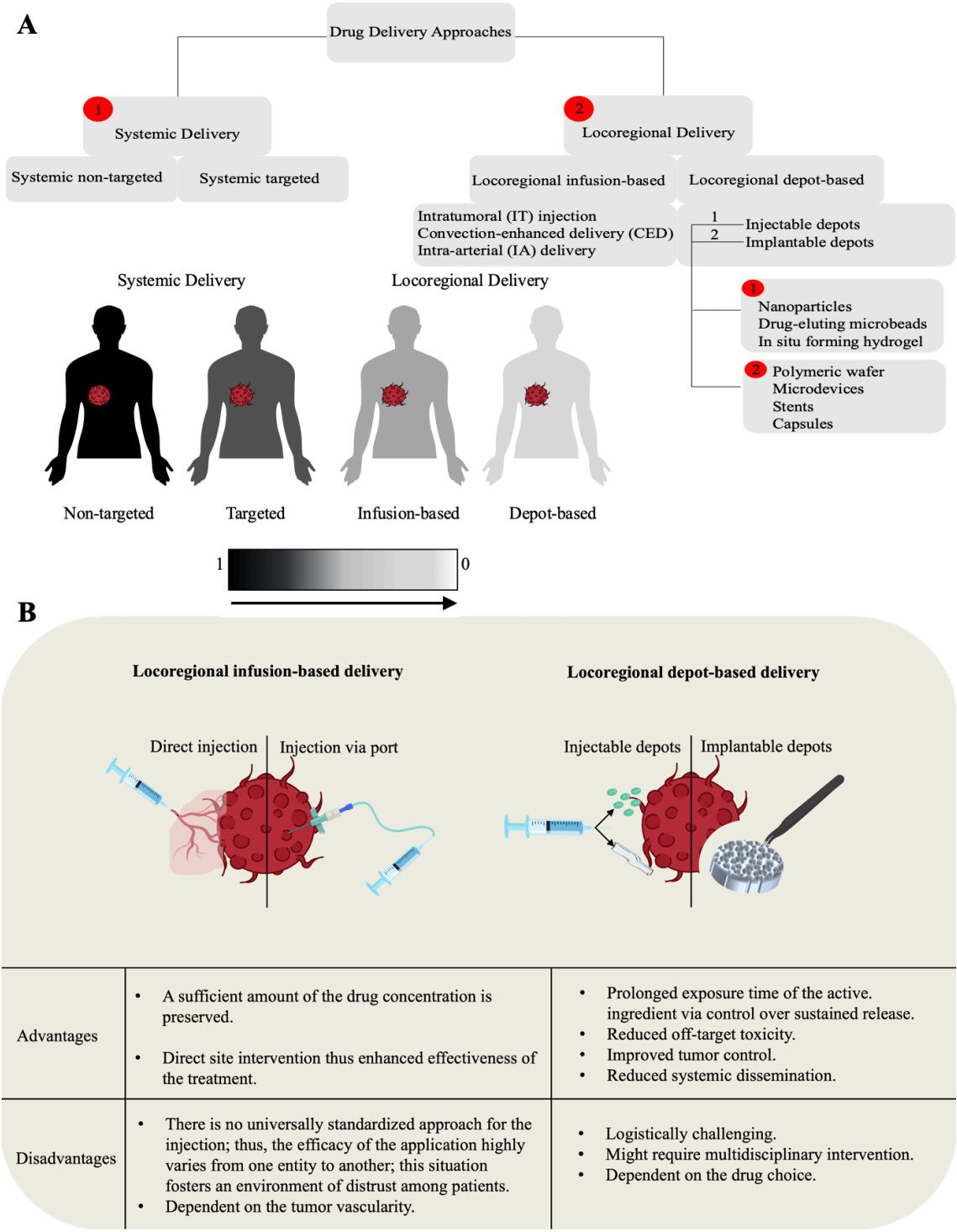
The direct injection of small- molecule drugs might diffuse quickly, variations in needle design (multi-side hole or endhole) might effect the efficacy and stability or any leakage, infection to the surrounding healthy tissue due to malposition of the catheter during the application is a substantial challenges needs to be overcome [68], [69]. Another method for delivering an active ingredient or drug near or at the tumor site is known as drug-loaded depot-based delivery [53]. This approach directly targets the tumor site and tumor cells, providing diminished side effects and avoiding reduced dosage regimens [70]. Additionally, over a systemic delivery, a depot-based approach enhances the stabilization and preservation of the embedded drug, and because of the direct delivery, drug waste is minimized [71]. Instead of bolus delivery, the controlled release of drugs through depot-based delivery systems ensures prolonged exposure to tumor cells; thus, this approach will remain active during multiple cell cycles [71]. This incidence is especially crucial for anti-neoplastic chemotherapy agents that target the cell replication pathways since only 10 to 15% of cancer cells are in the mitotic phase of cell division [53]. This might cause cancer cells to be unaffected if they are not in the division phase, and the effectiveness of the anti-neoplastic agent may be reduced due to the short exposure time of chemotherapy. In conjunction with depot-based controlled release, the efficacy of the drugs is improved, considering the prolonged presence of the drugs at the tumor site. Locoregional depot base delivery is analyzed under two main categories: injectable and implantable depots [53]. Injectable depots refer to a polymer or suspension that can be injected into the body, transforming into a depot or gel [72]. Generally, this innovative administration involves the dissolution of the biodegradable polymer within the organic solvent until a homogeneous solution is achieved. Alternatively, a suspension may be obtained, contingent upon the solubility level of the polymer [73]. Furthermore, certain depot formulations are created through the incorporation of a drug-containing alcohol functional group, which is esterified in the presence of long-chain fatty acids, resulting in an oily solution. In contrast, others are designed as aqueous suspensions of soluble salts or drug complexes [74].

Examples of injectable depots encompass nanoparticles, microspheres, drug-eluting microbeads, and in situ gels [53]. The installation procedure for injectable depots is more straightforward, as implantable depots necessitate surgical interventions for tumor lesion sites. Furthermore, injectable depots can be localized to targeted regions and enhance prolonged drug release due to their application characteristics [75]. Implantable depots consist of drug molecules combined with biocompatible polymers in the solid form [76]. This administration emphasizes the importance of delivering in a targeted manner and is considered desirable when it is essential for drug release to maintain a constant level. This method primarily involves encapsulation within a polymer based carrier in various forms. Additionally, it enhances drug delivery to effective locations without concerns regarding patient compliance [77]. This system primarily addresses challenges associated with drug stability, toxicity, duration of release, and issues pertaining to bioavailability as a result of oral administration [78]. The proposed therapy possesses the potential to overcome systemic side effects, enhance patient satisfaction, and extend the duration of action [79]. It offers several advantages, including the stability of the drug compound, which mitigates the rapid degradation of the drug within the gastrointestinal tract; a minimal effective dose, as it circumvents the first-pass hepatic metabolism, thereby improving systemic bioavailability; improved patient compliance, due to the absence of the need for repeated and timely administration; and the option for removal, as allergic reactions or adverse immune responses may be mitigated by the extraction of the implant. All these factors significantly enhance the ease of terminating drug delivery [78]. This approach must be meticulously designed for several specific occasions. For instance, the selection of materials must be biocompatible; otherwise, the implant may fail due to immune rejection or inflammation. Additionally, durability is crucial for treatments that necessitate long-term medication release. The compatibility of the implant is significantly important it must be capable of withstanding daily body movements without causing discomfort to the patient [80]. In addition to these advantages, it is prudent to take into account the biodegradability of the chosen material.

An implantable depot is designed to decompose naturally over time; to mitigate the potential loss of the drug, intelligent depots may be enhanced to release only in response to specific triggers, such as hormonal changes or temperature variations [81]. This approach encompasses a wide array of examples, including spheres, wafers, discs, films, rods, stents, and in-situ forming gels, all of which may be produced through methods such as solvent casting, extrusion, molding, or electrospinning [53].



Figure 1.1. Comparative Analysis of Drug Delivery Approaches with Representative Drug Distribution Profiles and Evaluation of Locoregional Delivery



Explanation: Classification of drug delivery approaches under two main headings with their sub-topics, along with a schematic representation of systemic non-targeted and targeted delivery, as well as locoregional infusion-based and depot-based methods, accompanied by a heat map illustrating the drug concentration required for the selected treatment application (A). An illustration of locoregional infusion-based and depot-based delivery, highlighting their main advantages and disadvantages (B).

1.3. Chemotherapeutic Agents in Breast Cancer Treatment

The treatment of breast cancer presents a significant challenge, as it involves numerous parameters to consider, such as heterogeneity, stage, and the presence or absence of specific hormonal receptors [82]. In order to select the most appropriate therapeutic strategy, it is essential that these parameters be thoroughly examined. In the treatment of BC, chemotherapeutics are utilized extensively and comprise several classifications, including antimetabolites, alkylating agents, antimitotic agents, and antitumor antibiotics [82]. These substances are typically recognized as agents that activate intrinsic apoptotic pathways within cancer cells [83]. Antimetabolites are agents that closely resemble typical intracellular components; however, they inhibit the activation of certain aberrant products that are incapable of fulfilling standard intracellular functions [82]. In short, they are categorized as agents that disrupt metabolic processes within cells; consequently, they represent promising candidates for application in cancer therapy. There are two primary pathways involved in these processes: purines and pyrimidines. Once inhibitors, known as antimetabolites, of these essential pathways are integrated into the deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), several consequences may arise, including stasis, chain termination, and cell death [84]. The working mechanism of antimetabolites involves targeting the proteins responsible for cellular metabolism. These agents are predominantly effective during the synthesis (S) phase, which is essentially the phase of DNA synthesis. They actively affect cells that are rapidly growing or dividing, or they function as building blocks for DNA and RNA, thereby inhibiting their formation and consequently halting cellular reproduction [85].

Methotrexate (MTX) is among the most widely utilized examples of antimetabolites. Main role of it is suppressing the folic acid that leads to the disruption of DNA synthesis thus destruction of cell division and tumors. widely utilized examples of antimetabolites. Main role of it is suppressing the folic acid that leads to the disruption of DNA synthesis thus destruction of cell division and tumors. It specifically binds to folic acid receptors, and the activity of dihydrofolate reductase will be inhibited. Consequently, thymidylate synthesis, which is crucial for DNA synthesis, is also affected. As a result of these inhibitions, cell proliferation is negatively impacted, ultimately resulting in apoptosis [82], [85]. Alkylating agents are among the earliest and most widely administered chemotherapeutic agents. They exert their effect by directly interfering with DNA and causing the degradation of DNA strands due to cross-linking. This circumstance leads to abnormal base pairing, thereby inhibiting cell division. Alkylating agents are present at each stage of the cell cycle, which renders them an effective choice for therapeutic applications; however, a limitation is that they are predominantly preferred for the treatment of slow-growing cancers [86]. In further detail, these molecular agents are regarded as highly reactive groups. They possess the capability to chemically bind with the nucleophilic centers, either nitrogen or oxygen, present in the DNA bases [87]. The biochemical structures of these agents are distinct from one another; however, the fundamental distinction between them is the capacity of the alkyl group to bind covalently through one or more saturated carbon atoms to the molecule. There remain several unresolved inquiries regarding the interaction that contributes to the detrimental effects on tumor cells. Since alkylating agents interact directly with DNA and possess the capability to react with nearly all molecular classes within the cell, it makes them an effective approach for treatment [88]. Cyclophosphamide and ifosfamide belong to the subclass of nitrogen mustards and have been utilized in the treatment of solid malignancies [89]. The mechanism of action of these drugs is activated through hepatic cytochrome P450 (CYP) and the process of 4-hydroxylation. The resulting compounds, following this activation, alkylate DNA, leading to DNA-DNA crosslinking, which subsequently inhibits DNA synthesis and induces apoptosis in the affected cells [90].

Antimitotic agents present great importance as chemotherapeutic agents by targeting the overactive cell cycle of cancer cells [91]. These agents disrupt mitotic progression primarily by interfering with microtubule dynamics. Microtubules, considered the cornerstone of eukaryotic cells, are polymeric structures composed of $\alpha\beta$ -tubulin heterodimers. They play key roles in cellular functions such as division and transport, making them a valid point of intervention for antimitotic agents [92]. Following the application of anti-mitotic agents, the cellular fate tends to reflect indecision during mitosis, as these agents chronically activate the spindle assembly checkpoints. This occurrence may result in various scenarios, including: direct cell death during mitosis, unequal division leading to the formation of aneuploid daughter cells, or the exit of the cell from mitosis without division, ultimately resulting in cell death during interphase or a temporary halt in that phase [93]. Antimitotic agents that are widely recognized include vinca alkaloids and taxanes, which are utilized in the treatment of various tumors, such as breast cancer, lung cancer, leukemia, and neuroblastoma. These agents primarily target mitosis by binding to β -tubulin, thereby disrupting microtubule dynamics. Specifically, while vinca alkaloids prevent the polymerization of microtubules, taxanes stabilize the already existing microtubules, thereby impeding the formation of the mitotic spindle [94]. The ineffectiveness of antimitotic agents is associated with a prevalent misconception that the proliferation rates of human cells *in vivo* are comparable to those observed *in vitro* with tumor cell lines. However, comparisons reveal that in patients, tumor doubling rates exceed those predicted by both preclinical models and cell lines. Furthermore, it has been estimated that the proportion of human cancer cells undergoing mitosis is less than 1%. In light of this information, it is imperative to explore alternative mechanisms needed [95]. Antitumor antibiotics, which serve as chemotherapeutic agents, are derived from a broader class of compounds referred to as antibiotics. It is well-established that antibiotics are agents that either kill or inhibit the growth of bacteria in the host [96]. In contrast to conventional antibiotics, anticancer antibiotics are specifically developed to possess anticancer properties aimed at combating cancer. Their primary effect is targeted at the uncontrolled proliferation and/or aggressive growth of malignant cells [97].

There are three primary mechanisms through which antitumor antibiotics exert their effects: the pro-apoptotic effect, the antiproliferative effect, and the inhibition of epithelial-mesenchymal transition (EMT). The mechanisms of action for these three include the increased expression of death receptor 5 (DR5) and fas-associated death domain (FADD), both of which are associated with apoptosis; decreased mitochondrial ribonucleic acid (mRNA) levels of SRY-Box transcription factor 2 (SOX2) and octamer-binding transcription factor 3/4 (OCT3/4), leading to a reduced ability of cancer cells; and decreased Wnt signaling and zinc finger E-box binding homeobox 1 (ZEB1), resulting in reduced tumor aggressiveness [97]. Antitumor and anticancer antibiotics, predominantly composed of anthraquinones and peptides, inhibit cellular growth by interfering with the genetic material of cells, thereby preventing the proliferation of cancer cells and the dysregulated expression of genes related to these processes overall [98]. A broad spectrum of antitumor antibiotics exists, each with various mechanisms of action. For instance, actinomycins and mithramycin function by inhibiting DNA-dependent RNA synthesis. In contrast, antibiotics belonging to the anthracycline class, such as daunomycin, adriamycin, and doxorubicin, intercalate between base pairs, which subsequently leads to the inhibition of both RNA and DNA synthesis [99]. Anthracyclines have been proven to be effective in both meta-analysis and cohort trials for breast cancer treatment [100]. The precise mechanism of action of anthracyclines is associated with two primary components: reactive oxygen species (ROS) and topoisomerase II (TOP II) however they react with cellular constituents in many different ways. These agents interfere with redox cycling, leading to the production of ROS, which subsequently results in DNA damage. More specifically, the quinones derived from anthracyclines have the capacity to convert into semiquinone radicals through the action of oxidoreductase enzymes, which facilitate the acceptance of an electron [101]. In the presence of molecular oxygen, these radicals generate superoxide anions. This process may perpetuate in a cyclic manner, culminating in the accumulation of superoxide anions [102].

If a cell has free cellular iron is present, there may be an elevation in the levels of reactive oxygen species (ROS). Additionally, the ferrous-ferric cycle may be augmented as a consequence [103]. A particular class of anthracyclines, such as doxorubicin, has the potential to form reactive nitrogen species or toxic radicals, which may ultimately result in mitochondrial dysfunction within cells [104]. The second primary component of anthracycline is Topoisomerase II (TOP II), which exists in two subtypes in mammalian: Topoisomerase II alpha (TOP II α) and Topoisomerase II beta (TOP II β) [101]. Their primary function in cancer treatment involves inducing structural alterations by facilitating the breakage of single or double strands during DNA replication, transcription, recombination, and chromatin remodeling [105]. However, their mechanisms vary. TOP II α , for instance, plays a crucial role primarily in cell cycle-related events such as DNA replication and sister chromatid segregation, as it is expressed only in proliferating and tumor cells. Therefore, there is a positive correlation between TOP II α and cell proliferation [106]. Conversely, TOP II β is expressed in all cell types throughout the cell cycle, including postmitotic and quiescent cells. In comparison to TOP II α , the precise mechanism of its action remains to be elucidated [107]. Its main role is determined to be DNA sequence rearrangements and the double-strand breakage, so without the TOP II β , antimitotic antibiotics/drugs can not be effective on the DNA directly, which highlights its importance [101], [108].

1.3.1. Mechanism of Action and Antitumor Activity of Doxorubicin

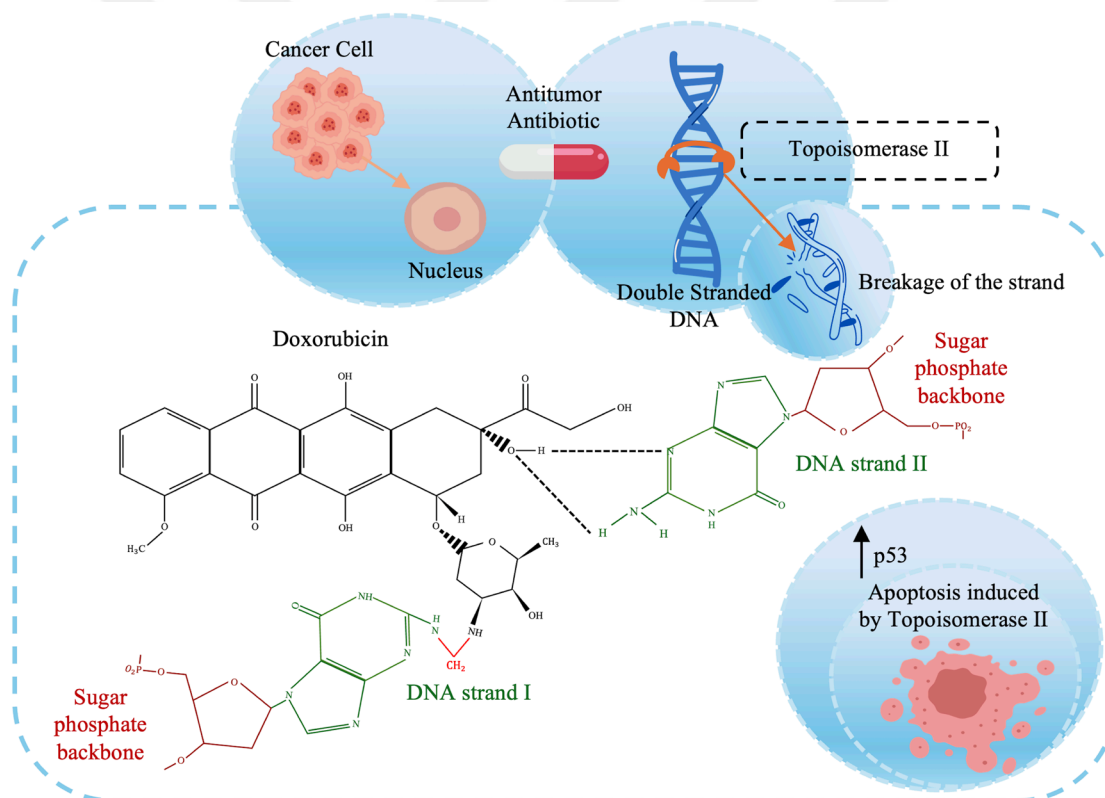
Doxorubicin (DOX), classified within the anthracycline functional group of antitumor antibiotics, is among the pioneering products employed as a chemotherapeutic agent, and its efficacy to treat solid or liquid tumors has been substantiated by numerous studies previously and currently [109]. Anthracycline groups generally exhibit planar molecular structures and induce intercalation between DNA base pairs by anchoring the sugar moieties that are positioned within the DNA minor groove [110].

DOX, commercially known as Adriamycin, possesses a molecular structure characterized by three functional domains: an anthraquinone ring, an amino sugar moiety (daunosamine), and an anthraquinone-like ring. These main components facilitate the intercalation of DNA, act as a binding agent within the minor groove, and serve as stabilizers for the DOX-DNA complex [111]. Upon the administration of DOX, its mechanism of action enables it to interact with the nucleus of cancer cells. Unlike certain other pharmaceuticals that cannot penetrate the nucleus directly, DOX is capable of entering through a concentration gradient. This phenomenon can be more comprehensively understood through the DOX-proteasome complex, which facilitates the effective accumulation of the drug within the nucleus of the cancer cell [112]. It is widely acknowledged that the precise mechanism by which DOX enters the nucleus remains inadequately characterized and controversial. Nevertheless, prior studies have explored a correlation between the proteasome and the binding of DOX, which contributes to this process [113], [114], [115]. One of the proposed mechanisms for DOX entering the nucleus works as follows: (1) DOX, a small molecule drug, diffuses across the cancer cell membrane; (2) the high binding affinity of DOX to the proteasome forms a DOX-proteasome complex instead of degrading it; (3) the presence of nuclear localization signals in the proteasome allows the complex to pass through the nuclear pores and enter the nucleus [115]. Following the entry of DOX into the nucleus, the subsequent critical step in the action mechanism of DOX involves its capacity to induce DNA damage via TOP II. The enzyme executes a critical function during essential biological processes, including DNA replication and transcription. It associates with the DNA supercoils and entangled DNA to facilitate the cleavage of the strands. Subsequently, it utilizes the gap formed to transfer to the adjacent DNA duplex and subsequently reseals the strand upon completion of the process. However, DOX molecules obstruct this activity and initiate a pathway leading to apoptosis [110]. The fundamental mechanism of DOX bears resemblance to that of the Topoisomerase II (TOP II) poison, etoposide.

It stabilizes the cleavage complex at the sites of DNA breakage by effectively trapping TOP II; consequently, the religation of the DNA strands is inhibited, leading to a disruption of DNA integrity [116]. Even though TOP II is regarded as a cornerstone, the existing literature underlying DOX's working mechanism contains studies demonstrating that cell death resulting from the administration of DOX occurs independently of TOP II [110]. The formation of DNA adducts is categorized under this subject. Rather than the impact of TOP II, the binding between DOX and DNA serves as a fundamental mechanism [117]. Owing to the specific hydrogen bonding interactions between guanine and DOX, the compound exhibits a preference for intercalation sites associated with adjacent GC base pairs, functioning as a DNA intercalator [118]. Consequently, this selective binding leads to the formation of stable DOX-DNA adducts, which activate DNA damage responses, ultimately resulting in cell death that is independent of the TOP II. Furthermore, these binding interactions are stabilized by cellular formaldehyde generated from carbon sources through free radical reactions [110], [119]. The binding complex comprises two types of bonding: covalent bonds and hydrogen bonds. Specifically, where DOX and guanine participate in formaldehyde-mediated covalent bonding, while hydrogen bonds are formed in the complementary strand [110]. The extent of formaldehyde-driven stabilization and cellular sensitivity has been observed. Elevated levels of formaldehyde have been found in DOX-sensitive tumor cells compared to normal cells. Since tumors contain formaldehyde, the formation of DOX-DNA adducts will increase, leading to the enhanced effectiveness of DOX [120], [121]. In addition to the formation of TOP II and DNA adducts, the torsional stress exerted on DNA topology warrants attention as a critical aspect of the mechanism of action of DOX. In the most stable configuration of DNA, known as B-DNA, base pairs are stacked sequentially with a counterclockwise rotation of 36 °C [110]. The application of DOX, measured at a single-molecule level, impacts DNA intercalation by inducing relaxation of the strands, accompanied by a reduction in temperature of 27 °C [122].

Consequently, this notable degree of underwinding instigates a structural alteration in the nucleosome, potentially leading to further destabilization and disassembly [110]. Although DOX is primarily recognized for its effect on TOP II inhibition effect as aforementioned, its nuclear effects are multifaceted, involving DNA adduct formation and torsional stress due to intercalation. This complexity makes the mechanism of action of DOX intricate and versatile, yet quite effective in inhibiting cancer cells.

Figure 1.2. Doxorubicin-Induced DNA Damage and Apoptosis through the Interaction with Topoisomerase II



Explanation: Illustration of the planar aromatic ring system of Doxorubicin and its covalent and hydrogen bonding with DNA strands I and II results in intercalation into the DNA due to the inhibition of Topoisomerase II, ultimately leading to the apoptosis of cancer cells with an increase in p53 protein.

Alongside the benefits of DOX mechanism of action mentioned above potential disadvantages should not be overlooked. One of the well established one is regarding dose dependent cardiotoxicity [123]. The underlying cause is linked to a few mechanism as; (1) decreased peroxidase, catalase and superoxide dismutase which involved by a oxidative stress and lowered oxidative pressure, (2) increased iron load due to impaired metabolism of iron, (3) altered homeostasis of Ca^{2+} , (4) deteriorated sarcomere structure composed of cardiac ankyrin repeat and titin protein [103], [123], [124], [125], [126].

In addition to its well-known cardiotoxicity, the cytotoxic effects of DOX also draw significant attention. The primary reason for this toxicity is the accumulation of DOX in the nucleus [123]. This phenomenon occurs due to the pH level of the drug, intrinsically referred to as 'ion trapping' [127]. DOX is classified as a weak basic drug, making it difficult to effectively target solid tumors. The acidic microenvironment of cancer cells causes DOX to uptake protons, resulting in ionization; this alteration diminishes its lipophilicity, thereby preventing the passive diffusion of DOX into the cells [123], [128]. As with any other chemotherapeutics, DOX also has unwanted side effects, in addition to cardiotoxicity and cytotoxicity, including itching, photosensitivity, nausea, vomiting, and anorexia. However, its discovery marked a paradigm shift, and as stated in meta-analysis, it still retains its status as the preferred option for cancer treatment [129], [130].

1.4. Microspheres as Drug Delivery Vehicles: General Insights and Applications

In order to mitigate the detrimental effects of DOX, a quest for targeted, effective, innovative, and supportive systems, with one promising strategy being microsphere encapsulation. These alternative delivery vehicles are utilized as localized therapy through injectable depot-based delivery systems [53]. This technique facilitates the control of drug release rates by manipulating the particle size during production, with microspheres ranging between 1 and 1000 μm [131]. Similarly, these vehicles can also be fabricated in nano sizes, referred to as nanoparticles, with dimensions varying from 10 to 1000 nm [132]. However, in comparison to nanoparticles, the advantageous surface area to volume ratio is greater in microspheres due to their larger radius. The reduced

surface area yields several benefits, such as enhanced suspension stability, increased drug loading capacity, and minimized burst release [133]. Among these benefits, the minimized burst release is especially significant in controlled drug release compared to traditional methods. In this context, therapeutics are being designed to be released over extended periods, ranging from days to months, and drug-encapsulated polymeric microspheres are among these methods. Two critical considerations warrant detailed examination for this approach: (1) drug release rates, and (2) protection of the drug. Depending on the treatment requirements, drug release rates can be tailored to meet specific needs, as some diseases require a constant release of a drug, while others necessitate a burst release at determined intervals, such as with antibiotics and vaccinations. Additionally, protecting the drug is crucial to prevent rapid destruction by the body [134]. A multitude of determinants are implicated in influencing the delivery of drug-loaded microspheres. Generally, microspheres are directed to the target site either through passive mechanisms or active means. The former is characterized by size retention, while the latter involves magnetic targeting [135]. Furthermore, the bio-distribution of the encapsulated drug is pivotal since it determines how effectively the therapeutic agent will reach and be maintained at its target site. The factors influencing this distribution can be regarded as the size and percentage of drug entrapment. However, with respect to release kinetics, the microsphere matrix serves as the most significant factor, which encompasses the type and concentration of the polymer, the ratio of the drug to the polymer, and the solubility of the drug [135], [136], [137]. Collectively, these factors exert a considerable influence on the loading efficiency of the microspheres. There are several techniques to produce microspheres; however, the double emulsion technique is regarded as the conventional and widely used method due to its ease of production and its ability to encapsulate multiple components[138]. Water in oil (W/O) emulsion is prepared as a hydrophilic emulsifier as the first step, and to obtain double emulsion droplets, single emulsions are dropped on top of the aqueous or oil phases to be re-emulsified in the constant stir and the resulting droplets are determined by the physical properties of the liquids, the stress region, and the interface [138], [139]. As mentioned above, this versatile approach offers many benefits on behalf of drug release through tailored formulations. However, loading drugs into microspheres and other carriers, such

as hydrogel systems, further enhances localized and sustained drug release, making this combination a successful post-operative treatment [140], [141].

1.5. Hydrogel-Based Drug Delivery Systems: Structure, Function, and Polymer Selection

Hydrogels are categorized as water-swollen networks that can be produced from polymers, small molecules, or colloidal particles, and they may be crosslinked either chemically or physically [142]. Hydrogels are gaining significant attention in the fields of drug delivery and regenerative medicine. Furthermore, with regard to design properties and customization capabilities, hydrogels demonstrate remarkable functionality for programmable and controlled release mechanisms, as well as accessibility within micro and nanoenvironments [143], [144]. In contrast to conventional methods of drug administration, the necessary high dosages and repeated administrations are not required to elicit a therapeutic effect. This may result in lower overall efficacy, diminished patient compliance, and a greater incidence of severe side effects, including toxicity [145], [146]. Overall the hydrogel systems must fulfill enumerated common requirements, which include drug bioactivity, transportation, storage, and chemical and physical stability [144]. Regarding drug loading within the system, two primary options are available: (1) post-loading and (2) in-situ loading. The three-dimensional hydrogel network is fabricated as an initial step, following which the drug is subsequently incorporated into the system. The drug may be utilized directly as a hydrogel precursor solution, or its encapsulation may occur concurrently through direct coupling with the polymer [147]. In the production of hydrogels, both natural and synthetic polymers may be employed. However, depending on the treatment requirements, material selection must be conducted thoroughly, taking into consideration factors such as bioavailability, biodegradability, reduced toxicity, and cell interactive properties [148], [149]. Among the widely employed polymers in tissue engineering applications, Polyvinyl Alcohol (PVA), Pullulan (PUL), and Poly(ϵ -caprolactone) (PCL) are notable examples [150], [151]. PVA is known for its hydrophilicity, biodegradability, and biocompatibility features, and it is well established that its use is common in biomedical applications.

Moreover, due to its tissue-mimicking ability and ease of water solubility, this phantom material has similarities in terms of deformation rates of elasticity. Alongside structural stability and high water content, these characteristics make it suitable for hydrogel applications [152]. PUL is a natural linear polymer polysaccharide derived from *Aureobasidium pullulans*. It stands out from other polymers due to its ability to form hydrating networks, its use in tissue engineering applications, and its sustained release properties [153]. PCL consists of long chains with high molecular weight, which affects the sol-gel formation in hydrogel production, as it creates stronger interactions between the polymer chains and is recognized as a promising application material in hydrogel fabrication [154]. Apart from its role in hydrogel production, it could be utilized in drug delivery through encapsulation. While PCL is soluble in aromatic and chlorine solvents, it is only partially soluble in polar solvents, and due to its hydrophobic nature, it is not soluble in water or alcohols. Because of its hydrophobicity, it is a strong candidate for encapsulating water-soluble drugs such as DOX. Furthermore, the oily core of the PCL capsules enhances drug stabilization in terms of phase separation. Additionally, minimal contact within the particle microenvironment will be ensured between the acid terminal group of PCL and the water [155], [156]. The administered hydrogel system for a variety of treatments not only necessitates physical and chemical characterization but also requires biological characteristics. The system that will be implemented necessitates various testing mechanisms to demonstrate its efficacy, with cellular studies, particularly in vitro types, being among the most essential tests.

1.6. Three-Dimensional Cell Culture in Cancer Research

Cell culturing was first demonstrated by Harrison in 1907, where cells were grown as a monolayer on the flat surface inside the culture flask preferable, petri dishes were also used in the same manner, which included medium at 37 °C. This conventional technique requires an alternative method due to some drawbacks, such as cell-to-cell and cell-to-extra cellular matrix (ECM) signaling.

Since these signals are significant mostly in differentiation and proliferation, 3D culture models have emerged [157], [158]. 3D *in vitro* cell culture models are recognized as a fundamental platform for investigating tumor behavior and drug response [159]. Unlike monolayer cultures, 3D models facilitate a more *in vivo*-like cellular behavior due to their capacity to replicate the native tumor environment [160]. Nonetheless, while this technique exhibits superiority over 2D cell cultures, it continues to inadequately reproduce the macroscopic structure of *in vivo* tumors. Overall, several topics have attracted significant attention regarding the comparative analysis of 2D and 3D cell cultures, including the connection of tissues to ECM, the presence of heterogeneous tumors, and the restricted availability of tissue samples. Tissues encompass interactions such as those between cells (cell-to-cell) and between cells and the ECM (cell-to-ECM), where critical biochemical signals mediate communication between receptors on the cellular surface and their environment. However, the artificial geometry inherent in 2D cultures impedes a comprehensive understanding of cellular behavior [161]. Furthermore, the cell lines primarily utilized are often derived from tumors, predominantly metastatic in origin, where the existing heterogeneity remains inadequately characterized. This lack of detailed information, coupled with the significant adaptation of cell lines to *in vitro* laboratory microenvironments, alters their genetic, transcriptional, and protein expressions in 2D cultures [161], [162], [163], [164]. Additionally, the absence of isolated tissue samples from patients constrains the exploration of potential drug developments as well as research in biochemistry and molecular cell biology. Consequently, to address these shortcomings and mitigate their impact, 2D cultures and the tumor microenvironment in 3D cultures hold considerable potential significance [161]. In the context of spatial omics, the application of *in vitro* spheroid models as 3D culture technique presents substantial potential for enhancing the understanding of the tumor microenvironment in cancer research. The scientific advantages offered by these models include a controllable environment, high throughput, accelerated processes, and a reduction in ethical concerns [165].

Building upon the comprehensive discussions presented in the preceding sections, it is essential to address the prevailing challenges associated with conventional breast cancer treatments and limited drug delivery, while minimizing systemic toxicity. Consequently, the implementation of a controlled drug-releasing hydrogel system as a postoperative treatment for breast cancer has been proposed. Within this work, a porous PVA/PUL hybrid hydrogel was produced via the lyophilization process, incorporating DOX-loaded microspheres produced via the emulsion technique with PCL into its pores. The characteristics of the system were evaluated in terms of morphology and physicochemical properties. The anti-cancer efficacy of the DOX-loaded microspheres was assessed on both the MCF-7 breast cancer cell line in 2D monolayer and 3D tumor spheroid models. Additionally, to promote healthy tissue growth within the hydrogel system while inhibiting MCF-7 cell proliferation, the L929 mouse fibroblast cell line was examined for attachment and proliferation.

MATERIALS AND METHOD

Poly(ϵ -caprolactone) (PCL; $M_w = 70.000 - 90.000$ g/mol), Poly (vinyl alcohol) (PVA; $M_w = 72.000$ g/mol), doxorubicin hydrochloride (DOX), hydrochloric acid (HCl), dichloromethane (DCM), glutaraldehyde (GA, 25% solution; $M_w = 100.12$ g/mol) and lysozyme (LYS) were the products of Sigma-Aldrich (New Jersey, U.S.A.). Pullulan (PUL; $M_w = 200$ kDa) was provided by Hayashibara Inc. (Okayama, Japan).

2.1. DOX-loaded Microsphere Production

DOX-loaded microspheres were produced by dissolving 300 mg of PCL in 6 ml of DCM (5% w/v). A concentration of 5 mg/mL of DOX was dissolved in phosphate buffer saline (PBS) (pH 7.5), and 100 μ L of this solution was added to the polymer solution. It was sonicated with a 250 W probe sonicator for 30 seconds to disperse the drug homogeneously and facilitate microsphere formation. The surfactant polymer was prepared by dissolving 1.7 g of PVA in 50 ml (3% w/v) of dH₂O at 1000 rpm and 80°C. The initial DOX-loaded solution was added to the PVA solution dropwise using a Pasteur pipette while being stirred at 1000 rpm, and the solution was left stirring overnight. Microspheres were filtered after 24 hours and dried at room temperature for 2 days. After the microspheres were completely dried, they were scraped from the filter paper.

2.2. DOX-PCL-MSs Loaded Hydrogel Production via Lyophilization/embossing Ice Particulate

Ice particles containing DOX-PCL-MSs are loaded into the hydrogel for specific surface texture and porosity by combining embossed ice particles and freeze-drying methods. Encapsulated ice particles were created by evenly distributing 40 mg of DOX-PCL-MSs in dH₂O using a spray bottle and spraying it onto the surface of an aluminum-covered 12-well plate. It was sprayed exactly eight times during each production run to ensure a consistent average diameter for the ice particles. The hydrogel was produced with a blend

of PVA and PUL in a 1:1 weight ratio by dissolving 0.2 g of each polymer in 4 mL of dH₂O at 80°C. Once a homogeneous solution was obtained, 100 µL of 25% glutaraldehyde solution was added to the PVA/PUL blend and stirred at 60°C for 1 hour. The solution's pH was adjusted to 2 by adding 0.01 M HCl. The homogeneous PVA/PUL blend solution was cooled in the freezer just before it lost its liquid form to avoid the melting of ice particles that were being added. The previously formed ice particles containing DOX-PCL-MSs were added to the polymer solution in the 12-well plate, and the plate was stored at -80°C overnight before being freeze-dried (Hanil Scientific Inc., Korea).

2.3. Analysis of Degradation and Water Retention

10×10×5 mm³ of freeze-dried hydrogels were cut into rectangular prisms. The initial dry weight of the hydrogels was measured and placed in a PBS solution (0.1 M, pH 7.4, 5 mL) containing sodium azide and lysozyme enzyme at concentrations of 0.01% and 0.1%, respectively. Hydrogel samples were incubated in a shaker incubator (JSR JSSI-300C, Korea) at 37°C, with the media replenished every three days. Samples were lyophilized for 24 hours at various time points for weight recordings. The *in vitro* degradation rate was determined by the equation (2.1).

$$Weight\ loss\ (\%) = \frac{(W_0 - W_t)}{(W_0)} \times 100 \quad (2.1)$$

where W_0 represents the initial dry weight of the hydrogel sample and W_t is the dry weight of it after the degradation at time t in the equation as mentioned above. Water retention was evaluated by removing the superficial water from hydrogel samples with filter paper. The wet weight at time t was recorded (W_t), and the water retention was determined by equation (2.2).

$$Water\ retention\ (\%) = \frac{(W_t - W_0)}{(W_0)} \times 100 \quad (2.2)$$

2.4. Controlled Release Study of DOX and DOX-loaded Microspheres

DOX releasing from hydrogels and DOX releasing from microspheres were studied separately. First, comparison of the releasing activity of DOX from PVA/PUL hydrogels was conducted with various groups as free DOX-loaded hydrogel (DOX was encapsulated by embossing ice particulate, and surface pores of the hydrogel was loaded), hydrogel carrying DOX-PCL-MSs (DOX-PCL-MSs were encapsulated within the surface pores of the hydrogel through the process of embossing ice particulate) and for the physical release, hydrogel surface loaded with DOX-PCL-MSs (surface pores were loaded with DOX-PCL-MSs) were produced. Additionally, free DOX release from microspheres (DOX-PCL-MSs) was studied. For free DOX-loaded hydrogel and hydrogel carrying DOX-PCL-MSs groups, they were cut into quadrants and incubated in PBS (2 ml, pH 7.4, 0.01 % sodium azide) at 37°C in a shaking water bath for 16 days. Samples were collected from the release media at predefined time intervals and their optical density was measured at 570 nm via UV spectrophotometer (PG Instruments Ltd.,UK). The dependent physical release measurement for the hydrogel surface loaded with DOX-PCL-MSs (surface pores were loaded with DOX-PCL-MSs) group was studied in release media through 16 days as other hydrogel groups; however, at incubation time points, samples were taken out from the release tube and the media containing DOX-PCL-MSs were mixed with redispersing aggregated microspheres. 100 μ L of samples were collected from the media, and under an inverted microscope, microspheres were counted. For the study of DOX-loaded PCL microspheres (DOX-PCL-MSs), 50 mg of microspheres was resuspended in the release media and placed in a shaking water bath at 37°C for 16 days, during which their density was measured using a UV spectrophotometer set to the same wavelength as the hydrogel groups.

2.5. Analyses of Scanning Electron Microscopy

The microporous surface of the PVA/PUL hydrogel produced by lyophilization was analyzed using high-resolution field emission scanning electron microscopy (FE-SEM) with a SU7000 from HITACHI, Japan. Additionally, a 5 nm gold coating was applied using a high vacuum sputter coater (EM ACE 600, Leica, Germany). Meanwhile, PCL microspheres and PVA/PUL hydrogel loaded with PCL microspheres were analyzed with a benchtop scanning electron microscope (JCM-7000 NeoScope™, JEOL, Japan). The dimensions measured from SEM micrographs were analyzed using Image J software (National Institutes of Health, USA).

2.6. Analysis of Fourier Transform Infrared (FTIR) Spectroscopy

The chemical composition of the samples was analyzed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Using a Nicolet iS50 FTIR spectrometer equipped with an ATR accessory (Smart iTX, Thermo Fisher Scientific Inc., USA) the IR spectra were recorded. The mid-infrared region ($500\text{--}4000\text{ cm}^{-1}$) and resolution of 4 cm^{-1} was used in the absorbance mode for samples at room temperature while they were placed on the ATR crystal plate.

2.7. Studies of *In Vitro* Cell Culture

Optimal *in vitro* conditions for the MCF-7 cell line were prepared. DMEM medium with supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin was used for cell culture. A carbon dioxide incubator (New Brunswick, Galaxy 170 S, Eppendorf, Germany) was used at 37°C for the incubation of MCF-7 cells. To maintain the optical “in order to ensure the appropriate conditions for cell proliferation culture media was replenished every three days. Once 80% of the confluency has reached cells were subcultured to maintain growth culture.

2.7.1. Development of Multicellular Spheroids via the Hanging Drop Method: A 3D Cell Culture Approach

20 ml of PBS was poured down to the bottom of the 100 mm sterile culture dish to create a high-humidity environment for droplets. MCF-7 cells containing suspension were prepared at 5×10^5 cells/mL concentration. From the suspension 20 μ L (containing 1×10^4 cells per each) were taken out and seeded carefully onto the dish lid in a droplet formation. The lid containing MCF-7 cell droplets was inverted and placed onto the PBS-containing bottom. Culture dishes were placed inside the CO_2 incubator for approximately 3 to 4 days until cell aggregation (spheroid) formation was obtained. On a daily basis, the medium of the droplets was checked and replenished as necessary with 10 μ L of fresh cell medium to preserve the optimal conditions for spheroid formation.

2.7.2. Assessment of DOX and DOX-loaded Microspheres in 3D Tumor Spheroids

DOX and DOX-PCL-MSs were studied separately in 3D tumor spheroids. Inside DMEM, DOX concentrations were prepared as follows: 100, 75, 50, 25, and 12.5 μ g/mL. MCF-7 spheroids were seeded as droplets of 20 μ L. After they were well formed, 10 μ L was replenished with 10 μ L of the DOX concentration (for each concentration separately). Over a period of 14 days, images of the tumor spheroids' integrity were captured via an inverted microscope (OLYMPUS, CKX4, UK). Furthermore, to analyze and observe differences, DOX-loaded microspheres containing equivalent amounts of DOX were examined for their release concerning the spheroid's structure and integrity.

2.7.3. *In vitro* Evaluation of Cell Viability

In vitro evaluation was conducted using MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] and deep blue. Three different experiments were performed to assess cell viability on MCF-7 cells with plain DOX and DOX-loaded hydrogels, DOX-PCL-MSs carrying hydrogels, and free hydrogel (PVA/PUL).

The viability of the MCF-7 cells was measured with the MTT assay. Cells were seeded (1×10^4) in a 96-well plate with gradually increasing concentrations of DOX (1.5, 3.125, 6.25, 12.5, 25, 50, 75, 100 $\mu\text{g/mL}$). The plate was left inside the incubator at 37°C for 24 hours. The effect of DOX concentrations on MCF-7 were analyzed under the inverted microscope (OLYMPUS, CKX4, UK) after incubation period ended. Following this, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well in the plate and incubated for 4 hours in a CO_2 incubator at 37°C. After removing the MTT, 100 μL of DMSO was added to dissolve the formazan crystals of the dye. The 96-well plate was placed on a shaker at 350 rpm for 45 minutes to ensure even distribution of the MTT. Density measurements were performed using a multimode microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, USA) at 570 nm. The anticancer activity of the hydrogel groups was evaluated using a deep blue assay. MCF-7 cells were seeded on 12-well plates and incubated for 24 hours. Cell strainers were placed on top of the wells with varying concentrations of DOX-PCL-MSs (12.5, 25, and 50 $\mu\text{g/mL}$) in PVA/PUL hydrogel groups and incubated for an additional 24 hours to observe the release activity on MCF-7 cells. After removing the strainers, 10% of the deep blue solution was added and incubated for 4 hours. The deep blue-containing solutions were transferred to a new plate for fluorescence measurement with a microplate reader, measured at 535 nm excitation and 590 nm emission. The same steps were repeated after 1, 4, and 7 days of incubation. For an additional comparison of the anticancer activity of the hydrogel groups, the free hydrogel group (PVA/PUL), the DOX-loaded hydrogel group (with DOX encapsulated into the surface pores of the hydrogel at 50 $\mu\text{g/mL}$), and the hydrogel carrying DOX-PCL-MSs (the hydrogel/microsphere carrying 50 $\mu\text{g/mL}$ DOX) were compared by measuring cell viability using the Deep Blue assay, following the same procedure explained above.

2.7.4. Cell Attachment Analysis

The surface morphology of the hydrogel, with L929 cell attachment, was analyzed using FE-SEM (SU7000, HITACHI, Japan) after 7 days of incubation. After the incubation period, paraformaldehyde (4%) in PBS was used to fix the cells. The hydrogels were incubated at room temperature for 10 minutes, then rinsed twice with PBS and discarded. Beginning with a 30% ethanol concentration, the hydrogels were incubated in 10-minute intervals until reaching 100%. Once the ethanol was removed, hexamethyldisilazane (HMDS) was applied for 20 minutes to facilitate the final drying stage of the hydrogel samples. In the final stage, the samples were coated with a 5 nm gold layer in a sputter coater (EM ACE 600, Leica, Germany) for SEM examination.

2.8. Statistical Analysis

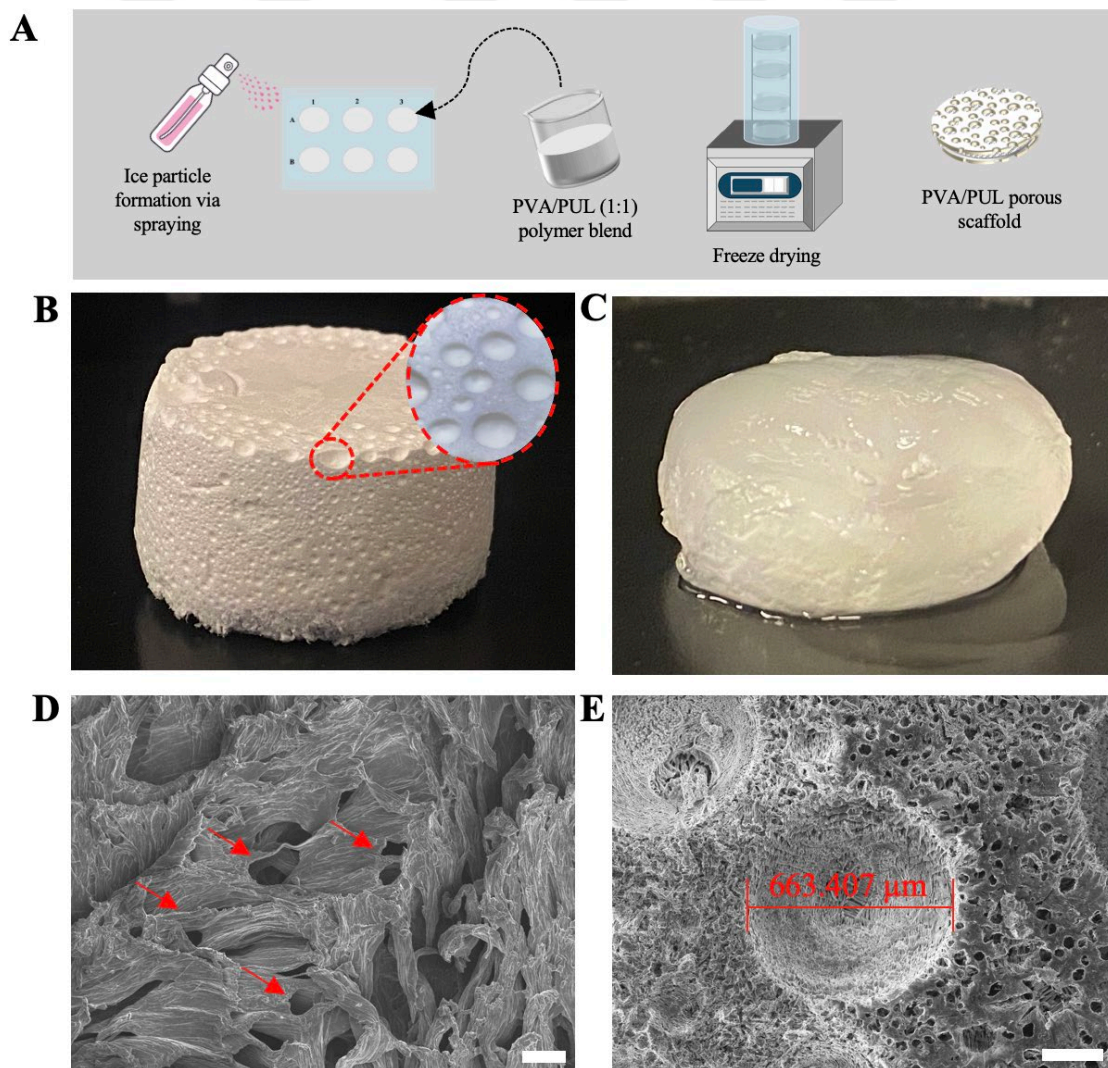
The quantitative evaluation of the results was performed using SPSS software (version 22, IBM, USA) and various statistical methods, including the independent samples t-test and One-Way Analysis of Variance (ANOVA). The comparison of mean values and the measurement of statistical significance between the groups were evaluated. The level of significance was set at $P < 0.05$.

RESULTS

The PVA and PUL are polymers prevalent in the field of drug delivery, according to the literature, due to their advantageous features as biodegradable supports, along with their robustness, high mechanical strength, and ease of preparation [153], [166]. In this study, PVA and PUL were selected for the production of a drug-releasing hydrogel, drawing upon findings from previous research. The porous structure of the PVA/PUL hydrogel was produced via lyophilization, and larger surface pores were formed using an embossing ice particulate technique which was illustrated as the production process (Fig.3.1A). The embossing of the hydrogel surface was accomplished by applying a spray method onto the surface of a well plate, resulting in the formation of ice spheres prior to the incorporation of the PVA/PUL polymer blend. The morphology of the PVA/PUL hydrogel, both in dry and wet states, is depicted (Fig.3.1B-C), where the porous structure of both the surface layer and the interior was meticulously examined, the micropores on the surface was shown with the red dotted circle (Fig.3.1B). The selected production techniques besides its ease of production, enabled convenient tailoring of the hydrogel's important characteristics, such as porosity, which is highly correlated with the storage and drug release rates [167]. Based on SEM analysis, the microstructurally aligned porous structure was observed, and the average pore size of the hydrogel was determined to be $67.5 \pm 21.9 \mu\text{m}$ (Fig.3.1D). According to existing literature, this pore size range is considered favorable for facilitating cellular penetration, promoting tissue ingrowth, and enhancing blood vessel development [168]. The larger formations of the microchambers, produced through the application of ice droplets onto the surface of a mold in contact with the hydrogels, were determined to have an average diameter of $271 \pm 172 \mu\text{m}$. However, larger pores were also observed as $663.407 \mu\text{m}$ (Fig.3.1E). In a study conducted by Ghosh et al., it is observed that larger pore sizes serve as more accessible vehicles for drug entrapment compared to smaller pore hydrogels. The findings of the study conclude that larger pores facilitate bolus delivery, whereas smaller pores provide a smaller and more consistent release profile [169].

Variations in pore size contribute differently to the overall functionality of the drug delivery system, impacting its efficacy. Considering all these values, the pore size parameter of the scaffold is of significant importance, as it serves as the primary determinant of cell penetration, cell viability within a 3D network, nutrient and oxygen diffusion, waste removal, as well as cell-to-cell interaction, transmigration, and cellular ingrowth [170].

Figure 3.1. Schematic Representation and Morphological Analysis of the PVA/PUL Hydrogel

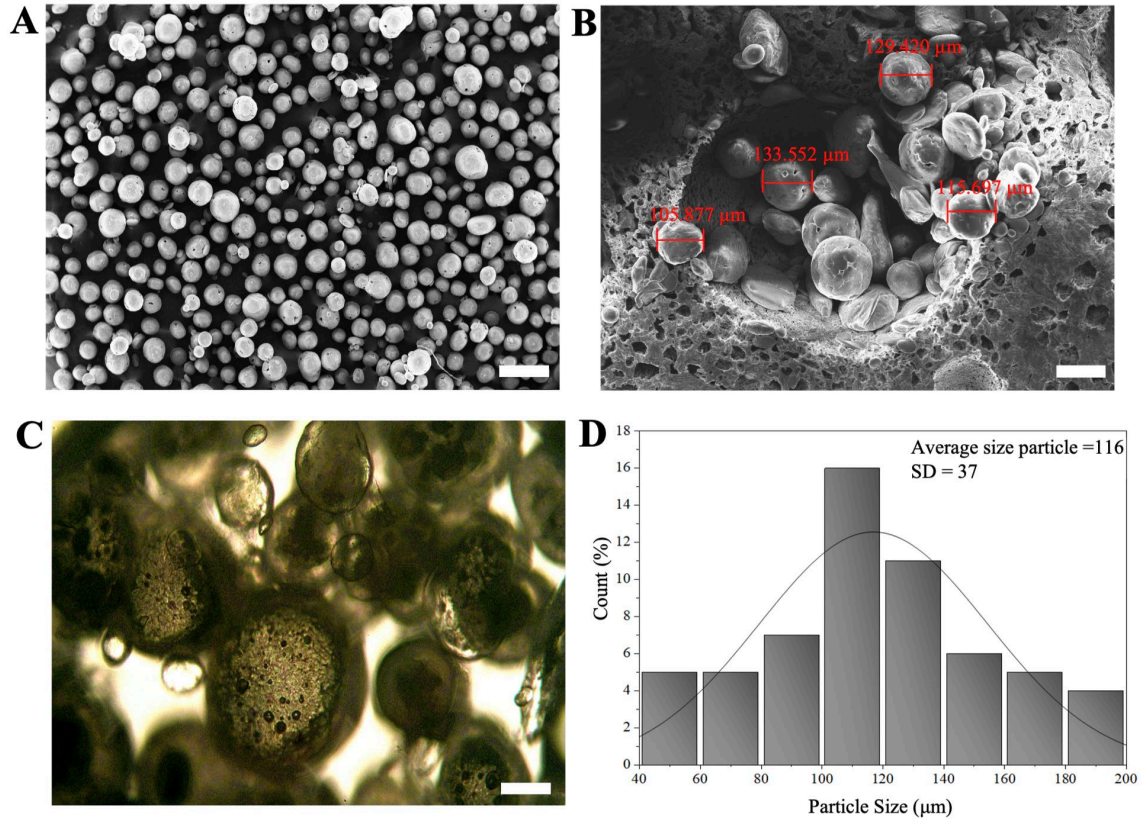


Explanation: Illustration of the production steps for the PVA/PUL blend hydrogel (A). The macro morphology of the freeze-dried hydrogel formation and the macropores achieved through lyophilization/ice embossing are presented within a red circle by light microscopy under 30x magnification (B). The wet/non-freeze-dried morphology of the hydrogel (C). Scanning electron microscopy image of the inner pores at 500x magnification of the hydrogel, indicated by red arrows (scale bar: 100 μ m) (D). Scanning electron microscopy image of the macropore on the surface of the hydrogel at 75x magnification; length analysis was performed using ImageJ software (scale bar: 200 μ m) (E).

The morphology of empty and DOX-PCL-MSs was examined using both SEM and an inverted microscope (Fig.3.2). The SEM analysis of PCL-MSs has revealed a non-uniform size with predominantly spherical morphology (Fig.3.2A), however, the close diameter of spheres has shown a structural consistency and suggests reproducibility. The average diameters of the PCL-MSs were found to be relatively close to each other: 105.877 ± 43.418 , 133.552 ± 28.971 , 129.420 ± 37.021 and 115.697 ± 52.207 μ m (Fig.3.2B). The smooth surface characteristics of the empty PCL-MSs indicate a successful production method: the water-oil-water emulsion technique. Furthermore, the choice of surfactant, PVA, has reduced the interfacial tension between the organic and aqueous phases, which is directly correlated with the smooth formation of the spheres. A non-porous, dense polymeric matrix, covering the outer surface of the PCL-MS is directly correlated with the kinetics of drug release [171]. To minimize the burst effect associated with DOX release, a dense matrix is necessary as opposed to a degraded matrix. Alongside these characteristics, the stability of PCL, with its extended degradation times, further contributes to the controlled release of the drug. The DOX-PCL-MSs produced via the embossing ice particulate technique were subsequently loaded into the surface-open micropore network of the lyophilized PVA/PUL hydrogel successfully (Fig.3.2B). The porous nature of the hydrogel enabled microspheres to be entrapped in the cavities, thus enhancing the localized and sustained release of DOX-PCL-MSs with this structural integrity.

This technique aimed at localized cancer treatment, and according to the literature, similar studies resulted in reduced systemic toxicity. The encapsulated drugs remained at the tumor site without harming the healthy surrounding tissues [172]. The non-uniform size distribution and spherical morphology of DOX-PCL-MSs were observed under an inverted microscope (Fig.3.2C). A faint pinkish color was observed due to the coloration of DOX itself, indicating successful encapsulation of the drug in PCL. Microspheres are well-established carriers capable of encapsulating both hydrophilic and hydrophobic drugs [173]. Additionally, characteristics such as polymer choice, solvent, and appropriate hydrophilicity enhance the encapsulation capacity [174]. In this study, a hydrophobic polymer, PCL, was selected as an outer surface for the hydrophilic anthracycline antibiotic DOX. The inherent hydrophobic and hydrophilic interaction elicits sustained drug release. The encapsulation efficiency of DOX-PCL-MSs was calculated to be $70.26 \pm 5.46\%$. For the water-in-oil (W/O) technique, a volume ratio of 1/60 was obtained, as 100 μl of DOX solution (500 $\mu\text{g/mL}$) was dispersed in 6 ml of DCM containing 300 mg of the organic phase with PCL. The final encapsulation concentrations of microspheres are associated with several characteristics, including the time interval between emulsion steps and the number of rinsing steps. Previous studies employing the oil-in-water (O/W) technique indicated that the encapsulation efficiency of PCL microspheres measuring between 50-100 μm and 100-200 μm was observed to be as low as 0.97%. In light of these findings, the challenge of incorporating hydrophilic drugs into hydrophobic carriers has been acknowledged [175]. The average size distribution of DOX-PCL-MSs was measured using ImageJ software found to be $116 \pm 37 \mu\text{m}$ and resulting data was analyzed with a histogram using Origin software (Fig.3.2D). The particle size of the microspheres significantly affects sustained drug release. Smaller diameters of microspheres, around 100 μm , demonstrate excellent sustained release and high encapsulation efficiency, ranging from 70% to 100% [176]. According to the results obtained (Fig.3.2), well-defined spherical microspheres with a great encapsulation efficiency were achieved for facilitating efficient sustained release.

Figure 3.2.PCL Microsphere Morphology and Size Analyses



Explanation: The scanning electron microscopy image of empty PCL microspheres with 100x magnification (scale bar: 200 μm) (A). The scanning electron microscopy image of PCL microspheres entrapped in a PVA/PUL hydrogel with 100x magnification (scale bar: 100 μm) (B). Inverted microscope image of DOX-loaded PCL microsphere (100 μg/ml) morphology at 20x magnification (scale bar: 50 μm) (C). Size distribution of PCL microspheres; analysis was performed using OriginPro software (D).

The ATR-FTIR spectral results of PUL, PVA, and the PVA/PUL hydrogel are presented (Fig.3.3B) alongside a table of absorption bands and vibrational assignments (Fig.3A) [177], [178], [179], [180], [181]. Each relevant peak number has been highlighted with an asterisk, where peaks 1 to 4 correspond to PUL, 5 to 9 correspond to PVA, and 10 to 11 correspond to the PVA/PUL hydrogel (Fig.3.3B). The characteristic stretching mode of $O-H$ bond corresponding to the PUL was obtained at 3297 cm^{-1} (peak number 4), alongside the $SP^3\text{ C-H}$ bond at 2926 cm^{-1} (peak number 3). Additionally, a notable peak of the $O-C-O$ bending was detected in both PUL and PVA/PUL groups. Furthermore, bendings were observed at 753 cm^{-1} and 927 cm^{-1} for $\alpha-(1,4)$ and $\alpha-(1,6)-D-$ glycosidic bonds, respectively [182]. The stretching vibration of the $C-O-C$ glycosidic bond at 1146 cm^{-1} (peak number 1), was observed in the PUL structure. In light of this information, the increased intensity at 1146 cm^{-1} (peak number 11), for the PVA/PUL hydrogel indicates successful blending; with the addition of the GA crosslinker the intensity of the $C-O-C$ stretching is enhanced due to the formation of aldehyde groups ($-CHO$) and hydroxyl groups $-OH$ present in PUL [183]. For PVA the stretching characteristic peaks $O-H$ were observed at 3276 cm^{-1} . Additionally, both asymmetric and symmetric stretching of the $C-O$ bonds were observed at 1420 cm^{-1} , as well as stretching shoulder of $C-O$ at 1142 cm^{-1} . Furthermore, the stretching of $C=O$ and the bending of OH were recorded at 1085 cm^{-1} (peak number 5) [184]. The spectral peaks observed at 1085 cm^{-1} (peak number 5), and 1018 cm^{-1} (peak number 10), within the PVA/PUL spectrum signify the overlap of $C-O-C$ vibrations, with a shoulder at 1018 cm^{-1} (peak number 10), indicating potential coupling among polymeric components. The characteristic peaks of GA are identified between at 3000 cm^{-1} and 3600 cm^{-1} corresponding to the $O-H$ stretching and around 1600 cm^{-1} related to the $C=O$ stretching. The concentrations of GA and the polymers employed are not equivalent, resulting in overlapping stretching peaks of $O-H$ and $C-H$ with those of PVA and PUL. However, the decrease in transmission at 1637 cm^{-1} in the PVA/PUL hydrogel spectrum may support the evidence of unreacted $C=O$ groups from GA [185]. Additionally, the peak representing $C-O$ at 1085 cm^{-1} (peak number 5) for PVA spectrum exhibited a broadening between 1100 cm^{-1} and 1000 cm^{-1} following the GA

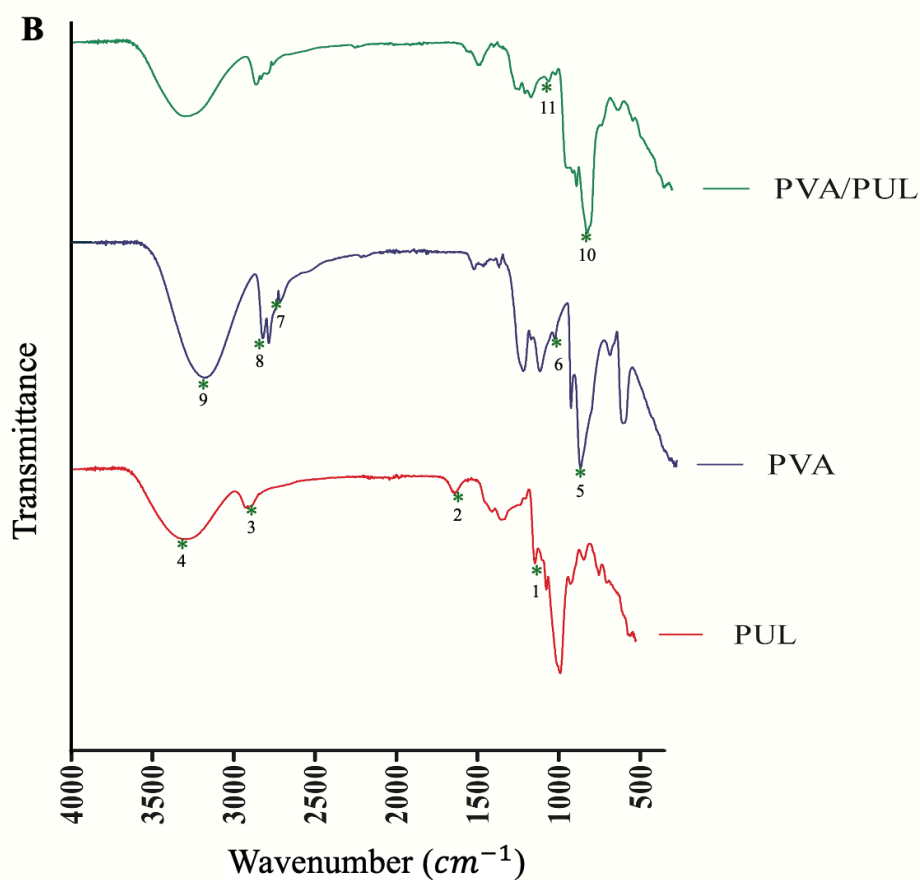
crosslinking; which correlates with the formation of acetyl bonds ($C - O - C$) through the crosslinking process. The evidence presented through the combined spectral features indicates the occurrence of GA crosslinking and the blending of polymers were shown.

Figure 3.3. ATR-FTIR Spectra of PUL, PVA and the PVA/PUL Hydrogel

A

FTIR absorption bands and vibrational assignments of PVA, PUL, and PVA/PUL hydrogels

Peak Number	Absorption band (cm^{-1})	Mode of vibration	Functional group/assignment
1	1146.71	C-O-C stretching	Glycosidic linkage
2	1637.21	O-C-O stretching	Carboxylate group
3	2926.14	C-H stretching	Aliphatic C-H
4	3297.89	-OH stretching	Hydroxyl group
5	1085.95	C=O stretching	Carbonyl group
6	1142.04	C-O stretching	C-O single bond
7	2904.30	C-H stretching	Aliphatic C-H stretch
8	2926.14	C-H stretching	Aliphatic C-H stretch
9	3276.68	-OH stretching	Hydroxyl group
10	1018.63	C-O-C stretching	Glycosidic linkage
11	1146.72	C-O-C stretching	Glycosidic linkage



Explanation: Table of peak numbers for the polymers (PVA,PUL) and the PVA/PUL hydrogel, along with their representative absorption bands, modes of vibrations, and functional groups (A). FTIR spectra of the PVA,PUL, and PVA/PUL hydrogel scaffold (B).

The cumulative weight loss, water retention, and cumulative DOX microsphere release graphs are presented alongside a schematic representation of the varying samples (Fig.3.4). The structural stability and fluidic management were evaluated under aqueous conditions. Although the fabrication techniques of PVA/PUL hydrogel groups maintained the exposure time of GA was changed to 30 minutes, and prolonged to 1 hour, this critically influenced the behavior of degradation and water retention is discussed. The degradation behaviors of PVA/PUL hydrogel were systematically examined over a duration of 12 days while being maintained within a shaking water bath, supplemented with PBS and lysozyme (Fig.3.4B). The hydrogel exposed to 30 minutes of GA (30 min-GA) exhibited a rapid degradation of approximately 40% of its initial weight during the first three days. In contrast to this aggressive loss, no significant variation was detected during the subsequent nine-day period. The cumulative weight loss of the hydrogel that was exposed to 30 min-GA stabilized at $35.85 \pm 8.47 \%$. This result may suggest that the limited exposure time to GA influences the degradation process and may result from a burst of hydrolytic degradation due to the limited cross-linking density. According to prior studies in the literature, GA has been recognized as an inexpensive and rapid stabilizer, and in terms of rheological properties, it significantly affects the characteristics of hydrogels [186]. Furthermore, when compared to the results of this study, the cumulative weight loss of the 1 hour of GA (1-h-GA) exposed hydrogel is consistent with the findings in the literature. Within the first three days, approximately 5% weight loss from its initial mass was observed. Following the third day, a significantly more gradual reduction was observed, culminating in a loss of only $16.08 \pm 7.03 \%$ by day 12. The initiation of gelation processes of hydrogels exhibited variability; specifically, a duration of 30 min-GA was insufficient to immediately initiate gelation upon the addition of HCl. Conversely, a duration of 1-h-GA resulted in gelation occurring promptly following the

addition of HCl, which effectively lowered the pH 2. These findings suggest that prolonged duration of GA results, indicates an improvement in stability attributable to the denser polymeric network, which provides a substantial advantage for controlled release studies [187]. The overall results regarding weight loss of the PVA/PUL hydrogels indicate that the 30 min-GA group exhibited a greater loss compared to the 1-h-GA group.

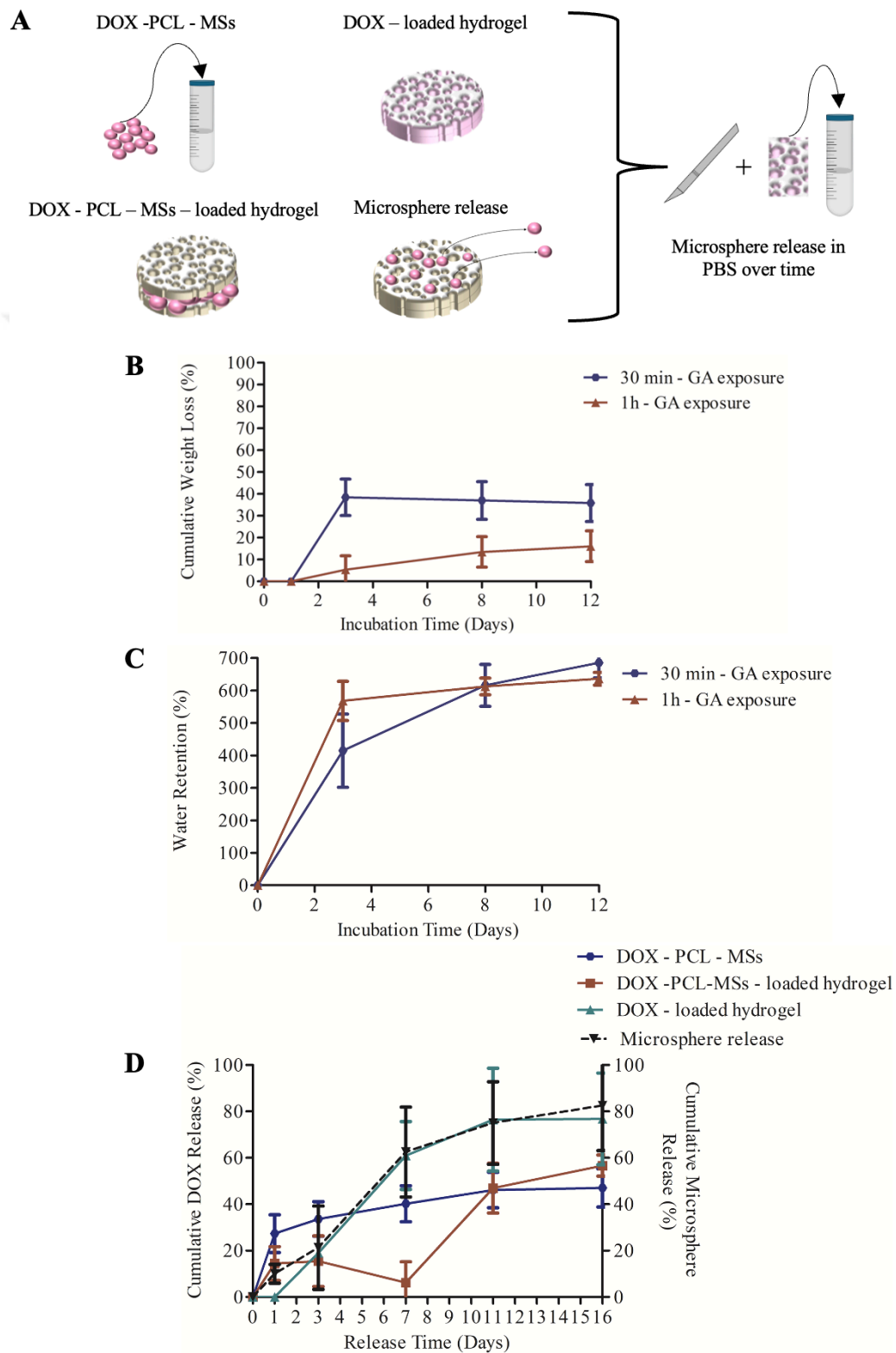
The water retention capacity of the PVA/PUL hydrogels is presented in Figure 3.4C. The groups were significantly influenced by the exposure time during GA crosslinking. Over the first three days, the groups exposed to 1-h-GA demonstrated a higher water retention of 568.35 ± 60.42 %, whereas the group exposed to 30-min GA exhibited a lower retention of 414.92 ± 112.93 %. However, between days 8 and 12, the difference between the hydrogel groups narrowed slightly. Beginning on day 8, the 30-min GA group demonstrated a higher water retention capacity of 616.25 ± 64.45 % compared to the 1-h-GA group, which was recorded at 612.96 ± 25.56 %. This behavior may be attributed to the influence of exposure time related to GA. The longer duration of crosslinking exposure resulted in a more resilient and intact structure for the 1-h-GA group. According to similar studies, it has been demonstrated that prolonged exposure times or increased amounts of GA significantly influence the morphology, stability, and mechanical properties, which in turn affects the water retention ability of the system [188], [189]. With these findings, it was demonstrated that (Fig.3.4C) the exposure time of the crosslinker impacted the swelling behavior of PVA/PUL hydrogels. The 30-min GA group resulted in higher water uptake, leading to increased swelling behaviour due to the shorter exposure time of GA, which created a loosely crosslinked network. In contrast, the 1-h-GA group, having been exposed longer to the crosslinker, formed a denser and more rigid hydrogel structure, thereby limiting its capacity to absorb water. The cumulative release behaviors of DOX from several formulations, including microspheres and hydrogels, over 16 days are presented in Figure 3.4D. Several release behaviors were studied, as illustrated in Figure 3.4A. There are four different groups, which are explained in detail under Section 2.4. The percent cumulative release of DOX-PCL-MSs showed a sustained and controlled release over the incubation period. On the first day, a burst

release of $27.35 \pm 8.11\%$ was observed; however, on day 11, the release percentage increased to $46.13 \pm 7.63\%$. The inherent characteristics of PCL, including hydrophobicity and degradation resistance, limited the release rate, thereby promoting sustained release [175]. Additionally, the slow and sustained release of DOX-PCL-MSs indicated that structural integrity was maintained.

The encapsulation with PCL has created a diffusion-limiting barrier, promoting a gradual release. Consequently, the encapsulated drug was released either through diffusion across the PCL matrix or through surface erosion [190]. In addition to the DOX-PCL-MSs, two distinct hydrogel systems were studied as well. These were in the forms of DOX-PCL-MSs-loaded hydrogel and plain DOX-loaded hydrogel. The first hydrogel group was investigated to analyze the dual barrier system, featuring an outer layer of PCL and a hydrogel matrix, to enhance the understanding of the microsphere release. The second group was incorporated to observe the behavior of free DOX under the same polymeric conditions without the presence of an outer barrier. DOX-loaded microspheres embedded within the hydrogel system (of DOX-PCL-MSs-loaded hydrogel) exhibited a delayed release, with no burst release observed. The release on the first day reached $14.44 \pm 7.29\%$, and it remained below 20 % throughout the 7 period. The presence of sequential barriers influenced the behavior of DOX release. A significant increase was observed on day 11, with a release of $46.93 \pm 10.73\%$ and at the end of day 16 it reached to $56.6 \pm 4.5\%$ only. Free DOX-loaded hydrogels exhibited a greater and more linear release profile in comparison with other groups since there are no barriers and no control over the system. Starting from day 7 ($60.98 \pm 14.60\%$), it showed a superior release reaching up to $76.79 \pm 19.72\%$. Since there is no outer protective layer (encapsulation) for DOX and both PVA and PUL have a hydrophilic nature, additionally, the hydrogel has pores, DOX molecules diffused readily compared to PCL-MSs groups [191], [192], [193]. Therefore, this system may be beneficial as prompt therapeutics; however, the lack of a secondary diffusion barrier limits its use for sustained and controlled applications. These results highlight the need and importance of a secondary barrier for drug release, such as

polymeric shells, as fabricated for this study. In addition to the cumulative DOX release analysis from both microspheres and hydrogel, a physical release of microspheres from the hydrogel matrix was investigated over time. The quantitative physical detachment was performed. Unlike other groups, instead of using UV-Vis spectrophotometry, released microspheres were counted under a microscope and analyzed using ImageJ software, depicted as a dotted line (Fig.3.4D). On the first day, only 10.00 ± 4.08 % of the microspheres were released into the medium. However, after day 7, similar to the other groups, the release percentage increased to 62.50 ± 19.36 %. At the end of the incubation period (day 16), the release of microspheres was observed to be the highest among all groups at 82.50 ± 19.36 %. These results demonstrate that a progressive and gradual physical release from the pores of the hydrogel was achieved. Moreover, in addition to the encapsulated DOX, the carrier itself, which is the PCL microsphere, has been proven effective in the drug release system.

Figure 3.4. Cumulative Weight Loss, Water Retention, and Cumulative DOX Release from the PVA/PUL Hydrogel



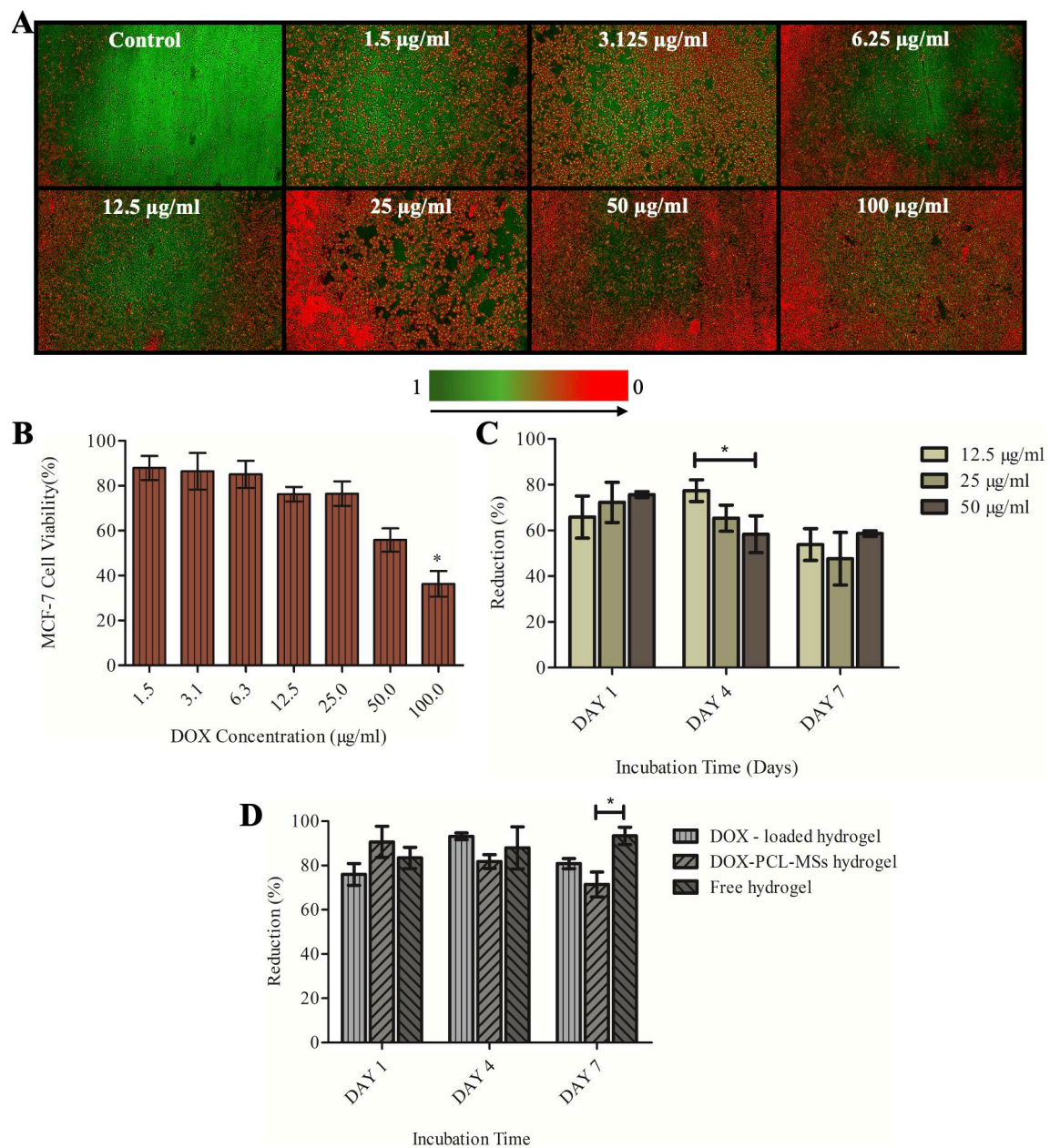
Explanation: The illustration depicts the release groups (DOX-PCL-MSs, DOX-PCL-MSs loaded hydrogel, DOX-loaded hydrogel, and microsphere release from the hydrogel by order) (A). The water retention graph of GA-crosslinked PVA/PUL hydrogels with 30-minute and 1-hour exposure times was measured over 12 days (in PBS with 0.01% sodium azide at pH 7.2) (B). The cumulative weight loss graph of the of GA-crosslinked PVA/PUL hydrogels with 30-minute and 1-hour exposure times was measured over 12 days (in PBS, supplemented with 2 mg/mL of lysozyme and 0.01% sodium azide at pH 7.2) (C). The percentage of cumulative release of DOX from different groups of hydrogels (C) was measured over 16 days of incubation (in PBS with 0.01% sodium azide at pH 7.2), with the cumulative release of microspheres through the open pores on the hydrogel's surface depicted as a dotted line on the graph using a secondary y-axis (D).

The morphological evaluations and possible cytotoxic effects of the DOX and DOX-PCL-MSs and carrier hydrogels were performed by inverted microscopy images and quantitative cell viability assays including MTT and deep blue assay on our target MCF-7 breast cancer cells in Figure 3.5. The cytotoxic dose ranges of DOX on monolayer MCF-7 were analyzed using microscopic images with ImageJ software (Fig. 3.5A). A control group without DOX treatment and varying concentrations of 1.5, 3.125, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$ of DOX were applied. The density of the cells changed with the increasing concentrations. Images were processed using a red–green Look Up Table (LUT), and according to the color distribution, it was observed that green regions correspond to the metabolically active areas, whereas red regions indicate lower viability due to the cytotoxic effects of DOX treatment. The increase in redness on MCF-7 was consistent with the increased DOX concentrations. The red region became wider as the concentration increased. The cytotoxicity of doxorubicin (DOX) was assessed using the MTT assay and presented in Figure 3.5B. Increasing concentrations of DOX (1.5, 3.125, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) were administered to the MCF-7 cells, with the cell viability of the control group established at 100%. The percentage viability exhibited a concentration-dependent decline with the following values: 87.94 ± 5.36 , 86.48 ± 8.17 , 85.10 ± 6.06 , and $76.26 \pm 3.19\%$. Notably, no significant reduction was observed until a

concentration of 25 $\mu\text{g/mL}$. However, at 50 $\mu\text{g/mL}$, the reduction in viability reached $55.84 \pm 5.21\%$, nearly halving the cell viability. Concerning the findings in Figure 3.5A, increased cell death began to be observed after 25 $\mu\text{g/mL}$, accompanied by an enlarged red region. The highest concentration of DOX (100 $\mu\text{g/mL}$) diminished cell viability to $36.33 \pm 5.68\%$, demonstrating a statistically significant difference ($p < 0.05$) when compared to the control group at 100 $\mu\text{g/mL}$ of DOX. The Alamar blue reduction was applied to the MCF-7 cells with the DOX-PCL-MSs-hydrogels, which contained concentrations of 12.5, 25, and 50 $\mu\text{g/mL}$ of DOX, as presented in Figure 3.5C. In addition to the plain DOX, encapsulated versions were also utilized, with the concentrations of DOX determined based on the MTT results (Fig. 3.5B), and only those deemed effective were assessed. Previous studies have demonstrated that encapsulation techniques facilitate sustained drug delivery while simultaneously reducing the required dosage, as the outer layer protects the drug from premature degradation, thereby mitigating systemic toxicity in the human body [194], [195], [196]. The encapsulated DOX-PCL-MSs were incorporated into the surface pores of PVA/PUL hydrogels, and reductions were analyzed over a period of seven days. On day one, the highest reduction was observed in the 12.5 $\mu\text{g/mL}$ group as $65.85 \pm 9.19\%$. Following day four, the viability rates began to decrease, except for the 12.5 $\mu\text{g/mL}$ group. A statistically significant difference ($p < 0.05$) was noted between the 12.5 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ carrying hydrogels, with the 50 $\mu\text{g/mL}$ group resulting in an approximate reduction in viability of 17%. By day seven, even the lowest concentration group (12.5 $\mu\text{g/mL}$) exhibited a reduction to 50%. According to these findings, the average reduction observed in the highest concentration group (50 $\mu\text{g/mL}$) was recorded at $64.20 \pm 9.93\%$. In summary, these findings indicate that the encapsulation of the treatment drug, DOX, significantly enhances the reduction in cell viability by preserving its cytotoxic efficacy against the target cell line, MCF-7, through the process of encapsulation, and our results align with previous studies documented. Cytotoxicity evaluation for different hydrogel groups were analyzed in Figure 3.5D. The potential toxic effect of DOX-PCL-MSs loaded hydrogel on MCF-7 cells was assessed by comparing this group with the DOX-loaded hydrogel and free hydrogel.

According to the findings (Fig.3.5C), the DOX-PCL-MSs loaded hydrogel groups contained microspheres carrying 50 $\mu\text{g/mL}$ DOX in cell media, and the same amount of free DOX was loaded into the DOX-loaded hydrogel group. On day 1, due to the burst release of the DOX, the DOX-loaded hydrogel exhibited the lowest viability at $75.96 \pm 4.93\%$. However, after day 1, the DOX-PCL-MSs loaded hydrogel group decreased by around 10% compared to its initial viability percentage. In contrast, after the burst release of DOX in the DOX-loaded hydrogel group, day 4 showed an increased viability of $93.16 \pm 1.52\%$. The DOX-PCL-MSs loaded hydrogel group demonstrated a statistically significant ($p < 0.05$) decrease on day 7 compared to free hydrogel at $71.42 \pm 5.60\%$. Reviewing the overall results, the free hydrogel's cell viability levels were significantly high on days 1, 4, and 7, at 83.42 ± 4.8 , 87.99 ± 9.50 , and $93.36 \pm 3.91\%$, respectively. According to ISO 10993-5, each hydrogel group (including DOX-loaded, DOX-PCL-MSs, and free hydrogel groups) was deemed non-cytotoxic on days 1, 4, and 7, as the reduction percentages remained above 70%. Furthermore, the controlled release of DOX, accomplished through encapsulation, enhanced the prolonged and increased effect on MCF-7 cells, with the DOX-PCL-MSs loaded hydrogel group exhibiting a significant reduction in cell viability.

Figure 3.5. Morphological Analyses, Quantification of Cell Viability and Alamar Blue Reduction (%) in MCF-7 Cells

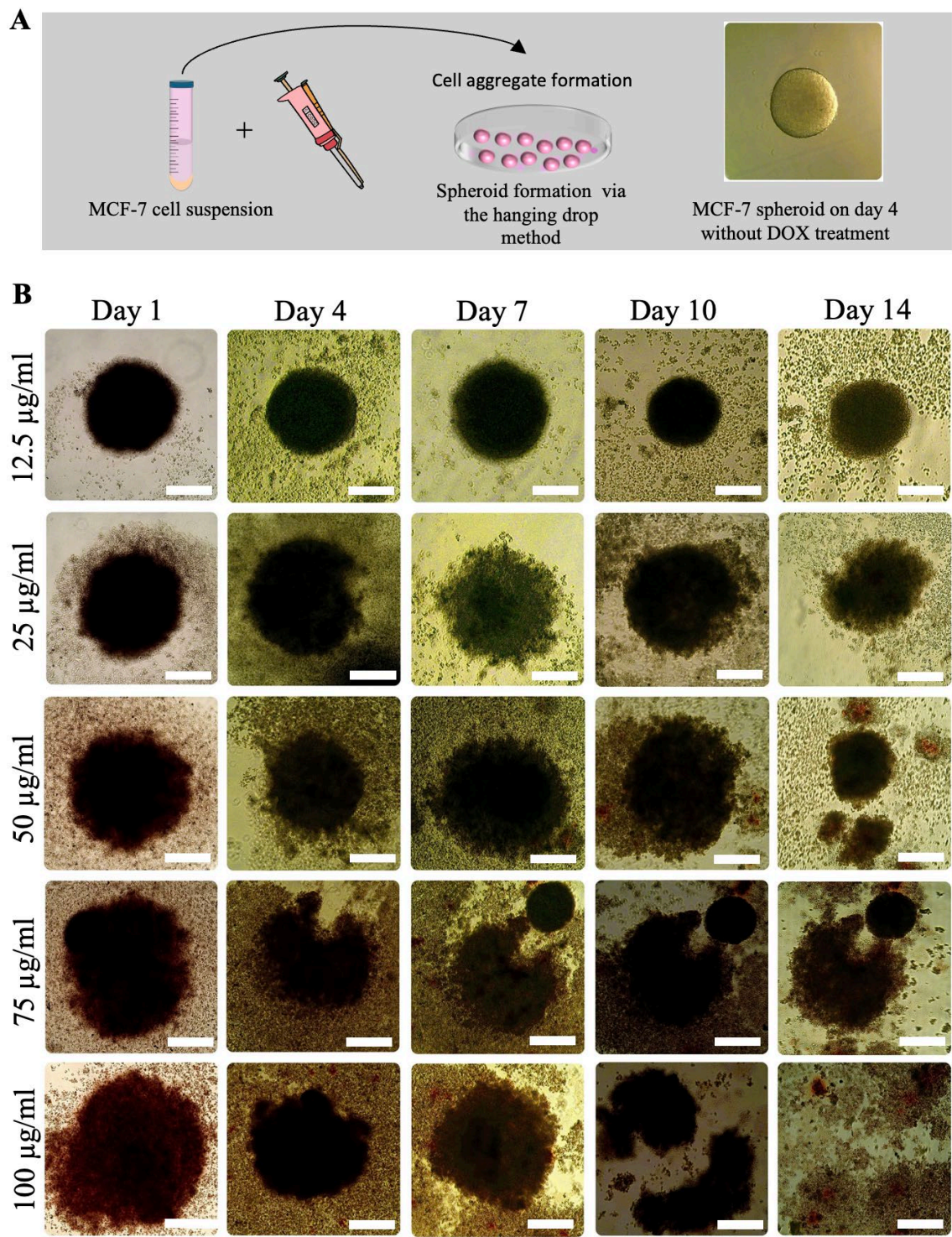


Explanation: The morphological evaluation of MCF-7 cells at varying concentrations of DOX (1.5, 3.125, 6.25, 12.5, 25, 50, 100 $\mu\text{g/mL}$) was conducted using ImageJ software with the application of red–green LUT pseudocolor. A heat map was generated to illustrate cell viability, wherein green indicates regions of active cells and red denotes regions of damaged or dead cells (A). The percent cell viability of MCF-7 cells with increasing concentrations after a 1-day incubation period. *: Indicates the statistically significant difference between the 100 $\mu\text{g/mL}$ concentration and the remaining concentrations ($p < 0.05$) (B). Percent alamar blue reduction assay of MCF-7 cells with different concentrations of hydrogels containing DOX-PCL-MSs (microspheres carrying 12.5, 25, and 50 $\mu\text{g/mL}$ DOX respectively in the incubation media) after 1, 4, and 7 days of incubation. *: Indicates the statistically significant difference between the 12.5–50 $\mu\text{g/mL}$ concentrations on day 4 ($p < 0.05$) (C). Percent alamar blue reduction assay of MCF-7 cells treated with different hydrogel groups (DOX-loaded, DOX-PCL-MSs microspheres carrying 50 $\mu\text{g/mL}$ DOX respectively in the incubation media and free hydrogel) after 1, 4, and 7 days of incubation: Indicates the statistically significant difference between the DOX-PCL-MSs hydrogel and the free hydrogel on day 7 ($p < 0.05$) (D).

To further support the findings obtained from 2D monolayer in vitro assays (Fig. 3.5.A-B), MCF-7 spheroids were additionally utilized as presented in Figure 3.6. A greater understanding of the tumor microenvironment, as opposed to relying solely on 2D models, was achieved through the fabrication of MCF-7 spheroids, enabling the attainment of more reliable and accurate results [197]. Two-dimensional cell culture remains insufficient in several respects; it fails to adequately mimic hypoxia, acidic pH environments, and barriers to drug transport. Consequently, it does not fully reflect the tumor-like concept. To address these limitations, MCF-7 microspheres could be administered, and as reported by Chen et al., they could be cultured in a dish as spheroids to better elucidate the nature of tumors and their relationship with drug testing [198]. As illustrated (Fig. 3.6A), MCF-7 cell suspensions were placed as droplets on top of the lid of a petri dish, and by using PBS, a hydration chamber was created. The cell aggregates

displayed a nice round form observed on day 4 (without DOX treatment) via an inverted microscope under 10x magnification. The efficacy of DOX has been previously documented concerning its anticancer activity across various cancer cell lines [199]. Therefore, the time course morphological evaluation of MCF-7 spheroids subjected to varying concentrations (12.5, 25, 50, 75, and 100 $\mu\text{g/mL}$) of DOX over a duration of 14 days, as observed through an inverted microscope, is illustrated in Figure 3.6B. On day 1, at the lowest concentration (12.5 $\mu\text{g/mL}$), all DOX-treated groups exhibited a well-formed round shape, and the density of apoptotic cells around the margin of the spheroid was narrower compared to other concentrations. However, the area increased with higher concentrations of DOX. By day 4, disruption of the peripheral cell dissociation started to become more noticeable, especially between the 25-100 $\mu\text{g/mL}$ of DOX treatment. In alignment with previous observations in a similar study, 16 $\mu\text{g/mL}$ of DOX after 72 hours of treatment was found to be effective on spheroid deterioration, and our study shows that the alteration of the formation of round shape began between 12.5-25 $\mu\text{g/mL}$ [200]. On day 7, a significant disruption of the spheroid was observed at a concentration of 75 $\mu\text{g/mL}$. Following this, substantial swelling and cell detachment were noted on the day 10 and 14, after administering 50 $\mu\text{g/mL}$ of DOX treatment across each group. In contrast, the group treated with 12.5 $\mu\text{g/mL}$ on day 14 remained relatively compact and retained partial integrity. Overall, the morphological findings of the spheroids, in conjunction with their time-dependent disruption results following DOX treatments, indicate that higher concentrations of DOX (50, 75, 100 $\mu\text{g/mL}$) are associated with more disturbed and scattered formations. Additionally, as time progressed and the duration of DOX exposure was extended, the disruption of the 3D spheroid models became increasingly dispersed within their respective concentration groups.

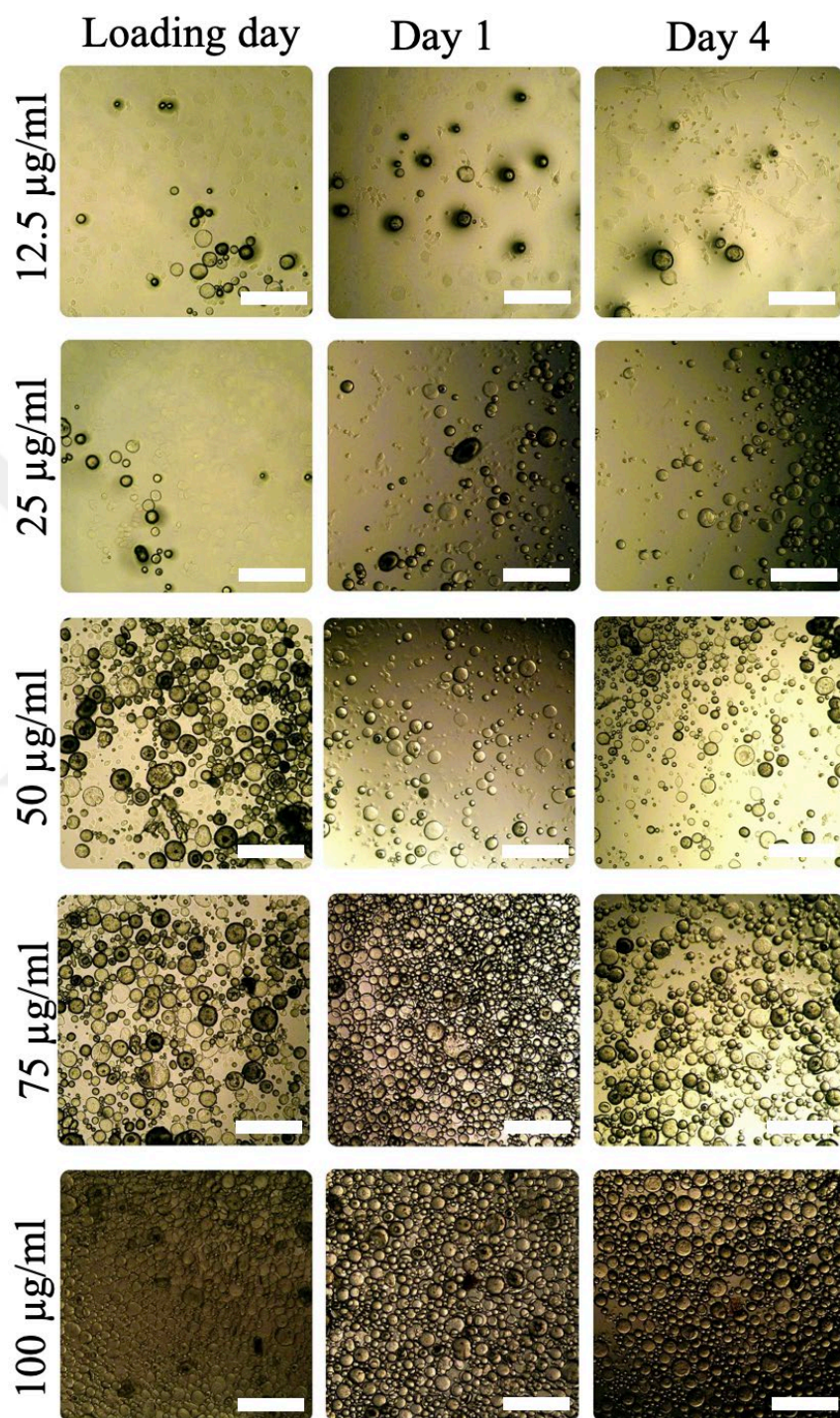
Figure 3.6. Morphological Changes in MCF-7 Spheroids Treated with Increasing Concentrations of DOX



Explanation: Schematic representation of MCF-7 spheroid formation using the hanging drop method (A). Morphology of MCF-7 spheroids was observed over a 14-day incubation period with increasing concentrations of DOX (12.5, 25, 50, 75, 100 $\mu\text{g/mL}$) under an inverted microscope at 10x magnification (scale bar: 200 μm) (B).

The cytotoxicity effect and the interaction between DOX-PCL-MSs and the MCF-7 monolayer were observed using the inverted microscope prior to the subsequent observation of DOX-PCL-MSs on MCF-7 spheroids. Increasing concentrations of DOX, determined based on the encapsulated quantity, were administered to the cells at concentrations of 12.5, 25, 50, 75, and 100 $\mu\text{g/mL}$ within the PCL-MSs. On the loading day, as well as on day 1 and day 4, their interaction with the cells was monitored (Fig. 3.7). The cellular interaction between the DOX-PCL-MSs and cells was more evident at a 12.5 $\mu\text{g/mL}$ concentration on day 4. Since the amount of DOX is the smallest around the PCL-MSs, cells expand around them, leading to cell-cell contact. However, with increasing concentrations, the morphology of MCF-7 cells altered; on day 4, the 25 $\mu\text{g/mL}$ concentration exhibited a more rounded shape and dead cells around the DOX-PCL-MSs compared to its loading day and day 1, and a significant number of dead cells were observed on day 4 at a 50 $\mu\text{g/mL}$ concentration. As a consequence of particle density at concentrations of 75 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of DOX encapsulation, it was almost impossible to accurately visualize the microscopic field of the surface of the well; thus, these two groups were excluded from further morphological analyses, including the effect of DOX-PCL-MSs on 3D spheroids. Although two groups were excluded (75, 100 $\mu\text{g/mL}$), as shown in Figure 3.6B, 50 $\mu\text{g/mL}$ of DOX was sufficient to be effective on MCF-7 cells. These observations prove the dose-dependent cytotoxicity and the effective release of DOX from the PCL-MSs. Given this preliminary data, the decided concentrations (12.5, 25, 50, 25, 50 $\mu\text{g/mL}$) were assessed for 3D MCF-7 spheroids.

Figure 3.7. Effect of DOX-PCL-MSs on MCF-7 Monolayer Cells

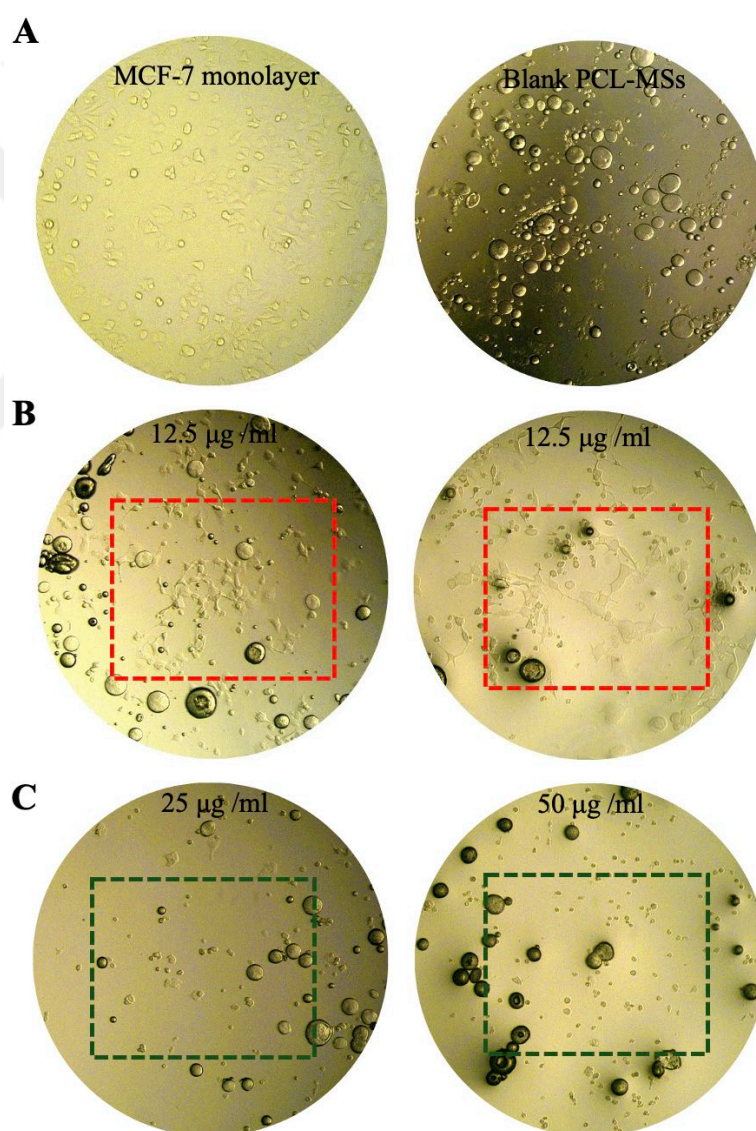


Explanation: The observation of increasing concentrations of DOX-PCL-MSs (12.5, 25, 50, 75, 100 $\mu\text{g/mL}$) on MCF-7 monolayer cells was conducted using an inverted microscope at 40x magnification during an incubation period starting from the loading day to day 4 (scale bar: 30 μm).

Increased dosages of the anti-cancer drug (DOX) are associated with cardiotoxicity and cytotoxicity, which pose a significant limitation to its clinical application. However, encapsulating the drug as a delivery vehicle can mitigate its off-target exposures while preserving the initial effective dosage. Consequently, this strategy offers protection against dose-dependent cardiotoxicity when compared to conventional drug administration [201]. Since the encapsulation of DOX has been proven to be an effective strategy, in addition to assessing the cytotoxic impact of DOX-PCL-MSs on monolayer MCF-7 cells (Fig. 3.7), an investigation of the blank PCL-MSs was also conducted to observe the intrinsic effect of the carrier itself. The morphological results of the two control groups as plain MCF-7 monolayer and blank PCL-MSs, as well as 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$ DOX-PCL-MSs (dosages determined based on the encapsulated DOX quantity), were evaluated under an inverted microscope, as illustrated in Figure 3.8. All groups were observed with the same amount of incubation period. Following the incubation period, no apoptotic MCF-7 cells were observed around the blank DOX-PCL-MSs. Conversely, at the lowest concentration (12.5 $\mu\text{g/mL}$), a significant accumulation of cell clusters around the microspheres was noted, suggesting that cell adhesion is enhanced in proximity to the microspheres (Fig. 3.8B, red squares). The interaction between blank PCL-MSs and the cells was presented in a similar study, which demonstrated enhanced cellular attachment attributed to the surface provided by PCL-MSs [202]. A more pronounced effect was observed at higher concentrations (25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$) of DOX-PCL-MSs (Fig. 3.8C, green squares). The epithelial morphology of MCF-7 cells exhibited a transition to a more rounded shape, accompanied by a noticeable loss of adherence. The interconnected and clustered cells shown in red squares, were significantly diminished when compared to the higher concentrations indicated by the green squares. The cytotoxic potential of these concentrations highlighted the efficacy of

DOX-PCL-MSs on MCF-7 cells. The overall results substantiate that, while increasing concentrations of DOX-PCL-MSs enhances the cytotoxic effect and disrupt the MCF-7 clusters, blank PCL-MSs do not exhibit a cytotoxic effect as a delivery system, thereby confirming the biocompatibility of PCL microspheres.

Figure 3.8. Cellular Interaction between DOX-PCL Microspheres and MCF-7 Monolayer Cells

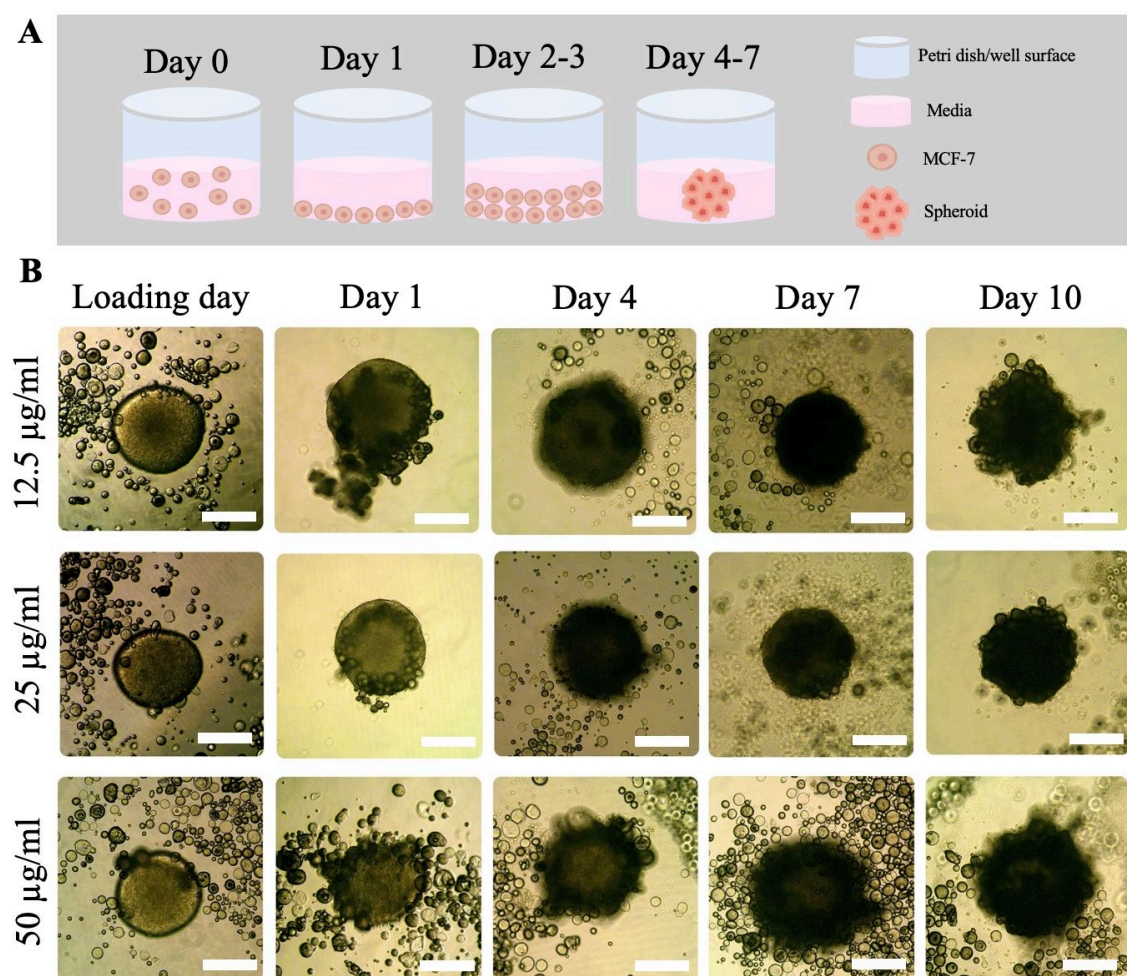


Explanation: The control groups include monolayer MCF-7 and blank PCL-MSs (A). The cellular interaction between DOX-PCL-MSs and MCF-7 occurs at concentrations of 12.5 $\mu\text{g/mL}$ (B), 25 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$ (C). Red squares represent the healthy, adhered MCF-7 cells, while green squares denote the unhealthy MCF-7 cells.

The formation of MCF-7 spheroids inside the petri dish/ well plate is illustrated in Figure 3.9A. Suspended small droplets of MCF-7 culture (10,000 cells per droplet) were placed on the surface of the petri dish/ well plate, and subsequently, cells started to proliferate. The hanging-drop method is a widely utilized technique that offers distinct growth conditions in comparison to monolayer surfaces. This in vivo-like approach emulates physiological tissues, thereby facilitating a deeper understanding of the tumor environment [203]. Due to gravity, the gradual sedimentation toward the bottom of the droplet began to be observed from day 1. Over time, aggregation of cells developed cell-cell interactions and formed a compact round shape as illustrated on days 4 to 7. The inverted microscopy observation on the morphological evaluation of MCF-7 spheroids with increasing concentrations of DOX-PCL-MSs (12.5, 25, and 50 $\mu\text{g/mL}$) was introduced into the medium of MCF-7 spheroids and observed over a period of 10 days, as presented in Figure 3.9B. Time-dependent penetration of DOX-PCL-MSs into the spheroids became visible after the loading day at all concentrations. This association suggests a potential interaction between the hydrophobic PCL and the spheroid. At the lowest concentration of DOX-PCL-MSs (12.5 $\mu\text{g/mL}$), the compact morphology was maintained over a period of 10 days. However, a slight deformation was observed on day 7. At a concentration of 25 $\mu\text{g/mL}$, evidence of the entering of DOX-PCL-MSs into the spheroid was clearly indicated on day 1, with the outer edge of the spheroids being surrounded by the DOX-PCL-MSs. Nevertheless, after day 4, the compact formation exhibited signs of distribution. Notably, a significant effect was observed at a concentration of 50 $\mu\text{g/mL}$, even after one day of incubation, as MCF-7 cells began to detach from one another due to the cytotoxic effects of DOX-PCL-MSs. A consistent dose-time dependent disintegration of MCF-7 spheroids was observed; this finding confirms that an increased amount of drugs within the carrier is effectively released,

leading to cellular disruption and subsequent cell death. Furthermore, since spheroids more accurately mimic the solid tumor microenvironment, the morphological disruption of the spheroid holds significant importance as it closely resembles in vivo-like system [204].

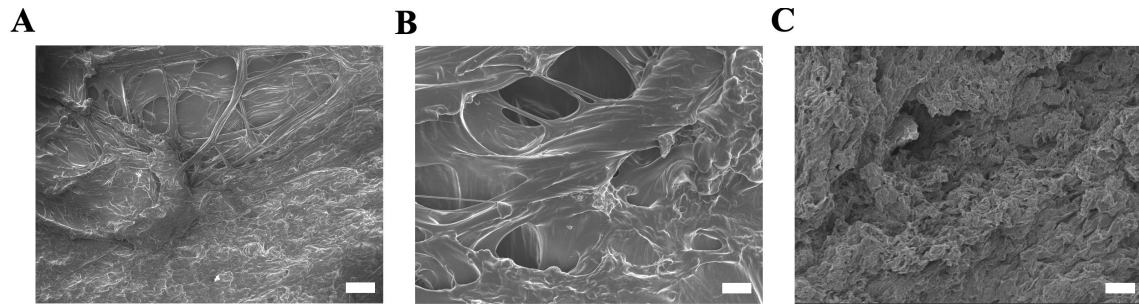
Figure 3.9. Morphological Changes in MCF-7 Spheroids Treated with Increasing Concentrations of DOX-PCL-MSs



Explanation: Schematic representation of the formation steps/morphogenesis of MCF-7 spheroids (A). The morphology of MCF-7 spheroids was observed over a 10-day incubation period with increasing concentrations of DOX-PCL-MSs (12.5, 25, 50 µg/mL) under an inverted microscope at 10x magnification (scale bar: 200 µm) (B).

The surface topography of the PVA/PUL hydrogel and the cell seeding with L929 fibroblast cells are presented in Figure 3.10. The porous and interconnected topography of the PVA/PUL scaffold, without cell seeding, is presented at different magnifications of 200x and 1000x, respectively (Fig. 3.10A-B). The presence of GA crosslinker as a chemical crosslinker in the hydrogel was observed to yield a denser and more stabilized network. The apparent large pores and interconnected structure of PVA/PUL hydrogel observed in Figure 3.10A-B, provides evidence for the presence of GA [205]. Additionally, cell viability analysis was conducted by seeding fibroblast cells L929 to evaluate the cytocompatibility of the material itself. This step plays a pivotal role in overall biomedical applications concerning biocompatibility and biological responses, aiming to investigate whether the material is inducing any toxicity [206]. The primary focus of the hydrogel system is the effective release of anticancer drugs; however, the system's safety for a healthy cellular environment holds nearly equal importance. The suitability of the hydrogel system as a drug delivery platform must be assessed using the aforementioned techniques, such as SEM imaging of healthy cell attachment. Although previous studies have reported that PUL causes reduced cell attachment, the addition of GA resulted in the attachment and proliferation of healthy cells on the PVA/PUL hydrogel was observed on Figure 3.10C [207], [208]. In contrast, a smoother surface was observed for the topography of the unseeded PVA/PUL hydrogel, depicted in Figures 3.10A-B. After seeding with L929 cells and an incubation period for 7 days, the surface transitioned to a more roughened and wavy morphology, as shown in Figure 3.10C. The interaction between L929 cells and the PVA/PUL hydrogel containing a GA crosslinker, which was developed for this study, demonstrates the potential interaction and enhanced cell viability between the selected materials and healthy cells. This combination of PVA and PUL polymers in our study indicated the possibility of cellular growth on the surface. However, PUL is regarded as less favorable for direct attachment; the incorporation of PVA and GA crosslinking has provided a more suitable structural environment for cells to attach and proliferate [207], [208], [209]. These findings indicate that while the PVA/PUL hydrogel containing the GA crosslinker demonstrates surface cytocompatibility, its suitability for tissue regeneration has also been validated through the proliferation of cells.

Figure 3.10. The Surface Topography of PVA/PUL Hydrogel with and without L929 Cell Seeding



Explanation: Scanning electron microscopy images of PVA/PUL hybrid hydrogel at 200x (scale bar: 100 μm) (A) and 1000x (scale bar: 20 μm) (B) magnification without L929 cell seeding, and 370x magnification with L929 cell seeding after 7 days of incubation period (scale bar: 20 μm) (C).

CONCLUSION

Breast cancer (BC) is recognized as one of the most prevalent malignancies, with its incidence rate affecting millions of individuals at an accelerated speed. Current BC therapeutic approaches for BC possesses various limitations. For example, total or partial mastectomy, a commonly utilized method, is contingent upon the presence of potential residual cancer cells. Hence, in this particular approach, the supportive application of localized drug delivery is of significant importance. In comparison to conventional drug delivery methods, including systemic delivery, locoregional delivery presents a promising and enhanced technique, characterized by its diverse delivery mechanisms, such as capsules, microparticles, and hydrogels. Researchers have recognized that systemic delivery may result in a toxic effect due to the distribution of the drug throughout body circulation. This toxicity can potentially be mitigated through locoregional mechanisms, as they specifically target designated areas. Consequently, a hybrid anticancer drug-loaded microsphere/hydrogel system was developed for this study, serving as a locoregional delivery system for postoperative treatment of breast cancer. The ice embossing method was successfully employed on the PVA/PUL hybrid hydrogel to create open pores on the surface, which facilitated an environment for DOX-loaded microspheres following freeze-drying, allowing for their anchoring and preservation. As a result, this combination of techniques has demonstrated that the embossing of ice particulates is a reliable method for loading microparticles onto the surface of the scaffold/hydrogel. While the structural aspects of the carrier are important, its release features are equally significant. The *in vitro* release study of DOX was evaluated through two methods: the release of DOX from DOX-PCL-MSs carrying hydrogel and the physical release of the microsphere from the pores on the surface of the hydrogel. The results have demonstrated that this hybrid anticancer drug-loaded microsphere/hydrogel system supports controlled release. The anticancer activity of DOX was investigated using both 2D monolayer MCF-7 cells and 3D MCF-7 spheroid models. The overall results suggest that the DOX-PCL-MSs, successfully decreased cell viability and disrupted the spheroid models in a dose-dependent manner through controlled release.

Furthermore, the controlled release was supported by the incubation of hydrogels (DOX-loaded hydrogel, DOX-PCL-MSs hydrogel) on MCF-7 cells. The results revealed that the DOX-PCL-MSs hydrogel demonstrates controlled and prolonged anticancer activity compared to the free DOX-loaded hydrogel. For the assessment of the ability to support L929 cell attachment and proliferation, the PVA/PUL hybrid hydrogel was examined both with and without the seeding of L929 cells. The results revealed that the hydrogel supports the growth of healthy cells, indicating its potential to facilitate healthy tissue regeneration, in addition to the releasing characteristics of DOX-PCL-MSs from the hydrogel. In summary, the presented system provides substantial evidence that the developed DOX-PCL-MSs loaded PVA/PUL hybrid hydrogel system represents a promising technique for mitigating the toxicity associated with systemic delivery and the risks of overdose. Consequently, this combinatorial approach supports its potential as a locoregional delivery system for postoperative treatment for BC patients.

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