

**DEVELOPMENT OF AN
APROTININ-BASED NOVEL
NANO-BIOCONJUGATE UTILIZING
MICROFLUIDICS VIA 3D CANCER
SPHEROID MODELS**

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MATERIALS SCIENCE & NANOTECHNOLOGY

By
Faiqa Nazir
August 2024

Development of an Aprotinin-based Novel Nano-bioconjugate Utilizing
Microfluidics via 3D Cancer Spheroid Models

By Faiqa Nazir

August 2024

We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

Amirhoushang Bahrami (Advisor)

Gurkan Yesiloz (Co-advisor)

Fatma Visal Okur

Aykut Erbas

Approved for the Graduate School of Engineering and Science:

Orhan Arikan
Director of the Graduate School

ABSTRACT

DEVELOPMENT OF AN APROTININ-BASED NOVEL NANO-BIOCONJUGATE UTILIZING MICROFLUIDICS VIA 3D CANCER SPHEROID MODELS

Faiqa Nazir

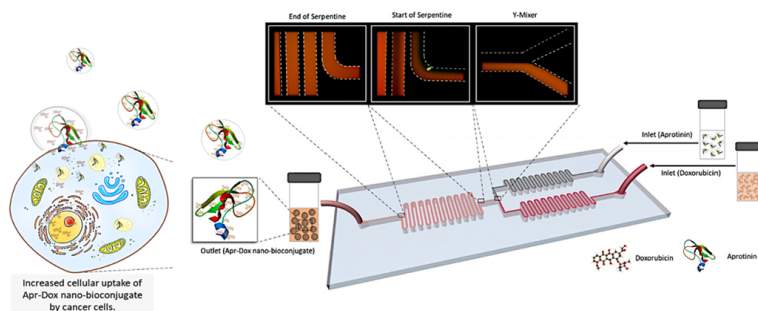
M.S. in Materials Science & Nanotechnology

Advisor: Amirhoushang Bahrami

Co-Advisor: Gurkan Yesiloz

August 2024

Proteins are promising substances for introducing new drug carriers with efficient blood circulation due to low possibilities of clearance by macrophages. However, such natural biopolymers have highly sophisticated molecular structures, preventing them from being assembled into nano-platforms with manipulable payload release profiles. Here, we announce a novel anti-cancer nano-drug carrier moonlighting protein, Aprotinin, to be used as a newly identified carrier for cytotoxic drugs. The Aprotinin-Dox orubicin (Apr-Dox) nano-bioconjugate was prepared via a single-step microfluidics co-flow mixing technique; a feasible and simple way to synthesize a carrier-based drug design with a double-barreled approach that can release and actuate two therapeutic agents simultaneously i.e., Apr-Dox in 1:11 ratio (aprotinin an anti-metastatic carrier drug and chemotherapeutic drug DOX).



With a significant stimuli-sensitive (i.e. pH) drug release ability, this nano-bioconjugate achieves superior bio-performances including high cellular uptake,

efficient tumor penetration and accumulation into acidic tumor microenvironment, as well as inhibiting further tumor growth by halting the urokinase plasminogen activator (uPA) involved in metastasis and tumor progression. Distinctly, in healthy human umbilical vein endothelial (HUVEC) cells, drastically lower cellular uptake of nano-bioconjugate has been observed and validated compared to anticancer agent Dox. Our findings demonstrate an enhanced cellular internalization of nano-bioconjugates towards breast cancer, prostate cancer, and lung cancer both in vitro and in physiologically relevant biological 3D-spheroid models. Consequently, the designed nano-bioconjugate shows a high potential for targeted drug delivery via natural and biocompatible moonlighting protein, thus opening a new avenue for proving aprotinin in cancer therapy both as an anti-metastatic and drug-carrying agent.

Keywords: Nanobioconjugates, carrier-protein, aprotinin, doxorubicin, drug delivery, microfluidics, cancer therapy.

ÖZET

3D KANSER SFEROİD MODELLERİ ARACIĞIYLA MIKROAKŞKANLAR KULLANILARAK APROTİNİN TABANLI YENİ BİR NANO-BİYOKONJUGATIN GELİŞTİRİLMESİ

Faiqa Nazir

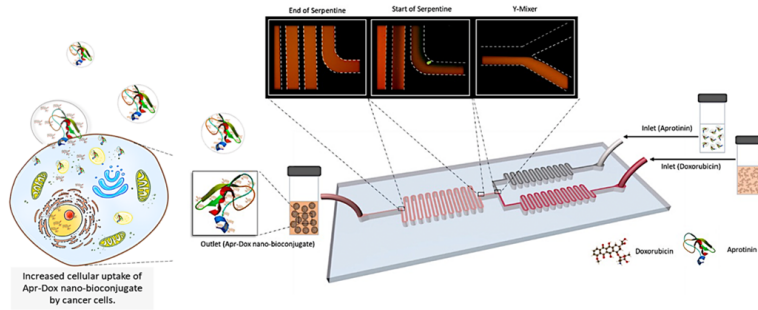
Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

Tez Danışmanı: Amirhoushang Bahrami

İkinci Tez Danışmanı: Gurkan Yesiloz

Ağustos 2024

Proteinler, makrofajlar tarafından temizlenme olasılıklarının düşük olması nedeniyle, etkili kan dolaşımına sahip yeni ilaç taşıyıcılarının tanıtılması için umut verici maddelerdir. Bununla birlikte, bu tür doğal biyopolimerler son derece karmaşık moleküler yapıya sahiptir ve bunların manipüle edilebilir yük bırakma profillerine sahip nano platformlarda bir araya getirilmesini engeller. Burada, sitotoksik ilaçlar için yeni tanımlanan bir taşıyıcı olarak kullanılacak yeni bir kanser karşıtı nano ilaç taşıyıcı ek iş proteini Aprotinin'i duyuruyoruz. Aprotinin-Dox orubisin (Apr-Dox) nano-biyokonjugatı, tek adımlı bir mikroakışkan ortak akışlı karıştırma tekniği yoluyla hazırlandı; iki terapötik ajanı aynı anda serbest bırakabilen ve harekete geçirebilen çift namlulu bir yaklaşımla taşıyıcı bazlı bir ilaç tasarımını sentezlemenin uygulanabilir ve basit bir yolu, yani 1:11 oranında Apr-Dox (aprotinin bir anti-metastatik taşıyıcı ilaç ve kemoterapötik ilaç DOX)).



Önemli bir uyarana duyarlı (yani pH) ilaç salma kabiliyetine sahip olan bu

nano-biyokonjugat, yüksek hücresel alım, verimli tümör penetrasyonu ve asidik tümör mikro ortamında birikim dahil olmak üzere üstün biyo-performanslara ulaşır ve ayrıca ürokinaz plazminojeni durdurarak daha fazla tümör büyümesini engeller. aktivatör (uPA) metastaz ve tümör ilerlemesinde rol oynar. Belirgin bir şekilde, sağlıklı insan göbek damarı endotelyal (HUVEC) hücrelerinde, antikanser ajanı Dox'a kıyasla nano-biyokonjugatın hücresel alımının önemli ölçüde daha düşük olduğu gözlemlenmiş ve doğrulanmıştır. Bulgularımız, nano-biyokonjugatların meme kanseri, prostat kanseri ve akciğer kanserine karşı hem in vitro hem de fizyolojik olarak ilgili biyolojik 3D-sfero modellerinde gelişmiş bir hücresel içselleştirmesini göstermektedir. Sonuç olarak, tasarlanan nano-biyokonjugat, doğal ve biyouyumlu ay ışığı proteini yoluyla hedefe yönelik ilaç dağıtımı için yüksek bir potansiyel gösterir, böylece aprotinin'in hem anti-metastatik hem de ilaç taşıyıcı bir ajan olarak kanser tedavisinde kanıtlanması için yeni bir yol açar.

Anahtar sözcükler: Nanobiyokonjugatlar, taşıyıcı protein, aprotinin, doksorubisin, ilaç dağıtımı, mikroakışkanlar, kanser tedavisi.

Acknowledgement

Above all, I am grateful for the opportunities and blessings that Allah Almighty has bestowed upon me.

I would like to express my sincere gratitude to my supervisor, Dr. Gurkan Yesiloz, who gave me the opportunity to prove myself in the field of academia and provided me with valuable guidance, feedback, and support throughout my research. I am truly thankful to him for trusting me and motivating me to aim for the highest, and with his supervision, I am capable of completing this thesis.

I am also extremely thankful to my Post-Doc fellow, Dr. Iqra Munir, who has been a great role model for me throughout my master's journey. She held my hand like a child and taught me everything about research. Her constant energy and love for science also inspired me to continue this science journey and do a Ph.D afterwards. In addition, I would also like to thank my group members Servin, Asli, Eylul, Ecem, Faruk, and Ali, who together make such a positive and productive lab environment. Their critical questions and comments pushed me to sharpen my thinking and brought greater depth to my knowledge and work.

In addition, I wish to acknowledge the generous financial support from the Scientific and Technological Research Council of Türkiye (TÜBİTAK) BİDEB International Outstanding Researchers Program under project No. 118C263 and ARDEB 1001 Program under Project No. 221M575.

My appreciation also goes out to all my friends who listened to my constant whining and were always there to put a smile on my face. I am grateful to my Bilkent Family (Dr. Iqra, Mohsin, Salah bhai, Shakir, Joya, Arooj, Musham, zain and Imran bhai's Family) who never let me feel alone and away from my home. I am truly thankful to my best buddy Hafsa for being my constant support from miles away and listening to my hours-long rants whenever I felt low. I am also thankful to Yasharah, Rimsha, Shanza, Fatima, and Rijja, who put up with me through thick and thin.

Without ongoing support from a few special people in my life, completing this thesis would not be easy. I want to thank my fiance Osama Aadil who endured my moods, crankiness, and endless amount of panic. I am grateful for the patience and kindness he has shown toward me. The most significant acknowledgment is reserved for my parents; if it weren't for them, I would be nothing today. I am deeply grateful to my parents, Nazir Ahmad and Razia Parveen, for supporting me and bringing me all the opportunities in the world to be successful and achieve greater milestones. They sent me miles away even though I am their only daughter and splendor of home. I can never pay back the gratitude, kindness, and love they have showered on me. I would also like to thank my siblings, Fahad, who supported my educational career at every step; my younger brother Faraz, the joker of our house; and my elder brother Faisal.

In the end, I would like to thank me for believing in me and doing all the hard work and sleepless nights.

Contents

1	Introduction	1
2	Scientific Background: Aprotinin as Moonlight Nanocarrier	5
3	Methodology	7
3.1	Materials	7
3.2	Isothermal Titration Calorimetric (ITC) Analysis of Apr-Dox Nano-bioconjugate	8
3.3	Synthesis of Apr-Dox Nano-bioconjugate using Microfluidic Platform	8
3.4	Characterization of Apr-Dox Nano-bioconjugate	9
3.5	Spectroscopic Analysis of Apr-Dox Nano-bioconjugate	9
3.6	Equilibrium Dialysis for the Removal of Unbound Drug	10
3.7	Percent Drug Loading (DL) and Drug Entrapment Efficiency (EE)	10
3.8	<i>In vitro</i> Drug Release and Release Kinetics	11
3.9	Hemolysis Assay	12

3.10	Cellular Uptake Studies of Apr-Dox Nano-bioconjugate	13
3.11	2D In vitro Cytotoxicity Assay of Apr-Dox Nano-bioconjugate . .	13
3.12	3D In vitro Cytotoxicity Assay of Apr-Dox Nano-bioconjugate . .	14
3.13	Live / Dead Cell Staining Assay	14
3.14	Microscopy and Imaging	15
3.15	Statistical Analysis	15
4	Results & Discussion	16
4.1	Aprotinin Binding Affinity to Dox	16
4.2	Synthesis and Characterization of Apr-Dox Nano-Bioconjugate .	18
4.3	Secondary Structure Analysis of Aprotinin and Apr-Dox Nano-bioconjugate	22
4.4	Drug Loading/Encapsulation Efficiency and pH Triggered Release of Dox from Apr-Dox Nano-bioconjugate	22
4.5	Details of Dox Loading into aprotinin conjugate and Entrapment efficiency of aprotinin with different drug/protein ratios for preparation of Apr-Dox nano-bioconjugate	24
4.6	Protein structure investigation under pH-stimuli	27
4.7	Comparative Cellular uptake of Apr-Dox Nano-bioconjugate . . .	28
4.8	<i>In Vitro</i> Compatibility of Apr-Dox Nano-bioconjugate with Blood Components	31

4.9	Cytocompatibility of Apr-Dox Nano-bioconjugate in Non-cancerous Cells	34
4.10	Enhanced Chemo-sensitizing Ability of Moonlight Drug Bioconjugate in Breast, Prostate and Lung cancer	36
4.11	Effect of Apr-Dox Nano-Bioconjugate on 3D Cancer Spheroids of Prostate Cancer C4-2 and Lung Cancer H-1299	39
5	Conclusion	43
A	Supplementary Data	59

List of Figures

4.1	Isothermal titration calorimetric analysis for aprotinin binding affinity to Dox molecules. Molar ratio between the amounts in moles of aprotinin and dox involved in a balanced chemical reaction are shown in graph below. Ka (equilibrium association constant) value is $8.5 \times 10^5 \text{ M}^{-1}$ showing strong binding	17
4.2	DLS analysis of aprotinin alone and after conjugation with Dox	19
4.3	Transmission electron microscopy (TEM) images of synthesized bioconjugate, i- Scale 50nm ii- Scale 20nm iii- mean graphical size obtained from TEM images	20
4.4	Zeta potential of aprotinin alone and after conjugation with Dox-orubicin molecules	20
4.5	UV-Visible spectrophotometric analysis of Apr-Dox nano-bioconjugate 21	
4.6	Circular Dichroism (CD) spectroscopic analysis of aprotinin alone and after its conjugation with Dox	23
4.7	Loading of Dox into aprotinin conjugate	24

4.8	Encapsulation efficiency % of aprotinin with different drug/protein ratios for preparation of Apr-Dox nano-bioconjugate. The concentration of aprotinin in all solutions is 0.1mM, whereas Dox concentration varies from 0.1mM to 1.5mM.	25
4.9	In vitro release analysis of Apr-Dox nano-bioconjugate in PBS and glycine buffer (pH 4.8, 6.5 and 7.3)	26
4.10	Structure alteration of aprotinin to release Dox in acidic conditions by CD spectroscopy	27
4.11	Cellular uptake analysis of MCF-7 breast cancer cells	29
4.12	Cellular uptake analysis of H-1299 small cell lung carcinoma	30
4.13	Cellular uptake analysis of C4-2 prostate cancer	31
4.14	Cellular uptake analysis of Human umbilical vein endothelial cells (HUVECs)	32
4.15	Hemolysis test of Apr-Dox nano-bioconjugate. (A) Images of centrifuged RBC solutions after incubation with different concentrations of nano-bioconjugate. (B) Percentage hemolysis of Apr-Dox nano-bioconjugate at different concentrations. PBS was used as negative control whereas Triton X-100 and deionized water were used as positive controls.	33
4.16	In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of HUVECs	34
4.17	In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of C4-2	35

4.18 In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of MCF-7	36
4.19 In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of H-1299	37
4.20 In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of HUVECs, MCF-7, C4-2 and H-1299	38
4.21 Schematics of spheroids fabrication for drug treatment studies . .	39
4.22 3D in vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate (Brightfield images of individual spheroids after drug treatment at day 5 for C4-2 with graphical description of size change)	40
4.23 3D in vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate (Brightfield images of individual spheroids after drug treatment at day 5 for H-1299 with graphical description of size change)	41
4.24 Stained images of spheroids after treatment with drug in C4-2 (left) H-1299 (right)	42
A.1 (A) The stability analysis of drug formulation after 8 weeks of formulation at different flow rates. The figure shows that the formulation was more stable at a 100µl/h flow rate than 50µl/h. (B) Stability analysis of the same formulation using DLS and (C) Reproducibility of zeta potential results to ensure the reliability of the obtained data	59
A.2 The removal of unbound drug from synthesized bioconjugate . .	60

A.3	The assembly utilized for drug release at different pH	60
A.4	QTOF data of Dox solution showing peak at 397nm and 544nm	61
A.5	The microfluidic setup for nano-bioconjugate synthesis	61
A.6	Brightfield images of C4-2 spheroids (A) control without treatment (B) Dox 2.5 μ M treated (C) Aprotinin 1.5 TIU treated (D) Apr- Dox 2 μ M treated (E) Apr-Dox 6 μ M treated (F) Apr-Dox 10 μ M treated	62
A.7	Brightfield images of H-1299 spheroids (A) control without treat- ment (B) Dox 2.5 μ M treated (C) Aprotinin 1.5 TIU treated (D) Apr-Dox 2 μ M treated (E) Apr-Dox 6 μ M treated (F) Apr-Dox 10 μ M treated	62
A.8	Simulation to elucidate the velocity profile of Y-mixer for synthe- sizing nano-bioconjugates using microfluidic chip	63
A.9	Numerical simulations showing mixing performance along the Y-mixer at optimal flow rate (100 μ l/h) for synthesizing nano- bioconjugates using microfluidic chips	63
A.10	Combined graph for dox internalization and fluorescence emission determined via flow cytometry	64

List of Tables

A.1	Drug conjugates' hemolysis activity and absorbance values compared to the positive and negative control.	65
A.2	Details of various drug release models. where C_t = amount of drug dissolved in time t , C_0 = the initial amount of drug in the solution, K_0 = zero order release constant, K_t = first order release constant, C_s = drug solubility in the matrix media, D = diffusivity of drug molecules in the matrix, M_t/M = fraction of drug released at time t , K_p = release rate constant, n = release exponent	65

Chapter 1

Introduction

In the current scenario of cancerous research, 3D cell culture models outshine conventional 2D monolayer cells by closely biomimicking the in vivo microenvironment; including crucial features like gradient constituents, cell-matrix, and cell-cell interactions [1], [2], thus making them more physiologically relevant yet convenient in vitro models in the regime of pre-clinical drug testing. These advancements have also sparked growing interest in 3D cell culture system as noticeable tool across various biological disciplines, particularly in cancer research and comprehensive drug screening. While designing 3D culture models, both adherent and non-adherent substrates have been underlined, for instance, via scaffold-based methods in which cells can interact with a substrate or via non-adherent scaffold-free methods in which non-adherent substrates are used to culture cells [3], [4]. Within the realm of these models, organoids and spheroids have gained significant acceptance, where former represent self-organized models of complex organ-like tissue, [5]–[7] while later represents cells aggregates that form a cell sphere without adhering to any culture substrate. However, in order to attain highly efficient and controllable 3D tumor spheroid models, there is a dire need to generate a 3D-model system out of various cancer cell lines that offer a state-of-the-art growth pattern monitoring for each cell line as a benchmark setup in a controlled and precise manner.

Despite the fact that targeted therapy was first proposed almost a century ago, to date, only a limited number of biomolecules-based nano-carriers and strategies targeting the tumor have progressed to clinical trials. Notably, a few of these carriers have received clinical approval, for instance, Abraxane (albumin-bound paclitaxel, owing to the complexity of designing the particles that can meet intricate criteria of targeted drug delivery. Understanding the current need, herein, we designed a protein-drug nanoconjugate utilizing nature’s multifunctional universal carrier i.e., aprotinin (Apr) as drug delivery vehicle. Structurally, this protein is composed of a chain of 16 distinct amino acid types with 58 residues that folds into a stable, compact tertiary structure of the small chain SS-rich type. The chain has a molecular weight of 6512 Da and is made up of three disulfides, a twisted -hairpin, and a C-terminal -helix, ideal to encapsulate small drugs such as dox. Aprotinin, a mostly occurring biopolymer in living organisms, raises fewer concerns in clinical translation compared to synthetic nano-carriers. Aprotinin, a naturally abundant protein involved in iron homeostasis and storage, offers several advantages for biomedical use. Ferritin-based nanosystems possess favorable properties compared to other systems, including high solubility and stability in water and blood, as well as low toxicity. These characteristics make aprotinin an appealing nature’s universal choice for in vivo applications in humans. Besides structure, aprotinin also offers a wide range of attractive characteristics like aqueous solution dispersity, uniform nano-size, easy functionalization, and low cost for large-scale production[8]–[11]. Therefore, we aimed to design aprotinin-drug nanoconjugates as a single and/or dual drug carrier that can effortlessly bind to tumor cells in cancers cells, leading to its endocytosis for the targeted delivery. Hence, in contrast to conventionally fabricated nanoparticles, the synthesized aprotinin nanoconjugate system offers several advantages, including intrinsic tumor targeting ability, favorable pharmacokinetics and safety profiles, excellent antitumor activity, easy scaling-up and manufacturing with robust and reproducible procedures, universal drug- loading platform [12]. These unique properties make the aprotinin nanoconjugate an ideal vehicle for efficient anticancer drug delivery.

There is a growing demand for new cancer treatments nowadays including

agents that can enhance the effectiveness of existing “anti-cancer” drugs[13]. Proteins have emerged as a promising nano-carrier molecules for drug delivery in various diseases, particularly for cancer[14]. A nano-carrier protein for drug delivery denotes a protein that transports therapeutic agents, such as small molecules, drugs, or nucleic acids to the targeted cells or tissues[15]. Such carrier proteins can improve the efficacy and specificity of drug delivery by protecting the therapeutic agents from degradation, enhancing their stability and bioavailability, and facilitating their targeting to the specific cells or tissues[16]. Moreover, these biocarriers can help target the anti-cancer drug to the tumor site, reducing the risk of off-target toxicity with increased drug concentration at the site of action[17]. Despite the controlled drug release can be achieved using some carrier biomolecules[18] such as BSA[19], HSA[16] and/or Fetuin[18] to reduce the dosing frequency and thus, potentially reduce the toxic side effects that come with systemic delivery of chemotherapeutic drugs[19]; however, recently, the focus has shifted to the involvement of some unique proteins *as therapeutics* called “moonlighting”[20] or “pleiotropic” proteins due to their distinctive ability to perform multiple, seemingly unrelated functions in the cell[20]. Since they can perform other activities in addition to their best-known or predominant activity, it thereby makes the drug delivery through carrier molecules more diverse[21], [22]. Interestingly, many moonlighting proteins are enzymes with a primary function of enzymatic catalysis, as well as having acquired secondary non-enzymatic roles such as autophagy, protein transport, or DNA maintenance[23]. For instance, a moonlighting protein exhibiting potential natural anti-cancer properties, can also offers to be a carrier molecule (due to its structural characteristics) to transport another therapeutic agent to the target tumor site[24], [25], thus simultaneously inhibiting cancer cell proliferation and inducing cell death, thereby exhibiting profound influence in cancer treatment [26].

One such model moonlighting protein is lactoferrin (from mammalian milk), which has been investigated to have anti-tumor activity[27] and also has been used as a carrier for a renown anti-cancer drug Doxorubicin (Dox) to target cancer cells[24], [25]. Moreover, metalloproteinases protein (MMPs), the enzymes that degrade components of the extracellular matrix and impact the invasion

and growth of cancer cells[27], process a wide variety of substrates, including chemokines and other cytokines have also opened new frontiers for moonlight protein studies. Out of various cell signaling pathways, in one mechanism, the plasminogen-plasmin system's proteolytic enzymes play a crucial role in the spread and invasion of cancer cells[28]. This is due to the high activity of plasminogen activators (PA) such as urokinase-type (uPA) and tissue-type (t-PA) in many primary tumors[29]. The expression of uPA, its receptor (uPAR)[30], and its inhibitor (uPAI) has been linked to cancer progression through invasion, cell proliferation, metastasis, and tissue remodeling[29] and via MMPs activation[26]. Consequently, blocking these proteolytic enzyme cascades can be a promising strategy to suppress the metastatic and invasive nature of tumor cells to distant sites; hence offering a promising approach for cancer therapy[31].

Chapter 2

Scientific Background: Aprotinin as Moonlight Nanocarrier

Aprotinin, a nonspecific serine protease inhibitor [32] with antifibrinolytic and anti-inflammatory properties of the drug as cited by [33], has only been shown to suppress the release of proinflammatory cytokines [34] and decrease the ability of leukocytes to pass through the vascular wall [33] for an alternative cancer treatment [35]. However, the limited number of *in vitro* and *in vivo* studies on the anti-tumor effects of aprotinin have produced varying results, depending on the type of cancer model investigated. Despite the significant role of the uPA-plasmin system and MMPs in cancer progression and prognosis, the anti-tumor effects of aprotinin in different cancers and its nano-drug carrier properties *have not yet been explored*. Hence, utilizing such moonlighting protein candidate as a nano-bioconjugate can be the key feature to explore.

The synthesized nano-bioconjugates were tested against various cancer cell lines, e.g., lung (H1299), breast (MCF-7) and prostate (C4-2) cancer cells, while also providing its comparison with healthy human umbilical endothelial cells (HUVECs) as a proof-of-concept to display non-toxic behavior of aprotinin against healthy human cells as a natural carrier protein. Our results have confirmed that the Apr-Dox nano-bioconjugates caused increased cytotoxic activity (e.g., up to 30-40%) in the tested cancer cell types, along with no signs of toxicity on healthy cells (HUVECs); thereby, showing a selective targeting approach of aprotinin towards cancer cells (Schematic 1). Furthermore, another interesting outcome obtained is that the synthesized Apr-Dox nano-bioconjugates showed a tremendous decrease (~ 100 times less) in the usual dose of Dox (i.e., $\sim 100\mu\text{M}$). As well, when testing this nano-bioconjugate formulation with a targeted pH release profile (at pH 4.0) it proved to be advantageous to release the drug specifically at the tumor site in the acidic environment. Thus, pH sensitive release improves its effectiveness and minimizes systemic toxicity by overcoming major factor in failure of cancer therapeutics where the microenvironment of solid tumors often exhibits distinct pH characteristics (typically acidic extracellular pH) compared to normal tissues.

In recent years, spheroid culture has been highlighted as a renowned three-dimensional (3D) technology that curates the complex architecture of tumors *in vivo*, and has been considered as an efficient model to predict chemotherapeutic drug treatment response in physiological systems [36]. 3D tumor cell models in the *in vitro* biological testing of nanotechnology-based cancer treatment techniques provide in-depth knowledge of the actual behavior of tumor cells within intricate biomimetic structures [37]. Thus, we also tested the chemotherapeutic effect of Apr-Dox nanobiocojugate in 3D tumor cell models; predicting a clear cytotoxic differentiation between normal and cancer cells.

Consequently, the Apr-Dox nano-bioconjugate appears to be a novel nano-formulation, fulfilling the co-drug delivery approach with nano-drug carrier properties, targeted drug release properties, low dosage applications as well as improved anti-cancer proliferative effect broadly applicable for drug targeting in multiple cancer models with great prospects for clinical applications.

Chapter 3

Methodology

3.1 Materials

Drugs such as Aprotinin (99%) was ordered from Cayman Chemical Ltd. (Michigan, USA) and it was used without further purification. Dox-HCL lyophilised injection was purchased from Saba East Pharma Company (Ankara, Turkiye) and it was used as such without purification. RPMI 1640 with L-Glutamine medium, DMEM With High Glucose, L-Glutamine, and Sodium Pyruvate medium, penicillin-streptomycin solution fetal bovine serum (FBS) and trypsin-EDTA (0.25%) were all supplied from Serana Europe GmbH (Brandenburg, Germany). The water used in all the experiments was ultrapure (18.2 M).

Staining dye; Calcein-AM was obtained from Invitrogen, Thermo Fisher Scientific Inc. (Massachusetts, US) and it was used after appropriate dilutions according to manufacturer's instructions. Propidium iodide solution was provided by Merck KGaA (Darmstadt, Germany) and it was used according to instructions provided by manufacturers. All other reagents were used without further purification.

3.2 Isothermal Titration Calorimetric (ITC) Analysis of Apr-Dox Nano-bioconjugate

Isothermal Titration Calorimetry (MicroCal VP-ITC calorimeter) was utilized to study the thermodynamics of molecular interactions between Dox (drug) and Aprotinin (protein). Furthermore, the optimum number of Dox molecules that can integrate in Aprotinin structure were also identified. Briefly, the sample solution contains Aprotinin (1.5TIU), while the titrant solution contained Dox solution (1mM). The ITC experiment was performed at 25°C with a titration program of 30 injections and individual injection volume to be 2µl. The titration was initiated after establishing an equilibration time of 300s to reach a baseline, followed by 2µl aliquots injection of Dox solution at 1min intervals into the sample cell containing the protein of interest (aprotinin). Then, 50µl of Dox (1mM) was titrated to 380µl of aprotinin (0.1mM) by 30 injections. The heat changes were recorded and analysed using the data acquisition software provided with the calorimeter.

3.3 Synthesis of Apr-Dox Nano-bioconjugate using Microfluidic Platform

The microfluidic device was fabricated according to standard soft-lithography protocols. The microfluidic device consists of a polydimethylsiloxane (PDMS) mold with the designed microchannels for fluid transport and a glass substrate carrying the mold. For the fabrication of microfluidic chips, SU-8 masters were fabricated on silicon wafers using the similar soft-lithography technique we used in our previous works [38], [39]. Briefly, to make PDMS replica molds, PDMS prepolymer was mixed at a 10:1 ratio of base to curing agent, degassed, and molded against the SU-8/silicon master and then cured at 95 °C for 2 hours. The molds were then peeled off from the master, and fluidic access holes were made using a 1.5 mm biopsy punch. The PDMS mold was then treated with oxygen

plasma at 100 W, 400 mTorr for 30 s and bonded to a glass substrate.

The microfluidic chip was connected to the syringe pumps using Teflon tubes inserted in microfluidic chip at inlets. For the one step synthesis of Apr-Dox nano-bioconjugate, Apr (0.01mM) was dissolved in PBS (5mL), and Dox (1.1mM) was dissolved in water (5mL). Later, the Dox and aprotinin solutions were separately fed into two inlets of the microchannels at the same flow rate of

100 μ l/h via syringe pumps. The synthesized product was obtained from the outlet, followed by the dialysis process (dialysis membrane-MWCO 1KDa) to remove excess/unbound drug from the Apr-Dox nano-bioconjugate solution. The microfluidic setup for synthesis is shown in Fig:A5.

3.4 Characterization of Apr-Dox Nano-bioconjugate

Microfluidic-based Apr-Dox nano-bioconjugate was further confirmed by various characterization techniques. The particle size and zeta potential of Apr-Dox nano-bioconjugate was characterized using DLS Malvern (Malvern Zetasizer Nano) at 25°C. The particle diameter and morphology was further confirmed through transmission electron microscope (TEM) by pouring a drop of nano-bioconjugate formulation onto a carbon-coated copper grid, dried in air at room temperature and imaged immediately.

3.5 Spectroscopic Analysis of Apr-Dox Nano-bioconjugate

The conformational properties and secondary structure of aprotinin alone and after conjugation with Dox was determined by CD Spectroscopy, using Chiral scan Spectrophotometer. The Apr-Dox nano-bioconjugate sample was measured in triplicate at the far UV-spectrum wavelength ranging from 190 to 250nm, with

1nm step size and 1nm bandwidth. The data was analysed quantitatively using SpectraMax Pro analysis software provided with the instrument.

The UV-Vis spectroscopic analysis of the sample solutions was performed using quartz cells with a path length of 1cm. The concentration of aprotinin (0.1mM) used was same as it was used in synthesis of Apr-Dox nano-bioconjugate to give comparative spectra results.

3.6 Equilibrium Dialysis for the Removal of Unbound Drug

The unbound drug was removed by equilibrium dialysis technique conducted in a Tube-O-DIALYZER™ 1kDa MWCO as explained by Wallace *et al.*[40]. To briefly explain, a 3ml Apr-Dox nano-bioconjugate was filled in the dialysis tube and it was suspended in distilled water as release media (Fig:A2). Dialysis membrane was pre-soaked in water before use, according to manufacturer's instructions.

3.7 Percent Drug Loading (DL) and Drug Entrapment Efficiency (EE)

The percentage of drug loading refers to the amount of drug present in the nano-bioconjugate, while the drug entrapment efficiency refers to the amount of drug that is effectively entrapped in the nano-bioconjugate. These parameters are essential for determining the efficacy of the nano-bioconjugate and its potential for clinical use. DL and EE studies of the synthesized formulation were also performed using the dialysis method. The Dox-loaded nano-bioconjugate (1ml) was poured in the dialysis bag (MWCO 1KDa). The dialysis bag was then placed in a small beaker containing 5ml PBS (pH 7.3), and kept on the magnetic stirrer for continuous stirring up to 3h. The assembly for drug release analysis is

shown in Fig:A3. After 3h, sample was withdrawn from the beaker (containing eluent diffusing out of dialysis membrane), and the sample was quantified for the presence of Dox using LCMS-QTOF. The peaks observed for the presence of Dox molecules are shown in Fig:A4. Further, the percent drug entrapment efficiency and percent drug loading were calculated using the formula below. The drug content that remained in the dialysis bag was also analyzed to confirm the results.

$$\% \text{ Drug Loading} = \frac{(\text{Drug conc.} - \text{Unentrapped Drug conc.})}{\text{Drug conc.}} \times 100 \quad (3.1)$$

$$\% \text{ Entrapment} = \frac{(\text{Dox Abd in BC} - \text{Dox Abd in RM})}{\text{Dox Abd in Stock Solution}} \times 100 \quad (3.2)$$

BC = Bioconjugate

Abd = Abundance

RM = Release Media

3.8 *In vitro* Drug Release and Release Kinetics

The drug release from the Apr-Dox nano-bioconjugate was studied *in vitro* under different pH conditions. The synthesized nano-bioconjugate formulation (1ml) was poured in dialysis bags. Each dialysis bag was suspended separately in 5ml of 0.2M Glycine buffer (pH 3.0), 0.2M Glycine buffer (pH 5.0), and phosphate buffer saline (PBS) pH 7.4, respectively. Next, 200µl of samples (eluent) were withdrawn from each dialysis assembly at regular intervals, and the volume of buffer was maintained with the similar amount (200µl) of respective buffer to maintain experimental conditions.

Then cumulative drug release was determined using ELISA microplate reader at an excitation and emission wavelength of 470nm and 560nm, respectively. The release kinetics of Dox from the nano-bioconjugate was analysed using mathematical models such as the zero-order, first-order, and Higuchi models with their details enlisted in Table A.2[41].

3.9 Hemolysis Assay

The hemolytic activity of the Apr-Dox nano-bioconjugate was determined using a spectrophotometric method adapted from Krishnamurthy *et al.*[42]. Briefly, for this assay, blood was withdrawn from healthy donors after institutional ethical clearance and appropriate informed consent and collected in 5ml heparin tubes. Blood samples were centrifuged at 2000rpm for 5min at RT to collect erythrocytes (RBCs), which were then washed with phosphate-buffered saline (PBS) three times to remove the plasma altogether. The cells were then suspended in PBS to a concentration of 2×10^8 cells/ml. Different concentrations of the Apr-Dox nano-bioconjugate (i.e., 5 μ M and 10 μ M) were added to the 1ml of erythrocyte suspension, and this mixture was further incubated for 1h at 37°C. PBS and SDS/Triton X100 were used as negative and positive controls, respectively. To prepare these positive controls, SDS and 0.1% Triton X100 solutions were added in 1ml of erythrocyte suspension each.

The extent of hemolysis was determined by measuring the release of hemoglobin from the erythrocytes using a spectrophotometer. The percentage of hemolysis was calculated as:

$$\% \text{ Hemolysis} = \frac{(\text{Sample Abs} - \text{Control Abs})}{(\text{Max Abs} - \text{Control Abs})} \times 100 \quad (3.3)$$

3.10 Cellular Uptake Studies of Apr-Dox Nano-bioconjugate

The cellular uptake was measured following flow cytometric analysis (LSRII, BD Biosciences). The experiment involved culturing three cancer cell lines (MCF-7, H-1299, and C4-2) and one healthy cell line (HUVECs) in a 96-well plate and incubating them at 37°C under 5% CO₂ for cell attachment. After approximately 12h of incubation (observing cell attachment) the cells were then treated with Dox alone, aprotinin alone and Apr-Dox nano-bioconjugate, followed by incubation for 24 hours.

Next, excess drug was removed by washing with PBS three times, and the cells were detached using TrypLE Express. Culture medium was added to stop trypsinization, and the cells were centrifuged. After discarding the supernatant, PBS (500l) was added to resuspend the cells for flow cytometry analysis. The intrinsic fluorescence ability of Dox was used to detect the fluorescence of Apr-Dox nano-bioconjugate at 470nm excitation and 560nm emission channels. Approximately 1000 gated cells were analyzed, and cells without any fluorescence labelling were used as the control. The FlowJo software was used to analyze the flow cytometry data.

3.11 2D In vitro Cytotoxicity Assay of Apr-Dox Nano-bioconjugate

The in vitro cytotoxic analysis was performed to evaluate the anti-cancerous efficacy of Apr-Dox nano-bioconjugate in both cancer and healthy cells. Two different concentrations of Apr-Dox nano-bioconjugate (5µM and 10µM), Dox alone (10µM), and aprotinin alone (5TIU) were prepared separately and cultured with each cell line for 48h. The data was analysed qualitatively using bright field imaging of a Zeiss Microscope.

3.12 3D In vitro Cytotoxicity Assay of Apr-Dox Nano-bioconjugate

The prostate cancer (C4-2) and non-small cell lung carcinoma (H-1299) spheroids synthesized in this study were established on the methodology described by Wei Liao *et al.*[36]. In short, the cells can form tight spheroids at day 3 of the culture with a defined border when seeded with 400 cell/microwell. The spheroids had an average diameter of 300µm when treated with Apr (1.5 TIU), Dox (2.5M) and Apr-Dox nano-bioconjugate (2-10M) at day 3. Media change was performed every second day and staining was done at day 7 to visualize the drug-treated and non-treated spheroids. The growth analysis was performed by measuring individual spheroids size. Images of Apr-Dox nano-bioconjugate drug diffusion were acquired in Zeiss microscope under Brightfield and fluorescence imaging filters respective to Calcein-AM and Propidium Iodide.

3.13 Live / Dead Cell Staining Assay

The qualitative analysis of the monolayer cells subjected to different drug treatments was investigated using live/dead cell staining assay. For live cells staining, samples were prepared according to standard staining protocols of Calcein-AM as provided by manufacturer's instructions. Briefly, the cells after incubation for 72h were subjected to staining process. The cell monolayers were rinsed with PBS solution to remove serum residues since, esterase present in Calcein-AM stain interferes with serum protein thus impacting the staining quality. Afterwards, Calcein-AM reagent (2µM) was added in each monolayer well to submerge cells completely. Incubation was performed for 2h with subsequent removal of dye and addition of cell culture medium and imaged under fluorescence illumination using LED illumination coupled with an Axio observer microscope.

Propidium iodide (PI) dye was used to stain dead cells according to manufacturer's instructions. The cells after fixation were submerged in 10mM PI dye and

were observed immediately after incubating it for 5min. Further imaging and analysis were performed accordingly.

3.14 Microscopy and Imaging

The cell morphology and/or changes in size and shape of cell were monitored using an inverted optical Axio observer microscope (Zeiss, Germany) under bright field imaging. Morphological changes were observed after treatment with drugs (alone) and nano-bioconjugate at every 12h interval.

Fluorescence illumination using LED coupled Zeiss microscope was used to obtain micrographs using a 10x objective. For the quantitative analysis of live/dead cells, ImageJ software was used to count the cells followed by graphical illustration of the obtained data.

3.15 Statistical Analysis

The in vitro experiments were repeated three times and analyzed statistically through the Student's t-test. The results were shown as the average and standard error of the three parallel measurements.

Chapter 4

Results & Discussion

4.1 Aprotinin Binding Affinity to Dox

To correlate the binding affinity of aprotinin (protein) with Dox (drug) molecules, herein, the protein binding affinity of aprotinin was examined through isothermal titration calorimetry (ITC) assay. In general, the analysis of protein's affinity to bind with small molecules such as drugs is usually performed through bioinformatics docking analysis [43] which is considered comprehensive and accurate but rather complicated, costly, and time-consuming. Thus, a simple isothermal reaction parameter is considered quick and valuable for analyzing the binding ability of a biomacromolecule. Principally, in the ITC assay, the K_a (equilibrium association constant) between aprotinin and Dox molecules was detected by drop-wise titration of aprotinin (0.1mM) into Dox solution (1mM) and the exothermic reaction of each drop was monitored where the integral of the titration data was set as the heat of each injection. To determine the value of K_a , the function between titration heat in kilocalories and the molar ratio of the concentration of aprotinin protein to that of Dox molecules were analyzed.

As shown in Fig: 4.1, the K_a values obtained were $8.5 \times 10^5 \text{ M}^{-1}$, and K_d (equilibrium dissociation constant) values were calculated to be 1.17×10^{-6} which

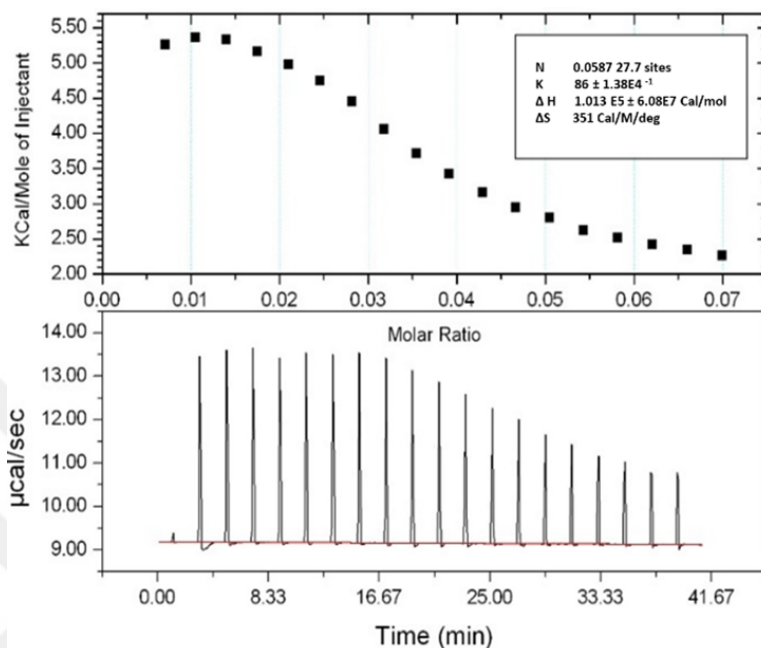


Figure 4.1: Isothermal titration calorimetric analysis for aprotinin binding affinity to Dox molecules. Molar ratio between the amounts in moles of aprotinin and dox involved in a balanced chemical reaction are shown in graph below. K_a (equilibrium association constant) value is $8.5 \times 10^5 \text{ M}^{-1}$ showing strong binding

shows a strong binding affinity at a ratio of 1:11 of protein-to-drug (i.e., 1mM Dox molecules with 0.1mM aprotinin biomolecule). The obtained data indicate that being a small protein, a single aprotinin molecule has the affinity to bind with approximately 10 Dox molecules. Therefore, these optimum concentrations were used in the proceeding experiments for the microfluidic-assisted synthesis of Apr-Dox nano-bioconjugate.

Theoretically, binding affinity usually refers to the binding strength between a single biomolecule (such as a protein or DNA) and its ligand/binding component (such as a drug or inhibitor), and it is typically measured and reported by the equilibrium association constant (K_a) or equilibrium dissociation constant (K_d), to assess the strength of bimolecular interactions. Thus, the larger the K_a value or smaller the K_d value is, the greater the binding affinity of the ligand to its target. Similar results were observed by Cao et al. [44] for small serum proteins binding with nanoparticles, complementing the ITC results obtained in these experiments. In another study by Delavari et al.33, the heat generated after each

consecutive injection of Dox solution gradually decreases but does not stabilize. This might indicate that the protein structure has been reorganized in addition to Dox binding [45]. Thus, it can be concluded that the binding affinity is affected by intermolecular interactions between noncovalent bonds such as hydrogen bonding, electrostatic interactions, hydrophobic forces, and van der Waals forces. Hence, the concluded data confirms the presence of bonding between aprotinin and Dox molecules showing the successful formation of Apr-Dox nano-bioconjugate.

4.2 Synthesis and Characterization of Apr-Dox Nano-Bioconjugate

In order to enhance the Dox loading capacity, tumor targeting specificity, and achieve optimum drug binding ratio to macromolecule, Y-mixer microfluidic platform was utilized for one step bioconjugate synthesis. The velocity profile of fluids from two inlets and mixing efficiency was optimized via simulation showing that the mixing was complete at 100 μ l/h (Fig: A8 and A9). As shown in Schematic 2.1, Dox was introduced from one inlet and aprotinin was introduced from the second inlet. The flow rate of synthesis was optimized as 100 μ l/h from simulations. After mixing two solutions in the serpentine portion of the chip design, the formulations for each flow rate were collected from the outlet to analyze and characterize the formation of nano-bioconjugate. Coincidentally, Dox molecules have intrinsic fluorescence properties thus, the mixing could be visualized in real time using fluorescence microscope at 530nm, giving red emission signal. Further, in order to monitor the effect of different flow rates on the stability of nano-bioconjugates, the synthesized product was observed for 8 weeks at 4°C. To much interest, the formulation developed with 100 μ l/h flow rate was stable for longer period of time as compared to the products obtained at other flow rates (Fig:A1-A). Principally, aprotinin, being a protein, is made of amino acid which provide various binding sites to the drug molecules mainly through COO⁻, NH₂⁺ and some other charged groups. The flow rate optimization at 100 μ l/h provided enough time for the small drug molecules to occupy appropriate binding sites

of the protein in a co-flow mixing technique unlike the low flow rates (30 μ l/h, 50 μ l/h) which resulted in concentrated precipitation of Dox molecules. These results are in line with the study of Hakala et al.³⁴ for HAS-drug nanoparticles formulation [46]. Moreover, the optimized 100 μ l/h flow rate provides sufficient throughput as compared to lower flow rates [47]. Proceeding to the microfluidic synthesis of Nano-bioconjugates, the unbound drug molecules were removed from the final formulation using dialysis membrane (MWCO 1KDa). Since, the weight of Dox molecule is 0.58KDa, the free/unbound drug could equilibrate in the external aqueous solution via equilibrium dialysis as also explained in previous study of Wallace et al.[40]. Following this, all the unbound drug was removed in 2-4h and the final nano-bioconjugate formulation was collected for further experiments

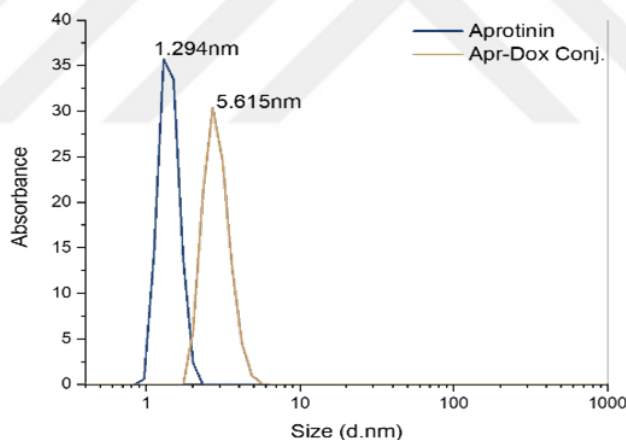


Figure 4.2: DLS analysis of aprotinin alone and after conjugation with Dox

The obtained nano-bioconjugates were characterized for its size, shape and charge using DLS, TEM and zeta potential, respectively. From DLS analysis, the size of aprotinin was found to be 1.41nm, however the bioconjugate showed an increased size of 5.65nm, thus indicating the encapsulation of Dox molecules by aprotinin (Fig: 4.2). Moreover, the synthesized nano-bioconjugate was also tested for stability upto 2 months (Fig. A1-B), depicting a stable formulation to be used as a drug carrier. Similar observations has been conducted previously for drug carriers, showing increase in the size of a carrier compound, upon holding the drugs molecules [48]–[50].

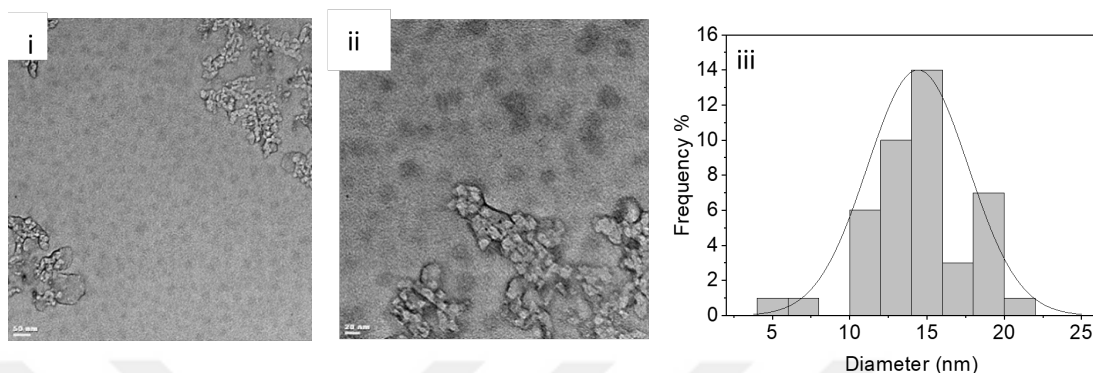


Figure 4.3: Transmission electron microscopy (TEM) images of synthesized bioconjugate, i- Scale 50nm ii- Scale 20nm iii- mean graphical size obtained from TEM images

To correlate the results obtained from DLS, Transmission electron microscopic (TEM) data was collected. TEM images showed spherical shaped nano-bioconjugates with a consistent size, comparable with DLS results (Fig: 4.3). Furthermore, the surface charge properties of aprotinin alone as well as Apr-Dox nano-bioconjugate was measured through zeta potential analysis. Aprotinin alone gave a charge potential of -10.1 mV, which was shifted to less potential of -5.48 mV in nano- bioconjugate formulation (Fig: 4.4, A1-C).

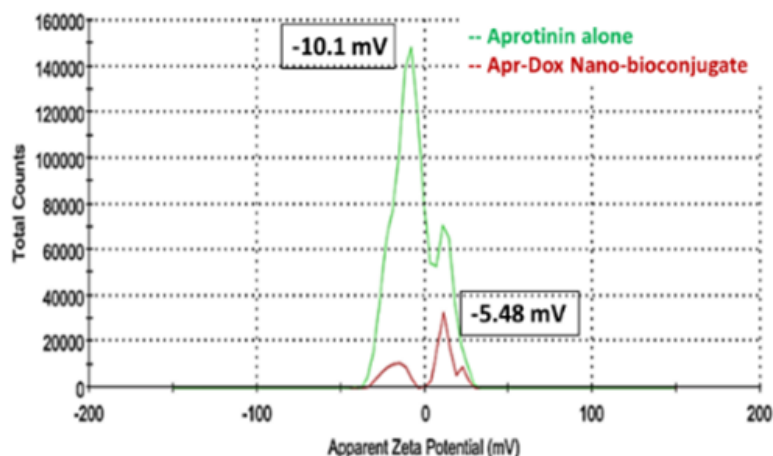


Figure 4.4: Zeta potential of aprotinin alone and after conjugation with Doxorubicin molecules

At this point, the surface charge was switched to less negative because amine

residues of Dox contributed to the additional protonation of protein moieties in Apr-Dox nano-bioconjugate. However, the overall negative surface charge of nano-bioconjugate has the potential to shield itself in blood circulation and provide stability in aqueous environment as indicated in the study of Li et al [50]. Moreover, the zeta potential results suggested that this nano-bioconjugate can easily release Dox molecules at lower pH [51].

The loading of Dox molecules to aprotinin was further confirmed through UV-Vis absorption spectra (Fig: 4.5). Aprotinin showed a typical protein spectrum at 280nm which gets shifted with the addition of Dox molecules in protein structure. Similarly, Dox alone gives a characteristic absorption peak around 480nm [52]. The spectra Apr-Dox nano-bioconjugate differ significantly from the native drug and protein spectra with respect to both intensity and peak shape. For instance, in the synthesized nano-bioconjugate spectra, a slight shift in the absorption peak of Dox was observed at 476nm from 480nm whereas the absorption peak of aprotinin was observed at 236nm (shifted from 280nm) thus confirming the successful bioconjugation of protein with Dox molecules[53].

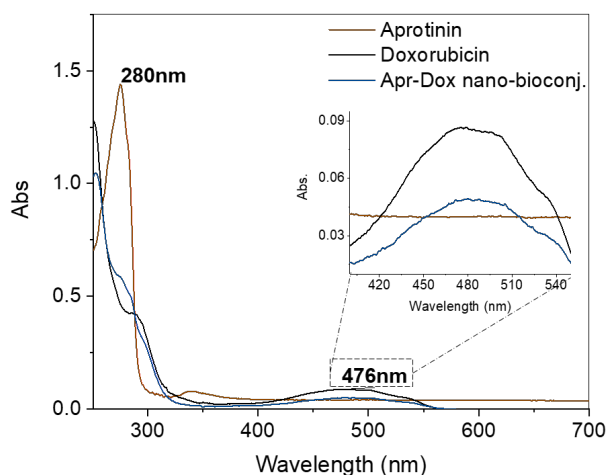


Figure 4.5: UV-Visible spectrophotometric analysis of Apr-Dox nano-bioconjugate

4.3 Secondary Structure Analysis of Aprotinin and Apr-Dox Nano-bioconjugate

In order to retain the anti-metastatic and anti-cancer activity by aprotinin protein, the structural stability of the protein remains a critical challenge. CD spectroscopy is considered a benchmark technique for the secondary structural characterization of proteins with respect to different stimuli (like pH, temperature, denaturants) and/or their binding with any other molecule. Thus, the synthesized Apr-Dox nano-bioconjugate was also analysed for the secondary structure of aprotinin. Figure: 4.6 indicates the far UV region (250-190nm) of CD spectra, that presented α -sheeted structure of aprotinin with a prominent peak at 260nm[54]. Interestingly, the addition of Dox molecules after nano-bioconjugate synthesis did not alter the spectrum signal of native aprotinin molecule, which confirms that the secondary structure of aprotinin was retained even after the formation of nano-bioconjugate (Fig: 4.6). Since the functionality of protein directly relies on its structure and native fold, no prominent alteration in the structure also confirms the maintained anti-metastatic activity of aprotinin even after nano-bioconjugate formation [35].

4.4 Drug Loading/Encapsulation Efficiency and pH Triggered Release of Dox from Apr-Dox Nano-bioconjugate

Dox is a standard first line chemotherapeutic drug and was selected in this study as a model drug owing to its therapeutic action in many cancer types, along with its fluorescence properties that allow real time fluorescence analysis. The effect of the unique single-step synthesis strategy and the drug loading capacity of aprotinin was investigated by the microfluidic synthesis of Apr-Dox nano-bioconjugate in varying concentrations of Dox. For instance, in order to find the maximum

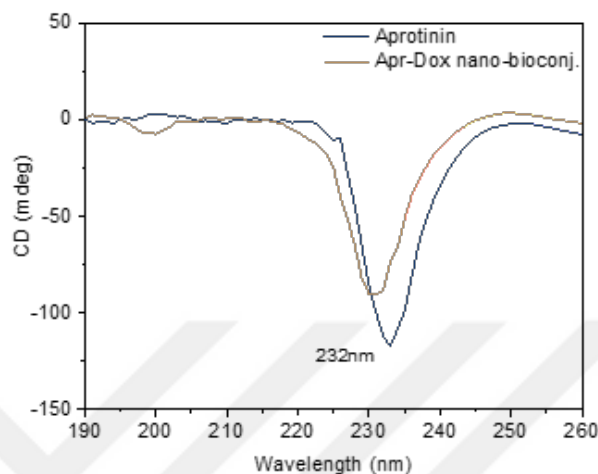


Figure 4.6: Circular Dichroism (CD) spectroscopic analysis of aprotinin alone and after its conjugation with Dox

carrying capacity of aprotinin as a carrier molecule, the concentration of aprotinin was fixed (0.1mM) while the concentration of Dox was increased sequentially (i.e., 0.2mM, 0.4mM, 0.6mM, 0.8mM, 1.1mM and 1.5mM). Afterwards, the formulations were subjected to dialysis (MWCO 1KDa) to eliminate unbound Dox molecules, followed by spectrophotometric analysis to estimate the maximum Dox loading onto aprotinin in a nano-bioconjugate form.

Fig: 4.7 shows that the amount of drug loading in nano-bioconjugate increases with the increased concentrations of Dox. However, at 1.1mM and 1.5mM concentrations the loading of drug is quite comparable, indicating the maximum drug loading capacity at 1.1mM. For a better comparison, Dox alone (in similar concentration range used for nano-bioconjugate formation) has also been measured to generate standard curve. According to the obtained results, the highest encapsulation ratio of Dox was found to be 77% as shown in Fig: 4.8, with an observation of almost similar drug loading capacity at 1.1mM and 1.5mM concentrations, highlighting the maximum carrying ability of aprotinin is utilized.

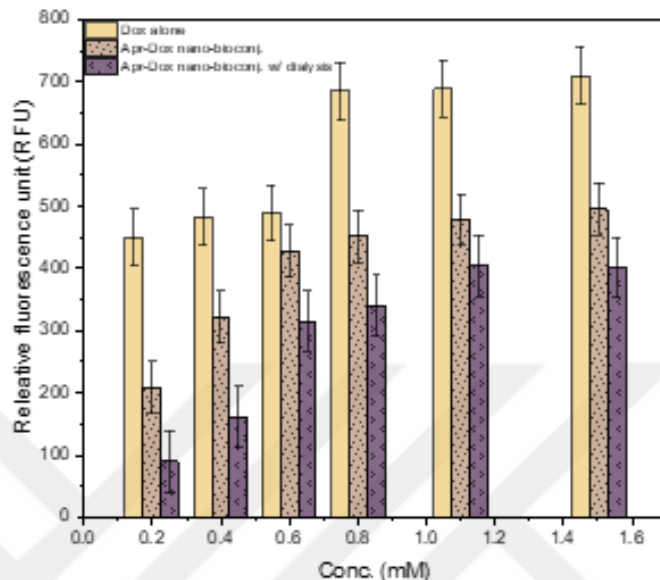


Figure 4.7: Loading of Dox into aprotinin conjugate

4.5 Details of Dox Loading into aprotinin conjugate and Entrapment efficiency of aprotinin with different drug/protein ratios for preparation of Apr-Dox nano-bioconjugate

To simplify the whole process, we first measured the fluorescent ability of Dox at five different concentrations (0.2, 0.4, 0.6, 0.8, 1.1 and 1.5mM) using microplate reader. Then each concentration was used to synthesize Apr-Dox nano-bioconjugate formulation. For instance, firstly 0.2mM Dox solution from one inlet and aprotinin 0.1mM from other inlet was introduced in the microfluidic chip (as mentioned in synthesis part) and product was collected from outlet. In the second experiment, we repeated the same experiment but instead of 0.2mM, we introduced 0.4mM Dox solution and rest of the conditions were same, and this was repeated with all the concentrations (i.e., 0.6, 0.8, 1.1 and 1.5mM). Each product obtained from varying concentrations of Dox was collected and studied for fluorescent intensity. In the third step, these products were dialyzed individually to remove all the unbound Dox molecules, and the resultant dialyzed product

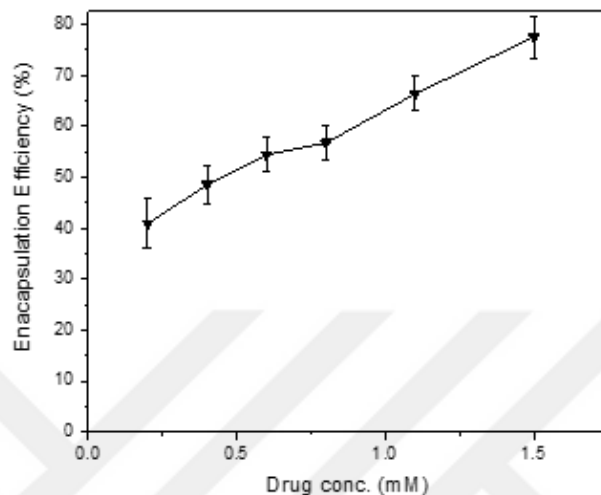


Figure 4.8: Encapsulation efficiency % of aprotinin with different drug/protein ratios for preparation of Apr-Dox nano-bioconjugate. The concentration of aprotinin in all solutions is 0.1mM, whereas Dox concentration varies from 0.1mM to 1.5mM.

was subjected to measure the fluorescence intensity again and thus plotted in the graph as shown in Fig. 3A. The aim of this experiment was to show how much Dox concentration was present at first and, when we synthesized the product, how much of the Dox molecules are loaded in the protein. We have used each Dox concentration as reference point to show the fluorescence after formation of nano-bioconjugate. This shows drug (dox) loading ability of aprotinin (as discussed in the manuscript). Further, in the drug loading ability study, we have discussed that 1.1mM and 1.5mM Dox concentration show same level of drug loading which indicates that after 1.1mM concentration, the protein gets saturated and there is no binding site left for more Dox to bind with current synthesis method. In Fig: 4.8, we are showing entrapment efficiency calculated by the formula given in manuscript and the resultant graph shows that with high concentration of Dox, there was more entrapment happening with the current method of synthesis.

Our loading/encapsulation efficiency results are in accordance with the similar trend found in the study by Li *et al.*[50]. Hence, these results confirms that aprotinin can act as a carrier protein and has the affinity to carry small drug

molecules as a drug delivery biocompatible macromolecule [55]. Moreover, the drug carrying capacity of the nano-bioconjugate system is quite favorable and thus assure high drug delivery to the targeted site where aprotinin acts as a biocarrier. Next, the *in vitro* Dox release from Apr-Dox nano-bioconjugate was monitored at varying pH conditions in PBS and glycine buffer; simulating human physiological pH (pH 7.3) and tumor microenvironment (pH 4-6) using dialysis membrane (MWCO 1KDa). As shown in Fig: 4.9, the release of cytotoxic drug Dox from nano-bioconjugate is highly sensitive to pH changes in the surrounding medium.

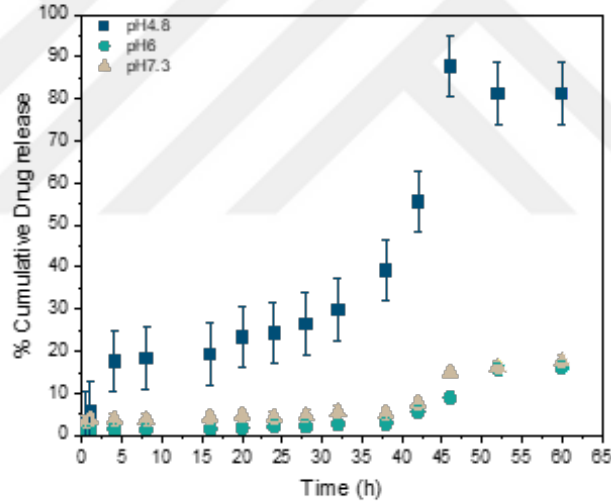


Figure 4.9: In vitro release analysis of Apr-Dox nano-bioconjugate in PBS and glycine buffer (pH 4.8, 6.5 and 7.3)

As the pH dropped from 7.3 to 4.8, the release of Dox molecules was prominently increased in 60 h release experiment, indicating 20% release in initial 10h, followed by gradual increase in the release pattern, with almost half of drug release i.e., 55% in 40h. Only after 50h there was a drastic release reaching to maxima of 90%. However, at pH 6.0 and pH 7.3, the release pattern remained static to <10% up to 40h, with a slight increase (<20%) up to 60h, indicating the expected high stability of the Apr-Dox nano-bioconjugate at higher pH. Apparently, the overall maximum release percentage of Dox was 91% at pH 4.8, 16%

at pH 6.5 and 17% at pH 7.4.

4.6 Protein structure investigation under pH-stimuli

Theoretically, the protonation of amine groups and hydrophilicity of Dox molecules under acidic conditions causes rapid diffusion of Dox moieties into surrounding media from Apr-Dox nano-bioconjugate. Moreover, aprotinin being a protein gets unfolded under acidic conditions, thereby releasing the carrying Dox molecules in the surrounding medium [56]. The unfolding and structural changes of aprotinin in acidic pH were also confirmed from CD spectroscopy as shown in Fig: 4.10. Besides, when the pH decreases from 7.3 to 4.8, aprotinin approaches its isoelectric point, and its surface potential decreases to zero. This diminishes the electrostatic attraction between aprotinin and Dox, resulting in the maximum release of Dox from Apr-Dox nano-bioconjugate .

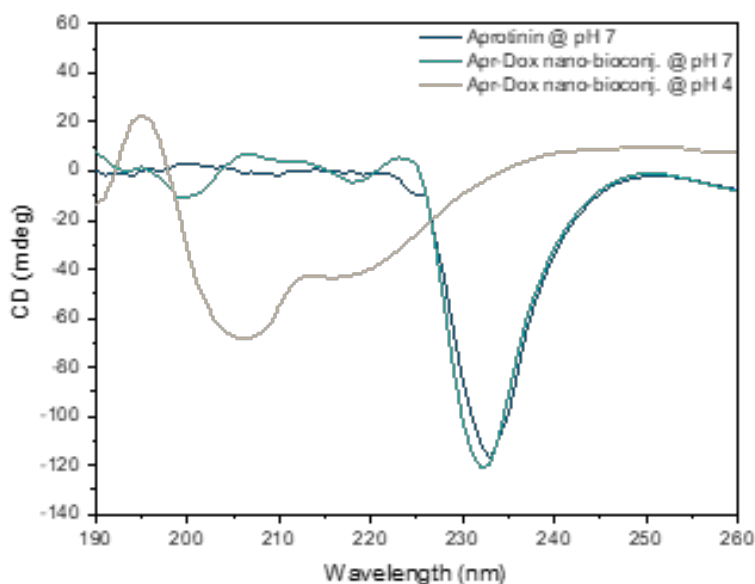


Figure 4.10: Structure alteration of aprotinin to release Dox in acidic conditions by CD spectroscopy

Lately, Stimuli-sensitive nanocarriers have gained considerable attention as they can respond to various stimuli including pH, temperature, metal ions, enzymes, light, etc.[57], hence, selectively releasing drug from the nanocarriers at the intended site[58]. The findings manifested that Apr-Dox nano-bioconjugate would be able to retain the majority of Dox molecules in a natural physiological pH, such as in blood circulation system[59]. However, in acidic tumor environment, it demonstrates more effective Dox release[60]. Thus, the pH-responsive drug release behavior of Apr-Dox nano-bioconjugate could aid in the specific release of Dox at tumor sites, reducing the unwanted side effects with normal cells[61].

4.7 Comparative Cellular uptake of Apr-Dox Nano-bioconjugate

Since Dox is an anti-cancer agent that targets nuclear DNA, it is essential to ensure its efficient internalization into cells, and subsequent release within the cells for optimal therapeutic efficacy. The impact of aprotinin as a moonlighting protein carrier on the cellular uptake of Dox in human healthy endothelial and epithelial cells (HUVECs), as well as in various cancer cell lines (C4-2, H-1299, and MCF-7) was investigated by flow cytometric analysis. This technique being a reliable method for measuring the fluorescence intensity of single cells, provides valuable information on the number of nanoconjugates internalized by each type of cells. The Dox molecules in the synthesized nano-bioconjugate acted as fluorescent dye owing to its internal fluorescent ability (Fig: 4.11, 4.12, 4.13 and 4.14) Apr-Dox nano-bioconjugate in two different concentrations (i.e., 2 μ M and 4 μ M) was incubated with C4-2, H-1299, and MCF-7 cancer cell lines, as well as HUVECs for 24h. Also, Dox alone in equivalent concentration of 2.5 μ M was incubated as positive control with same cell lines to study comparative internalization of bare drug and nano-bioconjugated drug. The nano-bioconjugated drug uptake was then determined by the side scattering signal (SSC), detected by FLR-3 fluorescence filter indicating the increasing uptake of nano-bioconjugate by the cells

[62], [63].

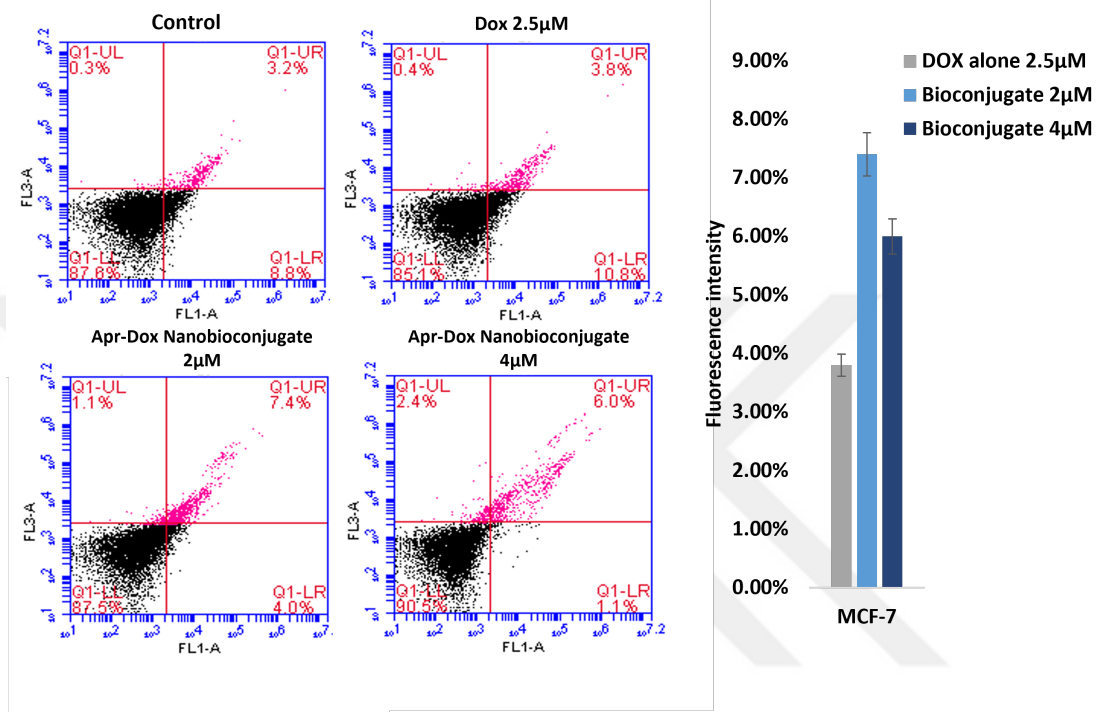


Figure 4.11: Cellular uptake analysis of MCF-7 breast cancer cells

Fig: 4.11, 4.12, 4.13 and 4.14 shows the fluorescent intensity graphs with individual histograms showing fluorescence intensity percentage measured from each treatment cell line and collectively shown in Fig: A10. As presented the fluorescence intensity was higher for Apr-Dox nano-bioconjugate as compared to Dox alone after 24h of incubation in C4-2, H1299, MCF-7 cells, indicating enhanced internalization of nano-bioconjugate by cancer cells. On the whole, the percentage of MCF-7 cells containing Dox molecules increased from 3.8% in Dox alone to 7.4% and 6% in nano-bioconjugate with respect to increasing concentrations (Fig: 4.11). Similarly, the cellular uptake of Apr-Dox nano-bioconjugate in H-1299 and C4-2 cancer cell lines showed comparable trend (Fig: 4.12 and 4.13) with cellular uptake percentages of 3.4% and 6.8% as compared to the cells treated with equivalent concentration of Dox alone (1.8% and 0.9%, respectively).

The high fluorescent intensity percentages with nano-bioconjugate incubation in cancer cells confirm the successful uptake of all Apr-Dox nano-bioconjugated

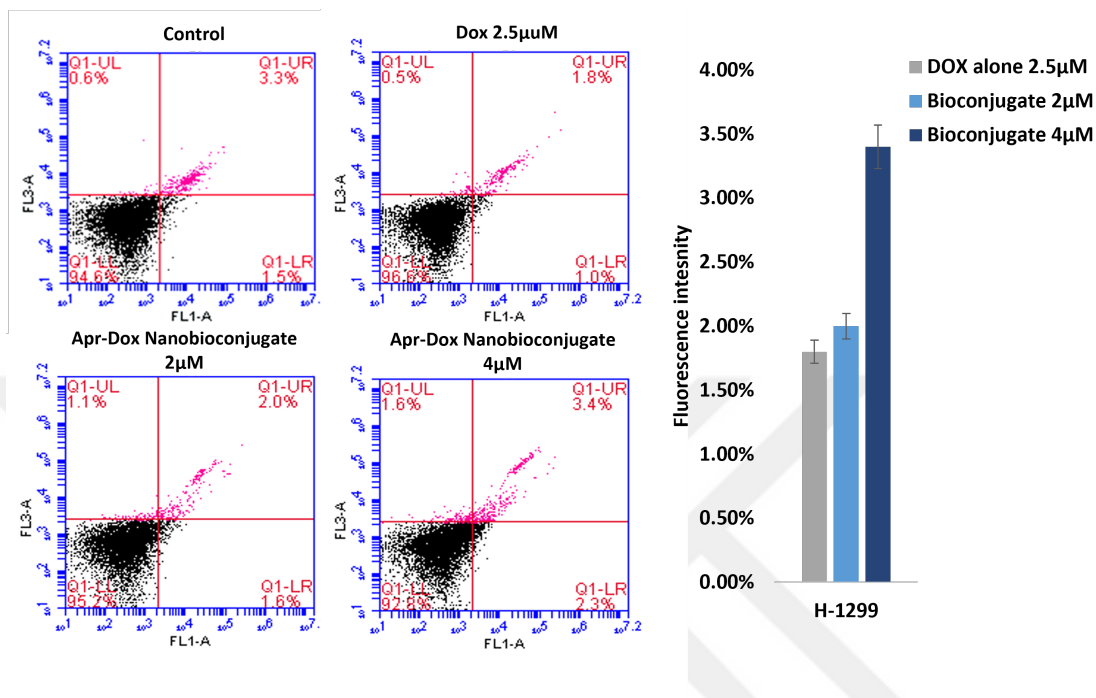


Figure 4.12: Cellular uptake analysis of H-1299 small cell lung carcinoma

molecules. In addition, the concentration dependent uptake was also found in these cell lines where increased concentration of bioconjugated drug led to increased internalisation of Apr-Dox nano-conjugates. For instance, the percentage fluorescence intensity was high for 4µM nano-bioconjugate concentration as compared to 2µM as shown in Fig: 4.11, 4.12 and 4.13.

These results were consistent with the study of Pan *et al.*[64] where aprotinin was administered along with radiolabelled FSH1 peptides to increase the uptake of labelling peptide in tumor. Since, aprotinin is a competitive serine protease inhibitor made up of a single peptide chain with three disulfide links, it is found to inhibit the active sites of enzymes by forming stable complexes with serine proteases including trypsin, chymotrypsin, plasmin, and other similar proteolytic enzymes [65]. Thus, this activity of aprotinin might be involved in the enhanced cellular uptake of Apr-Dox nano-bioconjugate in tumor cells. Remarkably, in comparison to all cancer cell lines, the internalization of Dox molecules in the normal (non-cancerous) cells i.e., HUVECs was significantly low when treated with Apr-Dox nano-bioconjugate as compared to Dox alone (Fig: 4.14). These

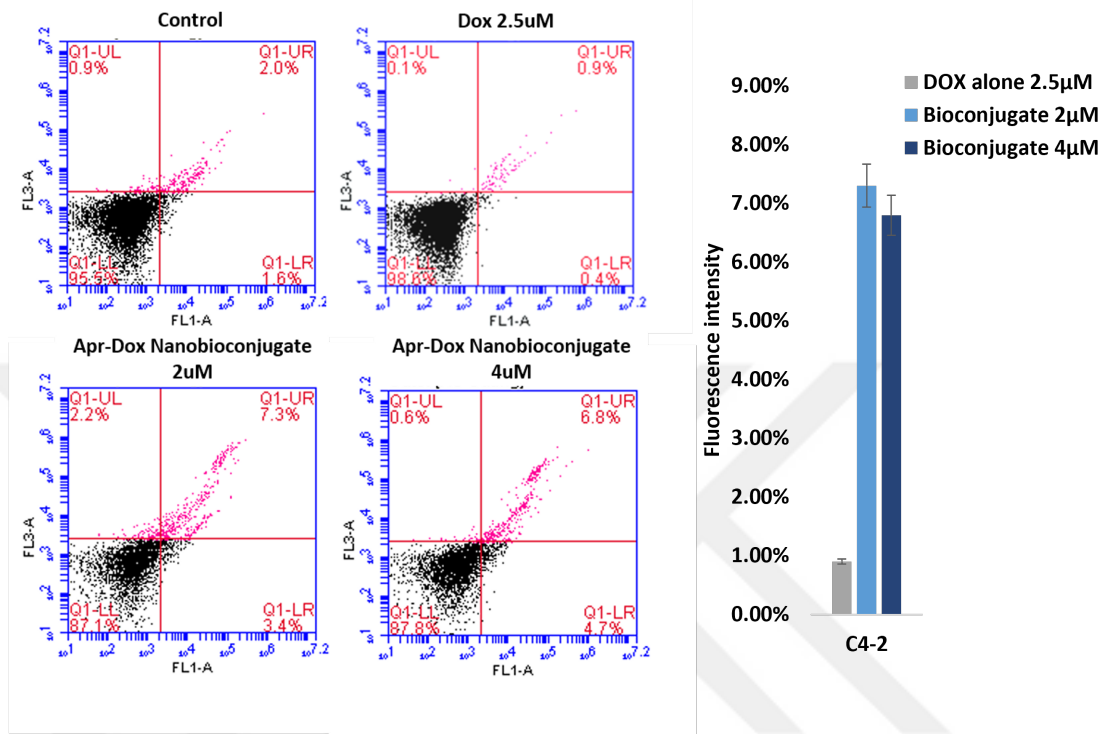


Figure 4.13: Cellular uptake analysis of C4-2 prostate cancer

observations indicated that aprotinin as a carrier protein can positively influence the internalization of Dox molecules in cancer cells by increased cellular uptake of Dox. However, restricted release of Dox in the normal cell lines further highlights the safety of this nano-bioconjugate to be used as a carrier protein in anti-cancer therapies. Thus, the finding of aprotinin as a new carrier moonlighting protein may have a significant impact on the development of more effective anti-cancer therapies

4.8 *In Vitro* Compatibility of Apr-Dox Nano-bioconjugate with Blood Components

The biocompatibility of drug-protein bio-nanoconjugate in systemic circulation is a crucial indicator for the potential use of drug carrier protein in living organisms. The determination of hemolytic properties conventionally analyze the

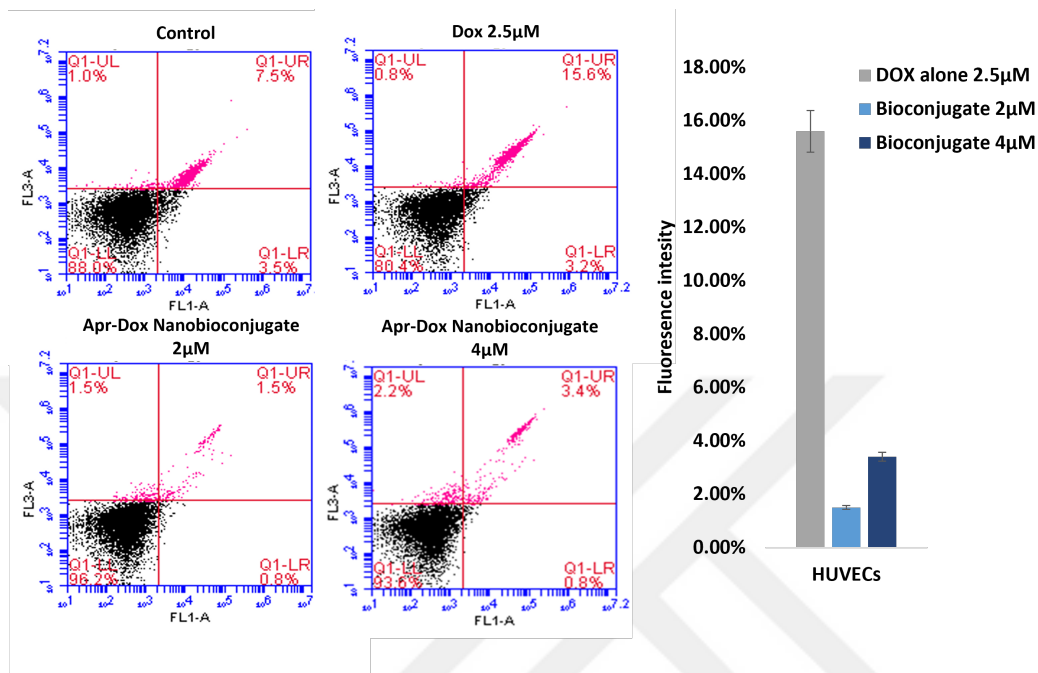


Figure 4.14: Cellular uptake analysis of Human umbilical vein endothelial cells (HUVECs)

nanoparticles' interactions with blood components, for instance, the interaction of erythrocytes with nanoparticles for biological applications [42]. In this study, hemoglobin release analysis was employed to evaluate the hemolytic potential of Apr-Dox nano-bioconjugate when entering into blood stream. In the experimental conditions, the red blood cells (RBCs) were exposed to different concentrations of nano-bioconjugate sample for 3h. Fig: 4.15 presents the images of the centrifuged RBC solutions and hemoglobin release analysis. It could be seen that addition of Apr-Dox nano- bioconjugate from 2µM to 8µM showed very low hemolytic toxicity (less than 5%), which is comparable to the negative control (PBS) showing 0% hemolysis. According to the literature, biomaterials are expected to have up to 5% hemolysis ratio that proves to be non-toxic for RBCs [57]. Therefore, the Apr-Dox nano-bioconjugate at the tested concentration did not exhibit significant hemolysis, indicating the reliability of the conjugates to be considered for further *in vivo* studies.

In principle, hemolysis occurs when water enters into RBCs resulting in a hypotonic environment, favorable for the lysis of cells. Thus, the resultant solution

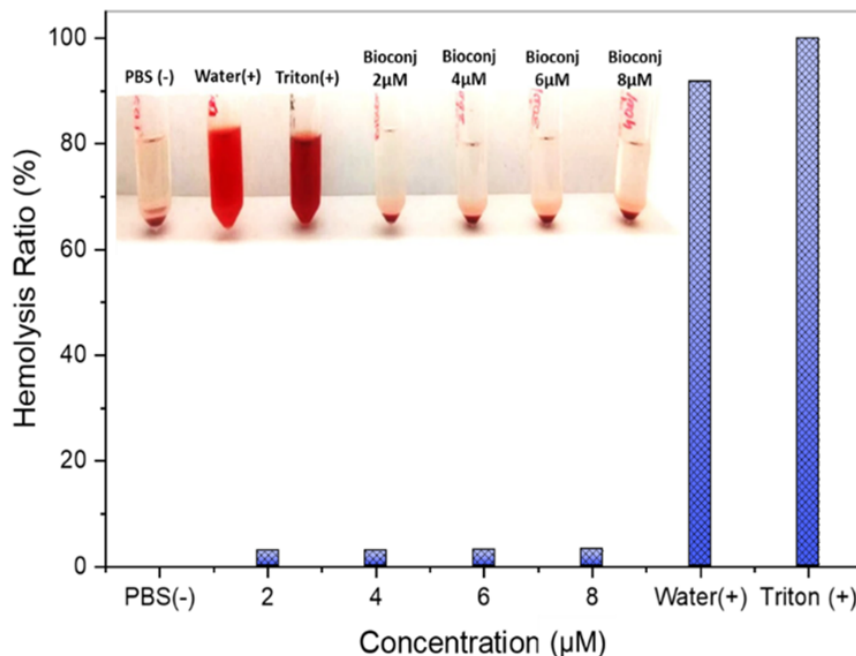


Figure 4.15: Hemolysis test of Apr-Dox nano-bioconjugate. (A) Images of centrifuged RBC solutions after incubation with different concentrations of nano-bioconjugate. (B) Percentage hemolysis of Apr-Dox nano-bioconjugate at different concentrations. PBS was used as negative control whereas Triton X-100 and deionized water were used as positive controls.

becomes turbid due to released hemoglobin, giving high absorption values when measured with colorimetric assay. To observe this phenomenon, two additional positive controls i.e., sodium dodecyl sulphate (SDS) and triton X-100 were used to treat RBCs suspension. Both the solutions had high absorption values, giving the calculated hemolytic percentage as $\sim 100\%$. However, the supernatant from the nano-bioconjugates at various concentrations did not show significant hemolytic activity as evidenced by their low absorbance values (Fig: 4.15; Table A.1). Thus, it can be concluded that the Apr-Dox nano-bioconjugate is safe for clinical application, with a higher biocompatibility with blood components.

4.9 Cytocompatibility of Apr-Dox Nano-bioconjugate in Non-cancerous Cells

The non-toxic behavior of Apr-Dox nano-bioconjugate in healthy cell lines was determined by incubating the nano-bioconjugate in non-cancerous, healthy umbilical vein endothelial cells (HUVECs). Briefly, the healthy cells were exposed to different concentrations of Apr-Dox nano-bioconjugate i.e., 2 μ M, 3 μ M, 4 μ M and 5 μ M, with equivalent concentration of Dox alone 2.5 μ M as positive control.

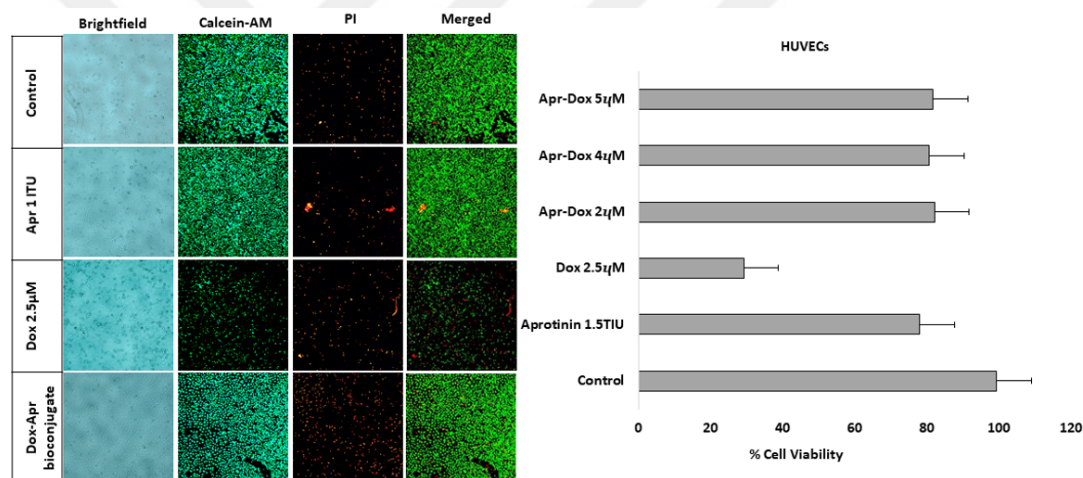


Figure 4.16: In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of HUVECs

Fig: 4.16 shows the representative images of live/dead cells staining of HUVECS after exposure to different concentrations of Apr-Dox nano-bioconjugate and Dox alone for 48h. The results indicate that the cells exposed to 2.5 μ M Dox showed signs of membrane rupturing and the morphology of cells exhibited round shape with fewer number of cells as compared with the control. Whereas the cell morphology of HUVECs was also elongated in shape with intact confluent monolayer when cultured with aprotinin alone as shown in the brightfield images (Fig: 4.16). Quantitative data showing % cell viability indicate that 78% of cells were alive after 72h of HUVECs incubation with aprotinin (1.5TIU). Remarkably, there was no significant evidence of cytotoxicity in HUVECs cultures

when they were exposed to varying concentration of Apr-Dox nano-bioconjugate (2 μ M, 4 μ M and 5 μ M) as compared to control with respect to cell morphology and cell density. The percentage viabilities remained 80%, 78%, and 79% respectively for each dose. These results suggested that aprotinin alone and even after conjugating with cytotoxic Dox molecules is not significantly cytotoxic to human endothelial cells within the range of tested concentrations (i.e., 2 μ M-5 μ M).

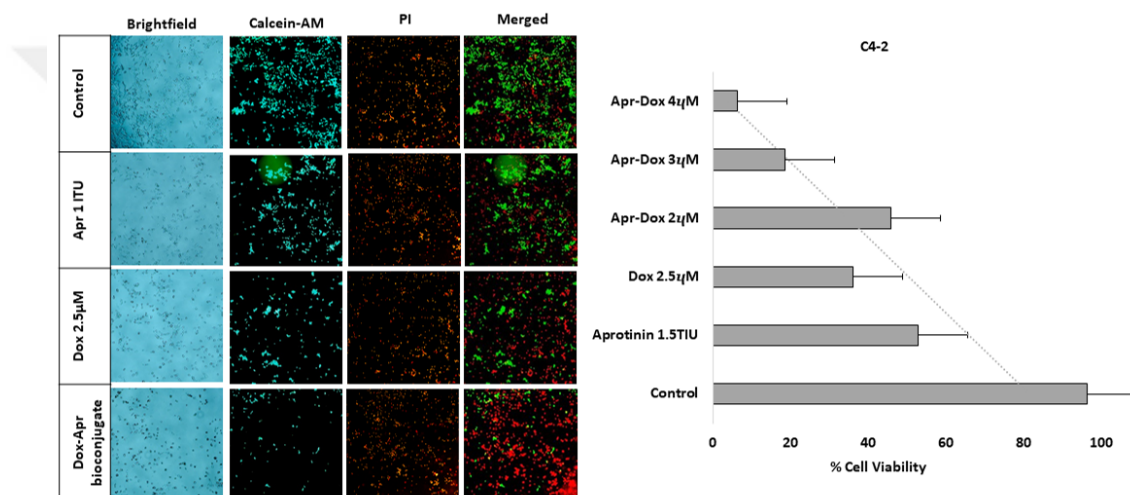


Figure 4.17: In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of C4-2

The major reason involved in the non-cytotoxic behavior of aprotinin towards healthy cells is the absence of urokinase plasminogen activator receptors which are abundant in cancer cells. Aprotinin bind to these receptors in cancer cells and reduce proliferation and metastatic activity [66]. Further studies highlighting the molecular pathway of this tumor specific response is yet to be studied.

4.10 Enhanced Chemo-sensitizing Ability of Moonlight Drug Bioconjugate in Breast, Prostate and Lung cancer

The chemo-sensitive potential of dual drug is based on the exploration that one drug might increase the action of another by modifying one or more resistance mechanisms [67], thus creating a synergistic effect. Therefore, the chemo-sensitizing potential of Apr-Dox nano-bioconjugate was also evaluated against the similar cancer cell lines. For this, the *in vitro* cell viability of Dox alone and Apr-Dox nano-bioconjugate was analyzed using C4-2, H-1299 and MCF-7 cells at 48h and 72h of incubation by qualitative analysis of live/dead cells staining images, obtained with Calcein-AM and propidium iodide (PI) dye, respectively. The untreated cells were used as baseline to calculate the percentage cell viability of each treatment cell.

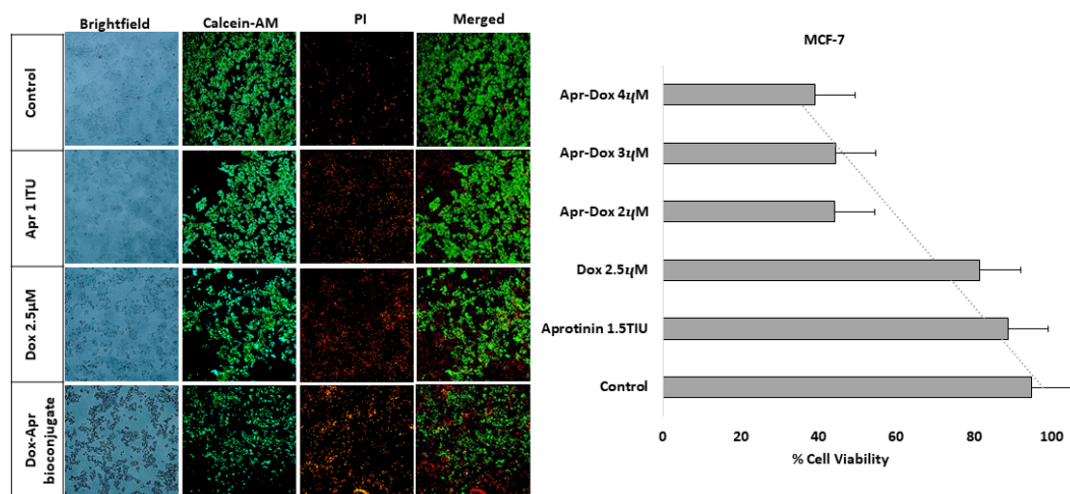


Figure 4.18: In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of MCF-7

The cytotoxicity of free Dox exposed to C4-2 with a comparison of Apr-Dox nano-bioconjugated drug in 2 μM, 3 μM and 4 μM concentrations is shown in Fig. 6B. The monolayers of C4-2 showed normal cell growth in control, where as in bioconjugate treated cells there was membrane rupturing with increased cell death

number as compared to Dox alone treatment. The cell viability assay showed that the percentage of viable cells with Dox alone was 30% whereas in Apr-Dox nano-bioconjugate drug treatment, the effect was pronounced upto 80% with only 19% living cells. Similar trend was observed in MCF-7 in which Dox alone showed cell viability of 82% whereas Apr-Dox nano-bioconjugate showed cell viability in 40% to 45% range which is comparable to the alone drug. The morphology of treated MCF-7 monolayers was significantly changed into circular shape showing dead cells as shown in Fig: 4.18.

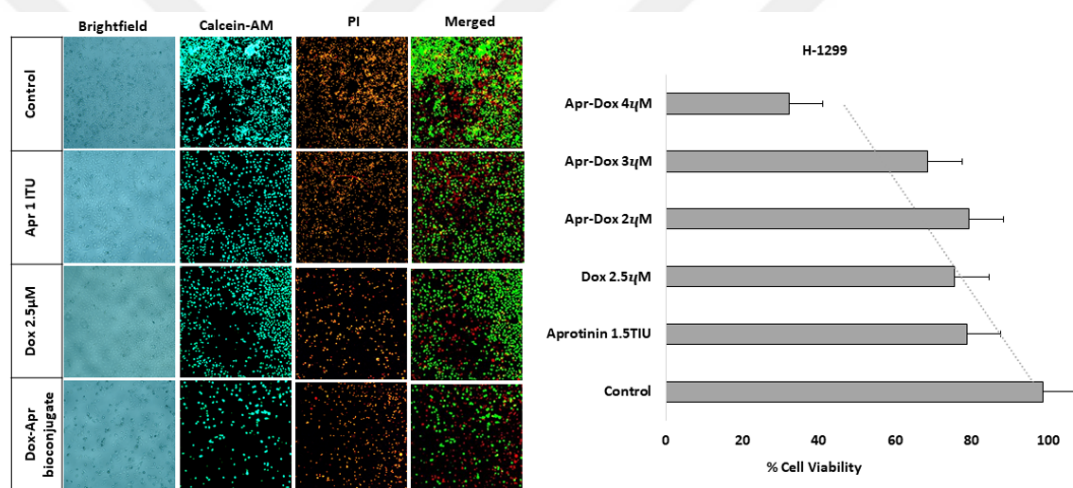


Figure 4.19: In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of H-1299

The results were comparable with the previously reported by [68] showing peptide having pronounced anti-cancer activity when used in combination with conventional chemotherapeutics. In the small cell lung carcinoma cell line, H-1299, the efficacy of Apr-Dox nano-conjugated drug was also significant with only 30% cell viability whereas with Dox alone treatment cytotoxicity was less and was comparable to aprotinin alone as shown in Fig: 4.19. In conclusion, the cell viability assay of cancer cell lines showed that the developed nano-bioconjugate demonstrated strong anti-cancer activity; highlighting the chemotherapeutic and chemo-sensitive potential of aprotinin (Fig: 4.20).

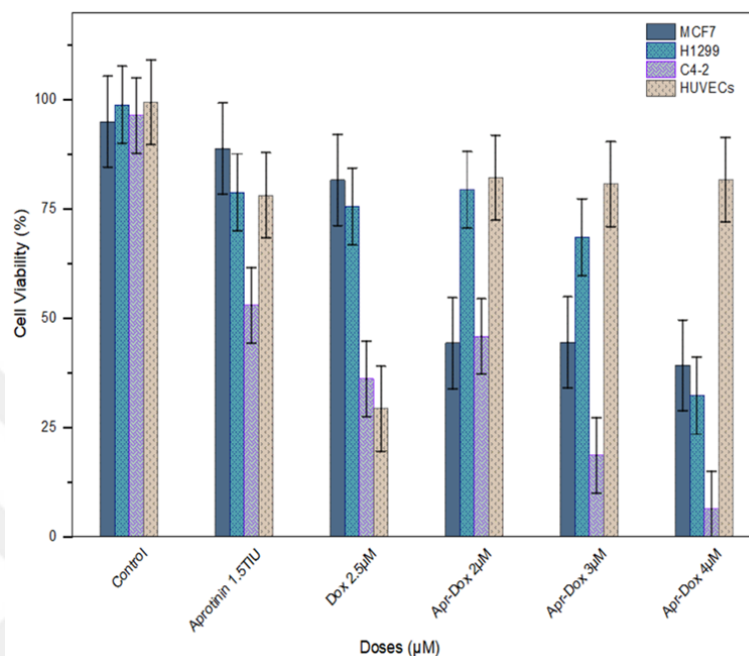


Figure 4.20: In vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate, Dox alone, aprotinin alone and PBS as control of HUVECs, MCF-7, C4-2 and H-1299

This also shows that the prostate cancer cell line C4-2 is highly sensitive to the drug-protein nano-bioconjugate, whereas its toxicity is least in healthy human umbilical cord endothelial cells (HUVECs). Comparatively, breast cancer and non-small cell lung carcinoma showed moderate chemo-sensitivity towards Apr-Dox nano-bioconjugate. However, detailed analysis of molecular and cellular interactions of each cancer cell line can be investigated further to understand the potential mechanism behind difference of efficacy in each cell line. Here, the potential of aprotinin as a carrier moonlighting protein with chemo-sensitive ability to carry conventional anti-cancer drug is highlighted as a proof of concept.

4.11 Effect of Apr-Dox Nano-Bioconjugate on 3D Cancer Spheroids of Prostate Cancer C4-2 and Lung Cancer H-1299

For the analysis of drug treatment on 3D-spheroid models, C4-2 and H1299 cell lines were tested, keeping in mind the maximum and minimum effect of the designed Apr-Dox nano-bioconjugate, respectively, on the 2D-monolayer models of these cell lines. The schematic of spheroids fabrication and their respective analysis is shown in Fig: 4.21.

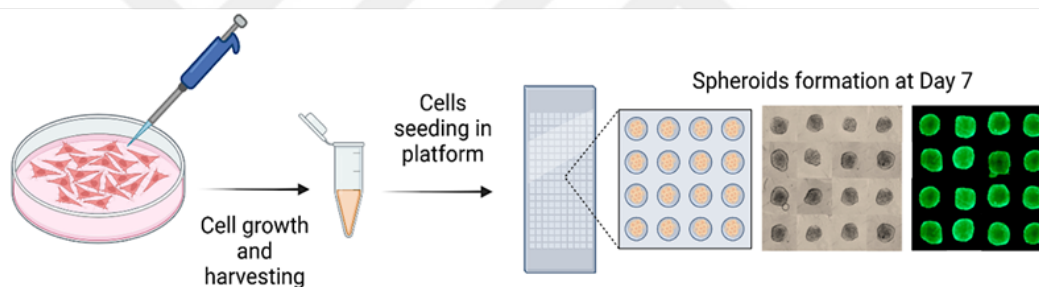


Figure 4.21: Schematics of spheroids fabrication for drug treatment studies

Experimentally, the following settings were used: 1) Spheroids incubated with no drug treatment acted as control for 48h 2) 48h incubation with aprotinin alone (1.5 TIU) 3) 48h incubation with 2.5 μ M Dox alone 4) spheroids incubated with Apr-Dox nano-bioconjugate at varying concentrations (i.e., 2 μ M – 10 μ M). The media was replaced with fresh media after 48h, and spheroids were incubated with no further drug addition up to 72h. For the analysis, individual spheroids were observed (Fig: A6 and A7) for their growth behavior and morphological characteristics throughout the study period.

Remarkably, the growth behavior of C4-2 spheroids showed a significantly effective response with 2 μ M Apr-Dox nano-bioconjugate drug treatment, whereas an equivalent concentration of Dox alone (2.5 μ M) did not show effective results while keeping the morphology of spheroids intact, thus suggesting an increased cytotoxic behavior of the devised nano-bioconjugate in spheroids (Fig: 4.22).

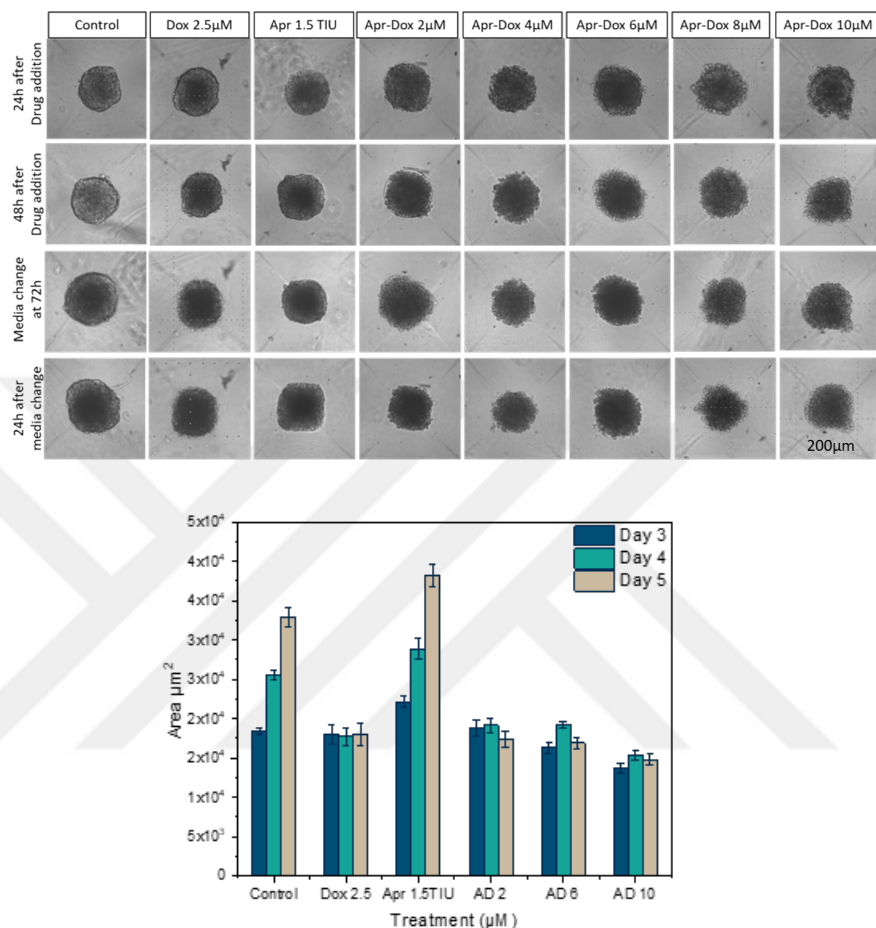


Figure 4.22: 3D in vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate (Brightfield images of individual spheroids after drug treatment at day 5 for C4-2 with graphical description of size change)

Relatively, for H-1299 spheroids, the effective concentration of Apr-Dox nano-bioconjugate to inhibit the regrowth of spheroids after media change was found to be $4\mu M$ (Fig: 4.23). This might be due to the fact that uPA increases the permeability of microvascular endothelial cells *in vitro*[69] and inhibiting uPA with aprotinin would affect the cellular uptake of conjugate in 3D spheroids with biomimetic tumor physiological environment.

Hence, the results of 3D spheroids were quite consistent with the 2D-monolayer data for each cell line, depicting that the efficiency of the Apr-Dox nano-bioconjugate is somehow related to the properties of the type of cancers, and

thus giving distinct results on each type of cancer cell lines.

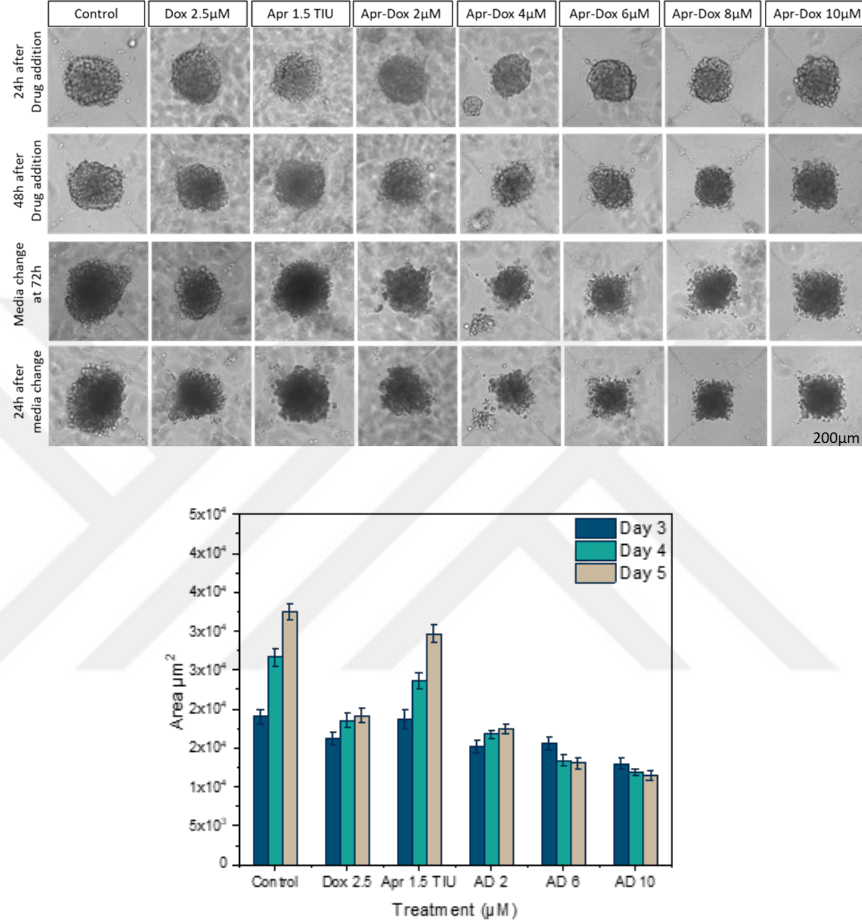


Figure 4.23: 3D in vitro cytotoxic analysis after treatment with Apr-Dox nano-bioconjugate (Brightfield images of individual spheroids after drug treatment at day 5 for H-1299 with graphical description of size change)

Moreover, the live-dead cells staining images emphasized a decreased viability of cells at the peripheral region of spheroids with Apr-Dox nano-bioconjugate treatment in both cell lines, when compared to non-treated (control) and Dox alone treatment as shown in Fig: 4.24 (Left and Right).

Here, the green fluorescent peripheral corona decreases with time after drug-conjugate treatment suggesting more cell death with Apr-Dox nano-bioconjugate, which is corroborated by the disintegration of spheroidal morphology as observed in brightfield images (Fig: 4.24 left and right). A decrease in viability and

spheroidal size is attributed to the increase cellular uptake of Apr-Dox nano-bioconjugate by spheroids via inhibition of uPA in extracellular environment which is a major contributing factor in tumor growth and progression[70].

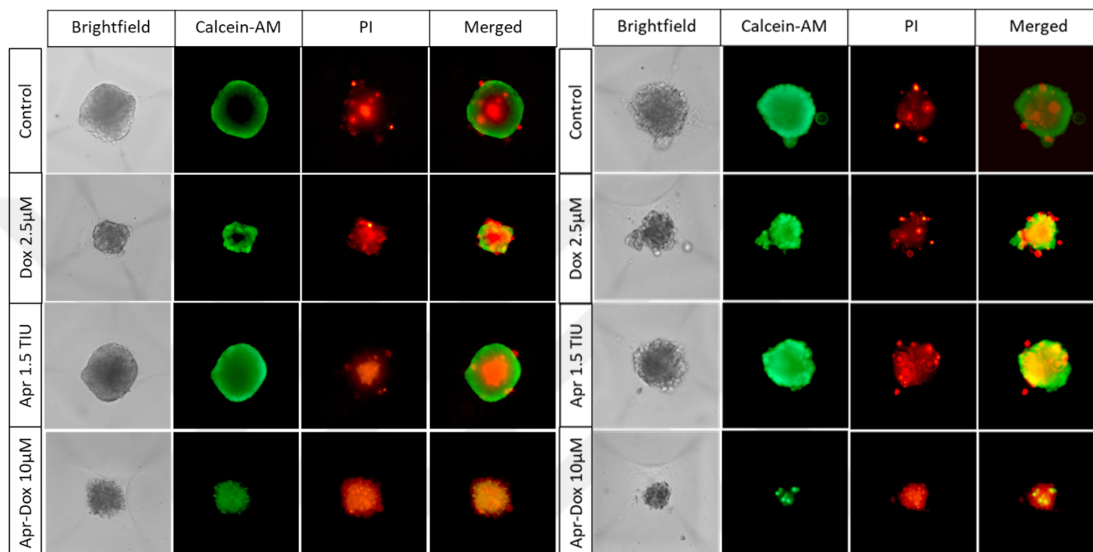


Figure 4.24: Stained images of spheroids after treatment with drug in C4-2 (left) H-1299 (right)

Chapter 5

Conclusion

This study complies designing a novel nano-bioconjugate-based combination therapies involving the use of anticancer therapeutic agent Dox, delivered using nano-carrier aprotinin (Apr-Dox nano-bioconjugate) to enhance the efficacy and reduce the toxicity of the drug, and hence introducing aprotinin as a moonlighting protein to carry a renowned cytotoxic drug Dox as a meaningful alternative with cancer treatment. Interestingly, the designed nano-bioconjugate offers tremendously reduced drug dose (~ 100 times), overcoming the off-targeted behavior (killing the normal cells along with cancer cells) of conventional anti-cancer drugs with no signs of toxicity on healthy cells (HUVECs) in 2D cultures, as well as proposing a targeted pH release profile (at pH 4.0) to be advantageous to release drug specifically at the tumor site in the acidic environment. Moreover, as a proof-of-concept; to observe the effectiveness of the Apr-Dox nano-bioconjugate on 3D spheroid models, the designed nano-bioconjugate was also tested on 3D spheroids, thus showing effective response in the preliminary 3D setups, and opening a way to be an effective candidate nano-drug for clinical applications in the future. However, a detailed study with respect to the effect of this nano-bioconjugate on 3D spheroid models is still in progress in our research group, to first understand the in-depth physiology of the spheroids generated from various cancer cell lines, followed by time-dependent addition of Apr-Dox nano-bioconjugate for a much reflective effect. Consequently, the designed nano-bioconjugate shows a selective

targeting approach of aprotinin towards cancer cells, thus improving the effectiveness and minimizing systemic toxicity observed in usual cancer treatments.



Bibliography

- [1] A. Riedl, M. Schlederer, K. Pudelko, *et al.*, “Comparison of cancer cells cultured in 2D vs 3D reveals differences in AKT/mTOR/S6-kinase signaling and drug response,” en, *Journal of Cell Science*, jcs.188102, Jan. 2016, ISSN: 1477-9137, 0021-9533. DOI: 10.1242/jcs.188102. [Online]. Available: <https://journals.biologists.com/jcs/article/doi/10.1242/jcs.188102/259005/Comparison-of-cancer-cells-cultured-in-2D-vs-3D> (visited on 11/23/2023).
- [2] K. M. Yamada and E. Cukierman, “Modeling Tissue Morphogenesis and Cancer in 3D,” en, *Cell*, vol. 130, no. 4, pp. 601–610, Aug. 2007, ISSN: 00928674. DOI: 10.1016/j.cell.2007.08.006. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0092867407010288> (visited on 03/05/2024).
- [3] J. C. Fontoura, C. Viezzer, F. G. Dos Santos, *et al.*, “Comparison of 2D and 3D cell culture models for cell growth, gene expression and drug resistance,” en, *Materials Science and Engineering: C*, vol. 107, p. 110 264, Feb. 2020, ISSN: 09284931. DOI: 10.1016/j.msec.2019.110264. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0928493119305934> (visited on 03/05/2024).
- [4] F. Sambale, A. Lavrentieva, F. Stahl, *et al.*, “Three dimensional spheroid cell culture for nanoparticle safety testing,” en, *Journal of Biotechnology*, vol. 205, pp. 120–129, Jul. 2015, ISSN: 01681656. DOI: 10.1016/j.jbiotec.2015.01.001. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0168165615000103> (visited on 03/06/2024).

- [5] S. Qian, J. Mao, Z. Liu, *et al.*, “Stem cells for organoids (Smart Medicine 1/2022),” en, *Smart Medicine*, vol. 1, no. 1, e44, Dec. 2022, ISSN: 2751-1871, 2751-1871. DOI: 10.1002/SMMD.44. [Online]. Available: <https://onlinelibrary.wiley.com/doi/10.1002/SMMD.44> (visited on 03/05/2024).
- [6] V. Velasco, S. A. Shariati, and R. Esfandyarpour, “Microtechnology-based methods for organoid models,” en, *Microsystems & Nanoengineering*, vol. 6, no. 1, p. 76, Oct. 2020, ISSN: 2055-7434. DOI: 10.1038/s41378-020-00185-3. [Online]. Available: <https://www.nature.com/articles/s41378-020-00185-3> (visited on 03/05/2024).
- [7] E. Fennema, N. Rivron, J. Rouwkema, C. Van Blitterswijk, and J. De Boer, “Spheroid culture as a tool for creating 3D complex tissues,” en, *Trends in Biotechnology*, vol. 31, no. 2, pp. 108–115, Feb. 2013, ISSN: 01677799. DOI: 10.1016/j.tibtech.2012.12.003. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0167779912002223> (visited on 03/05/2024).
- [8] E. Falvo, E. Tremante, A. Arcovito, *et al.*, “Improved Doxorubicin Encapsulation and Pharmacokinetics of Ferritin–Fusion Protein Nanocarriers Bearing Proline, Serine, and Alanine Elements,” en, *Biomacromolecules*, vol. 17, no. 2, pp. 514–522, Feb. 2016, ISSN: 1525-7797, 1526-4602. DOI: 10.1021/acs.biomac.5b01446. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acs.biomac.5b01446> (visited on 03/05/2024).
- [9] K. Fan, C. Cao, Y. Pan, *et al.*, “Magnetoferritin nanoparticles for targeting and visualizing tumour tissues,” en, *Nature Nanotechnology*, vol. 7, no. 7, pp. 459–464, Jul. 2012, ISSN: 1748-3387, 1748-3395. DOI: 10.1038/nnano.2012.90. [Online]. Available: <https://www.nature.com/articles/nnano.2012.90> (visited on 03/05/2024).
- [10] M. Bellini, S. Mazzucchelli, E. Galbiati, *et al.*, “Protein nanocages for self-triggered nuclear delivery of DNA-targeted chemotherapeutics in Cancer Cells,” en, *Journal of Controlled Release*, vol. 196, pp. 184–196, Dec.

- 2014, ISSN: 01683659. DOI: 10.1016/j.jconrel.2014.10.002. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0168365914006762> (visited on 03/05/2024).
- [11] E. Falvo, E. Tremante, R. Fraioli, *et al.*, “Antibody–drug conjugates: Targeting melanoma with cisplatin encapsulated in protein-cage nanoparticles based on human ferritin,” en, *Nanoscale*, vol. 5, no. 24, p. 12278, 2013, ISSN: 2040-3364, 2040-3372. DOI: 10.1039/c3nr04268e. [Online]. Available: <http://xlink.rsc.org/?DOI=c3nr04268e> (visited on 03/05/2024).
- [12] I. Inoue, M. Chiba, K. Ito, *et al.*, “One-step construction of ferritin encapsulation drugs for cancer chemotherapy,” en, *Nanoscale*, vol. 13, no. 3, pp. 1875–1883, 2021, ISSN: 2040-3364, 2040-3372. DOI: 10.1039/D0NR04019C. [Online]. Available: <http://xlink.rsc.org/?DOI=D0NR04019C> (visited on 03/05/2024).
- [13] S. Senapati, A. K. Mahanta, S. Kumar, and P. Maiti, “Controlled drug delivery vehicles for cancer treatment and their performance,” en, *Signal Transduction and Targeted Therapy*, vol. 3, no. 1, p. 7, Mar. 2018, ISSN: 2059-3635. DOI: 10.1038/s41392-017-0004-3. [Online]. Available: <https://www.nature.com/articles/s41392-017-0004-3> (visited on 02/08/2023).
- [14] G. M. Ngandeu Neubi, Y. Opoku-Damoah, X. Gu, Y. Han, J. Zhou, and Y. Ding, “Bio-inspired drug delivery systems: An emerging platform for targeted cancer therapy,” en, *Biomaterials Science*, vol. 6, no. 5, pp. 958–973, 2018, ISSN: 2047-4830, 2047-4849. DOI: 10.1039/C8BM00175H. [Online]. Available: <http://xlink.rsc.org/?DOI=C8BM00175H> (visited on 02/08/2023).
- [15] A. Bolhassani, “Potential efficacy of cell-penetrating peptides for nucleic acid and drug delivery in cancer,” en, *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1816, no. 2, pp. 232–246, Dec. 2011, ISSN: 0304419X. DOI: 10.1016/j.bbcan.2011.07.006. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0304419X11000448> (visited on 02/08/2023).

- [16] X. Li, J. Mu, F. Liu, *et al.*, “Human Transport Protein Carrier for Controlled Photoactivation of Antitumor Prodrug and Real-Time Intracellular Tumor Imaging,” en, *Bioconjugate Chemistry*, vol. 26, no. 5, pp. 955–961, May 2015, ISSN: 1043-1802, 1520-4812. DOI: 10.1021/acs.bioconjchem.5b00170. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.5b00170> (visited on 04/14/2023).
- [17] F. Kratz and B. Elsadek, “Clinical impact of serum proteins on drug delivery,” en, *Journal of Controlled Release*, vol. 161, no. 2, pp. 429–445, Jul. 2012, ISSN: 01683659. DOI: 10.1016/j.jconrel.2011.11.028. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0168365911010820> (visited on 02/08/2023).
- [18] I. Munir, S. Ajmal, M. R. Shah, A. Ahmad, A. Hameed, and S. A. Ali, “Protein–drug nanoconjugates: Finding the alternative proteins as drug carrier,” en, *International Journal of Biological Macromolecules*, vol. 101, pp. 131–145, Aug. 2017, ISSN: 01418130. DOI: 10.1016/j.ijbiomac.2017.03.095. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0141813016324898> (visited on 03/06/2024).
- [19] E. N. Hoogenboezem and C. L. Duvall, “Harnessing albumin as a carrier for cancer therapies,” en, *Advanced Drug Delivery Reviews*, vol. 130, pp. 73–89, May 2018, ISSN: 0169409X. DOI: 10.1016/j.addr.2018.07.011. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0169409X18301777> (visited on 02/08/2023).
- [20] C. J. Jeffery, “Moonlighting proteins: Old proteins learning new tricks,” en, *Trends in Genetics*, vol. 19, no. 8, pp. 415–417, Aug. 2003, ISSN: 01689525. DOI: 10.1016/S0168-9525(03)00167-7. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0168952503001677> (visited on 02/08/2023).
- [21] P. G. Jobin, G. S. Butler, and C. M. Overall, “New intracellular activities of matrix metalloproteinases shine in the moonlight,” en, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1864, no. 11, pp. 2043–2055, Nov. 2017, ISSN: 01674889. DOI: 10.1016/j.bbamcr.2017.05.013.

- [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0167488917301301> (visited on 03/24/2023).
- [22] C. J. Morrison, G. S. Butler, D. Rodríguez, and C. M. Overall, “Matrix metalloproteinase proteomics: Substrates, targets, and therapy,” en, *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 645–653, Oct. 2009, ISSN: 09550674. DOI: 10.1016/j.ceb.2009.06.006. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S095506740900132X> (visited on 03/24/2023).
- [23] D. H. Huberts and I. J. van der Klei, “Moonlighting proteins: An intriguing mode of multitasking,” en, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1803, no. 4, pp. 520–525, Apr. 2010, ISSN: 01674889. DOI: 10.1016/j.bbamcr.2010.01.022. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0167488910000352> (visited on 03/25/2023).
- [24] K.-W. Min, S.-H. Lee, and S. J. Baek, “Moonlighting proteins in cancer,” en, *Cancer Letters*, vol. 370, no. 1, pp. 108–116, Jan. 2016, ISSN: 03043835. DOI: 10.1016/j.canlet.2015.09.022. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0304383515006199> (visited on 02/08/2023).
- [25] A. Cutone, L. Rosa, G. Ianaro, *et al.*, “Lactoferrin’s Anti-Cancer Properties: Safety, Selectivity, and Wide Range of Action,” en, *Biomolecules*, vol. 10, no. 3, p. 456, Mar. 2020, ISSN: 2218-273X. DOI: 10.3390/biom10030456. [Online]. Available: <https://www.mdpi.com/2218-273X/10/3/456> (visited on 02/08/2023).
- [26] A. Adamo, C. Frusteri, M. T. Pallotta, T. Pirali, S. Sartoris, and S. Ugel, “Moonlighting Proteins Are Important Players in Cancer Immunology,” *Frontiers in Immunology*, vol. 11, p. 613 069, Jan. 2021, ISSN: 1664-3224. DOI: 10.3389/fimmu.2020.613069. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.613069/full> (visited on 02/08/2023).

- [27] A. O. Elzoghby, M. A. Abdelmoneem, I. A. Hassanin, *et al.*, “Lactoferrin, a multi-functional glycoprotein: Active therapeutic, drug nanocarrier & targeting ligand,” en, *Biomaterials*, vol. 263, p. 120355, Dec. 2020, ISSN: 01429612. DOI: 10.1016/j.biomaterials.2020.120355. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0142961220306013> (visited on 02/08/2023).
- [28] A. G. Bharadwaj, R. W. Holloway, V. A. Miller, and D. M. Waisman, “Plasmin and Plasminogen System in the Tumor Microenvironment: Implications for Cancer Diagnosis, Prognosis, and Therapy,” en, *Cancers*, vol. 13, no. 8, p. 1838, Apr. 2021, ISSN: 2072-6694. DOI: 10.3390/cancers13081838. [Online]. Available: <https://www.mdpi.com/2072-6694/13/8/1838> (visited on 02/08/2023).
- [29] N. Mahmood, C. Mihalciou, and S. A. Rabbani, “Multifaceted Role of the Urokinase-Type Plasminogen Activator (uPA) and Its Receptor (uPAR): Diagnostic, Prognostic, and Therapeutic Applications,” *Frontiers in Oncology*, vol. 8, p. 24, Feb. 2018, ISSN: 2234-943X. DOI: 10.3389/fonc.2018.00024. [Online]. Available: <http://journal.frontiersin.org/article/10.3389/fonc.2018.00024/full> (visited on 02/08/2023).
- [30] H. Noh, S. Hong, and S. Huang, “Role of Urokinase Receptor in Tumor Progression and Development,” en, *Theranostics*, vol. 3, no. 7, pp. 487–495, 2013, ISSN: 1838-7640. DOI: 10.7150/thno.4218. [Online]. Available: <http://www.thno.org/v03p0487.htm> (visited on 02/08/2023).
- [31] S. Ullisse, E. Baldini, S. Sorrenti, and M. D’Armiento, “The Urokinase Plasminogen Activator System: A Target for Anti-Cancer Therapy,” en, *Current Cancer Drug Targets*, vol. 9, no. 1, pp. 32–71, Feb. 2009, ISSN: 15680096. DOI: 10.2174/156800909787314002. [Online]. Available: <http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1568-0096&volume=9&issue=1&page=32> (visited on 02/08/2023).
- [32] T. Lisman, J. Adelmeijer, D. Huskens, and J. C. M. Meijers, “Aprotinin Inhibits Thrombin Generation by Inhibition of the Intrinsic Pathway, but is not a Direct Thrombin Inhibitor,” en, *TH Open*, vol. 05, no. 03, e363–e375, Jul. 2021, ISSN: 2512-9465. DOI: 10.1055/s-0041-1735154. [Online].

Available: <http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1735154> (visited on 02/08/2023).

- [33] G. Asimakopoulos, R. Thompson, S. Nourshargh, *et al.*, “An anti-inflammatory property of aprotinin detected at the level of leukocyte extravasation,” en, *The Journal of Thoracic and Cardiovascular Surgery*, vol. 120, no. 2, pp. 361–369, Aug. 2000, ISSN: 00225223. DOI: 10.1067/mtc.2000.106323. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0022522300242384> (visited on 02/08/2023).
- [34] J. R. S. Day, R. C. Landis, and K. M. Taylor, “Aprotinin and the Protease-Activated Receptor 1 Thrombin Receptor: Antithrombosis, Inflammation, and Stroke Reduction,” en, *Seminars in Cardiothoracic and Vascular Anesthesia*, vol. 10, no. 2, pp. 132–142, Jun. 2006, ISSN: 1089-2532, 1940-5596. DOI: 10.1177/1089253206288997. [Online]. Available: <http://journals.sagepub.com/doi/10.1177/1089253206288997> (visited on 02/08/2023).
- [35] S. Soleyman-Jahi, F. Sadeghi, Z. Afshari, *et al.*, “Anti-neoplastic effects of aprotinin on human breast cancer cell lines: In vitro study,” *Advances in Clinical and Experimental Medicine*, vol. 28, no. 2, pp. 151–157, Feb. 2019, ISSN: 1899-5276. DOI: 10.17219/acem/89770. [Online]. Available: <http://www.advances.umed.wroc.pl/en/article/2019/28/2/151/> (visited on 08/07/2024).
- [36] W. Liao, J. Wang, J. Xu, *et al.*, “High-throughput three-dimensional spheroid tumor model using a novel stamp-like tool,” en, *Journal of Tissue Engineering*, vol. 10, p. 2041731419889184, Jan. 2019, ISSN: 2041-7314, 2041-7314. DOI: 10.1177/2041731419889184. [Online]. Available: <http://journals.sagepub.com/doi/10.1177/2041731419889184> (visited on 09/11/2023).
- [37] R. C. Popescu, V. Kopatz, E. Andronescu, D. I. Savu, and W. Doerr, “Nanoparticle-Mediated Drug Delivery of Doxorubicin Induces a Differentiated Clonogenic Inactivation in 3D Tumor Spheroids In Vitro,” en, *International Journal of Molecular Sciences*, vol. 24, no. 3, p. 2198, Jan. 2023, ISSN: 1422-0067. DOI: 10.3390/ijms24032198. [Online]. Available: <https://www.mdpi.com/1422-0067/24/3/2198> (visited on 09/11/2023).

- [38] W. Cui, G. Yesiloz, and C. L. Ren, “Microwave Heating Induced On-Demand Droplet Generation in Microfluidic Systems,” en, *Analytical Chemistry*, vol. 93, no. 3, pp. 1266–1270, Jan. 2021, ISSN: 0003-2700, 1520-6882. DOI: 10.1021/acs.analchem.0c04431. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acs.analchem.0c04431> (visited on 09/11/2023).
- [39] G. Yesiloz, M. S. Boybay, and C. L. Ren, “Label-free high-throughput detection and content sensing of individual droplets in microfluidic systems,” en, *Lab on a Chip*, vol. 15, no. 20, pp. 4008–4019, 2015, ISSN: 1473-0197, 1473-0189. DOI: 10.1039/C5LC00314H. [Online]. Available: <http://xlink.rsc.org/?DOI=C5LC00314H> (visited on 09/11/2023).
- [40] S. J. Wallace, J. Li, R. L. Nation, and B. J. Boyd, “Drug release from nanomedicines: Selection of appropriate encapsulation and release methodology,” en, *Drug Delivery and Translational Research*, vol. 2, no. 4, pp. 284–292, Aug. 2012, ISSN: 2190-393X, 2190-3948. DOI: 10.1007/s13346-012-0064-4. [Online]. Available: <http://link.springer.com/10.1007/s13346-012-0064-4> (visited on 03/27/2023).
- [41] N. Chaurawal, C. Misra, H. Abul Barkat, *et al.*, “Oral sorafenib-loaded microemulsion for breast cancer: Evidences from the in-vitro evaluations and pharmacokinetic studies,” en, *Scientific Reports*, vol. 12, no. 1, p. 13746, Aug. 2022, ISSN: 2045-2322. DOI: 10.1038/s41598-022-17333-6. [Online]. Available: <https://www.nature.com/articles/s41598-022-17333-6> (visited on 03/04/2023).
- [42] K. Shanthi, K. Vimala, D. Gopi, and S. Kannan, “Fabrication of a pH responsive DOX conjugated PEGylated palladium nanoparticle mediated drug delivery system: An in vitro and in vivo evaluation,” en, *RSC Advances*, vol. 5, no. 56, pp. 44998–45014, 2015, ISSN: 2046-2069. DOI: 10.1039/C5RA05803A. [Online]. Available: <http://xlink.rsc.org/?DOI=C5RA05803A> (visited on 03/03/2023).
- [43] A. Velazquez-Campoy and E. Freire, “Isothermal titration calorimetry to determine association constants for high-affinity ligands,” en, *Nature Protocols*, vol. 1, no. 1, pp. 186–191, Jun. 2006, ISSN: 1754-2189, 1750-2799.

- DOI: 10.1038/nprot.2006.28. [Online]. Available: <http://www.nature.com/articles/nprot.2006.28> (visited on 02/17/2023).
- [44] Z.-T. Cao, L.-Q. Gan, W. Jiang, *et al.*, “Protein Binding Affinity of Polymeric Nanoparticles as a Direct Indicator of Their Pharmacokinetics,” en, *ACS Nano*, vol. 14, no. 3, pp. 3563–3575, Mar. 2020, ISSN: 1936-0851, 1936-086X. DOI: 10.1021/acsnano.9b10015. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acsnano.9b10015> (visited on 03/26/2023).
- [45] B. Delavari, F. Mamashli, B. Bigdeli, *et al.*, “A biophysical study on the mechanism of interactions of DOX or PTX with -lactalbumin as a delivery carrier,” en, *Scientific Reports*, vol. 8, no. 1, p. 17345, Nov. 2018, ISSN: 2045-2322. DOI: 10.1038/s41598-018-35559-1. [Online]. Available: <https://www.nature.com/articles/s41598-018-35559-1> (visited on 02/17/2023).
- [46] T. A. Hakala, S. Davies, Z. Toprakcioglu, B. Bernardim, G. J. L. Bernardes, and T. P. J. Knowles, “A Microfluidic Co-Flow Route for Human Serum Albumin-Drug-Nanoparticle Assembly,” en, *Chemistry – A European Journal*, vol. 26, no. 27, pp. 5965–5969, May 2020, ISSN: 0947-6539, 1521-3765. DOI: 10.1002/chem.202001146. [Online]. Available: <https://onlinelibrary.wiley.com/doi/10.1002/chem.202001146> (visited on 03/25/2023).
- [47] A. Bogseth, J. Zhou, and I. Papautsky, “Evaluation of Performance and Tunability of a Co-Flow Inertial Microfluidic Device,” en, *Micromachines*, vol. 11, no. 3, p. 287, Mar. 2020, ISSN: 2072-666X. DOI: 10.3390/mi11030287. [Online]. Available: <https://www.mdpi.com/2072-666X/11/3/287> (visited on 03/27/2023).
- [48] A. Gupta, A. Dhiman, A. Sood, R. Bharadwaj, N. Silverman, and G. Agrawal, “Dextran/eudragit S-100 based redox sensitive nanoparticles for colorectal cancer therapy,” en, *Nanoscale*, vol. 15, no. 7, pp. 3273–3283, 2023, ISSN: 2040-3364, 2040-3372. DOI: 10.1039/D3NR00248A. [Online]. Available: <http://xlink.rsc.org/?DOI=D3NR00248A> (visited on 03/27/2023).

- [49] M. Assali, N. Jaradat, and L. Maqboul, "The Formation of Self-Assembled Nanoparticles Loaded with Doxorubicin and γ -Limonene for Cancer Therapy," en, *ACS Omega*, vol. 7, no. 46, pp. 42 096–42 104, Nov. 2022, ISSN: 2470-1343, 2470-1343. DOI: 10 . 1021 / acsomega . 2c04238. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acsomega.2c04238> (visited on 02/23/2023).
- [50] Y. Li, X. Wang, J. Yan, *et al.*, "Nanoparticle ferritin-bound erastin and rapamycin: A nanodrug combining autophagy and ferroptosis for anticancer therapy," en, *Biomaterials Science*, vol. 7, no. 9, pp. 3779–3787, 2019, ISSN: 2047-4830, 2047-4849. DOI: 10 . 1039 / C9BM00653B. [Online]. Available: <http://xlink.rsc.org/?DOI=C9BM00653B> (visited on 03/06/2024).
- [51] C. Y. Zhang, X. Dong, J. Gao, W. Lin, Z. Liu, and Z. Wang, "Nanoparticle-induced neutrophil apoptosis increases survival in sepsis and alleviates neurological damage in stroke," en, *Science Advances*, vol. 5, no. 11, eaax7964, Nov. 2019, ISSN: 2375-2548. DOI: 10 . 1126 / sciadv . aax7964. [Online]. Available: <https://www.science.org/doi/10.1126/sciadv.aax7964> (visited on 02/23/2023).
- [52] S.-M. Zhou, D.-K. Ma, S.-H. Zhang, *et al.*, "PEGylated Cu₃ BiS₃ hollow nanospheres as a new photothermal agent for 980 nm-laser-driven photothermochemotherapy and a contrast agent for X-ray computed tomography imaging," en, *Nanoscale*, vol. 8, no. 3, pp. 1374–1382, 2016, ISSN: 2040-3364, 2040-3372. DOI: 10 . 1039 / C5NR06041A. [Online]. Available: <http://xlink.rsc.org/?DOI=C5NR06041A> (visited on 03/27/2023).
- [53] J. Chen, J. Liu, Y. Hu, Z. Tian, and Y. Zhu, "Metal-organic framework-coated magnetite nanoparticles for synergistic magnetic hyperthermia and chemotherapy with pH-triggered drug release," en, *Science and Technology of Advanced Materials*, vol. 20, no. 1, pp. 1043–1054, Dec. 2019, ISSN: 1468-6996, 1878-5514. DOI: 10.1080/14686996.2019.1682467. [Online]. Available: <https://www.tandfonline.com/doi/full/10.1080/14686996.2019.1682467> (visited on 02/24/2023).
- [54] S. Li, Z. Peng, and R. M. Leblanc, "Method To Determine Protein Concentration in the Protein–Nanoparticle Conjugates Aqueous Solution Using

- Circular Dichroism Spectroscopy,” en, *Analytical Chemistry*, vol. 87, no. 13, pp. 6455–6459, Jul. 2015, ISSN: 0003-2700, 1520-6882. DOI: 10.1021/acs.analchem.5b01451. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acs.analchem.5b01451> (visited on 02/23/2023).
- [55] H. Zare-Zardini, H. Soltaninejad, A. Ghorani-Azam, *et al.*, “Slow release curcumin-containing soy protein nanoparticles as anticancer agents for osteosarcoma: Synthesis and characterization,” en, *Progress in Biomaterials*, vol. 11, no. 3, pp. 311–320, Sep. 2022, ISSN: 2194-0509, 2194-0517. DOI: 10.1007/s40204-022-00197-4. [Online]. Available: <https://link.springer.com/10.1007/s40204-022-00197-4> (visited on 04/16/2023).
- [56] L. Konermann, J. Pan, and Y.-H. Liu, “Hydrogen exchange mass spectrometry for studying protein structure and dynamics,” en, *Chem. Soc. Rev.*, vol. 40, no. 3, pp. 1224–1234, 2011, ISSN: 0306-0012, 1460-4744. DOI: 10.1039/C0CS00113A. [Online]. Available: <http://xlink.rsc.org/?DOI=C0CS00113A> (visited on 02/26/2023).
- [57] F. Nazir, T. A. Tabish, F. Tariq, S. Iftikhar, R. Wasim, and G. Shahnaz, “Stimuli-sensitive drug delivery systems for site-specific antibiotic release,” en, *Drug Discovery Today*, vol. 27, no. 6, pp. 1698–1705, Jun. 2022, ISSN: 13596446. DOI: 10.1016/j.drudis.2022.02.014. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S1359644622000721> (visited on 02/26/2023).
- [58] F. u. Din, W. Aman, I. Ullah, *et al.*, “Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors,” en, *International Journal of Nanomedicine*, vol. Volume 12, pp. 7291–7309, Oct. 2017, ISSN: 1178-2013. DOI: 10.2147/IJN.S146315. [Online]. Available: <https://www.dovepress.com/effective-use-of-nanocarriers-as-drug-delivery-systems-for-the-treatme-peer-reviewed-article-IJN> (visited on 02/26/2023).
- [59] P. Yingchoncharoen, D. S. Kalinowski, and D. R. Richardson, “Lipid-Based Drug Delivery Systems in Cancer Therapy: What Is Available and What Is Yet to Come,” en, *Pharmacological Reviews*, vol. 68, no. 3, E. L. Barker, Ed., pp. 701–787, Jul. 2016, ISSN: 0031-6997, 1521-0081. DOI: 10.1124/pr.

- 115.012070. [Online]. Available: <http://pharmrev.aspetjournals.org/lookup/doi/10.1124/pr.115.012070> (visited on 11/20/2023).
- [60] E. S. Lee, Z. Gao, and Y. H. Bae, "Recent progress in tumor pH targeting nanotechnology," en, *Journal of Controlled Release*, vol. 132, no. 3, pp. 164–170, Dec. 2008, ISSN: 01683659. DOI: 10.1016/j.jconrel.2008.05.003. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0168365908002514> (visited on 02/26/2023).
- [61] Y. Yang, Z. Wang, Y. Peng, J. Ding, and W. Zhou, "A Smart pH-Sensitive Delivery System for Enhanced Anticancer Efficacy via Paclitaxel Endosomal Escape," *Frontiers in Pharmacology*, vol. 10, p. 10, Jan. 2019, ISSN: 1663-9812. DOI: 10.3389/fphar.2019.00010. [Online]. Available: <https://www.frontiersin.org/article/10.3389/fphar.2019.00010/full> (visited on 02/26/2023).
- [62] R. M. Zucker, E. J. Massaro, K. M. Sanders, L. L. Degn, and W. K. Boyes, "Detection of TiO₂ nanoparticles in cells by flow cytometry," en, *Cytometry Part A*, vol. 77A, no. 7, pp. 677–685, Jun. 2010, ISSN: 15524922, 15524930. DOI: 10.1002/cyto.a.20927. [Online]. Available: <https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20927> (visited on 03/23/2023).
- [63] J. Park, M. K. Ha, N. Yang, and T. H. Yoon, "Flow Cytometry-Based Quantification of Cellular Au Nanoparticles," en, *Analytical Chemistry*, vol. 89, no. 4, pp. 2449–2456, Feb. 2017, ISSN: 0003-2700, 1520-6882. DOI: 10.1021/acs.analchem.6b04418. [Online]. Available: <https://pubs.acs.org/doi/10.1021/acs.analchem.6b04418> (visited on 03/23/2023).
- [64] D. Pan, L. Wang, X. Wang, J. Yan, Y. Xu, and M. Yang, "Optimizing the performance of ⁶⁸Ga labeled FSHR ligand in prostate cancer model by co-administration of aprotinin," en, *International Journal of Radiation Biology*, vol. 98, no. 10, pp. 1571–1580, Oct. 2022, ISSN: 0955-3002, 1362-3095. DOI: 10.1080/09553002.2022.2063431. [Online]. Available: <https://www.tandfonline.com/doi/full/10.1080/09553002.2022.2063431> (visited on 04/30/2023).

- [65] P. S. Briquez, K. M. Lorentz, H. M. Larsson, P. Frey, and J. A. Hubbell, “Human Kunitz-type protease inhibitor engineered for enhanced matrix retention extends longevity of fibrin biomaterials,” en, *Biomaterials*, vol. 135, pp. 1–9, Aug. 2017, ISSN: 01429612. DOI: 10.1016/j.biomaterials.2017.04.048. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S0142961217302855> (visited on 04/30/2023).
- [66] V. M. Baart, R. D. Houvast, L. F. De Geus-Oei, *et al.*, “Molecular imaging of the urokinase plasminogen activator receptor: Opportunities beyond cancer,” en, *EJNMMI Research*, vol. 10, no. 1, p. 87, Dec. 2020, ISSN: 2191-219X. DOI: 10.1186/s13550-020-00673-7. [Online]. Available: <https://ejnmires.springeropen.com/articles/10.1186/s13550-020-00673-7> (visited on 01/06/2024).
- [67] S. C. Gupta, R. Kannappan, S. Reuter, J. H. Kim, and B. B. Aggarwal, “Chemosensitization of tumors by resveratrol: Resveratrol and cancer,” en, *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 150–160, Jan. 2011, ISSN: 00778923. DOI: 10.1111/j.1749-6632.2010.05852.x. [Online]. Available: <https://onlinelibrary.wiley.com/doi/10.1111/j.1749-6632.2010.05852.x> (visited on 03/28/2023).
- [68] P. A. Trinidad-Calderón, L. M. López-Castillo, S. Gallegos-Martínez, G. Trujillo-de Santiago, S. García-Lara, and M. M. Álvarez, “nurP28, a New-to-Nature Zein-Derived Peptide, Enhances the Therapeutic Effect of Docetaxel in Breast Cancer Monolayers and Spheroids,” en, *Molecules*, vol. 27, no. 9, p. 2824, Apr. 2022, ISSN: 1420-3049. DOI: 10.3390/molecules27092824. [Online]. Available: <https://www.mdpi.com/1420-3049/27/9/2824> (visited on 03/28/2023).
- [69] A. M. Makarova, T. V. Lebedeva, T. Nassar, *et al.*, “Urokinase-type Plasminogen Activator (uPA) Induces Pulmonary Microvascular Endothelial Permeability through Low Density Lipoprotein Receptor-related Protein (LRP)-dependent Activation of Endothelial Nitric-oxide Synthase,” en, *Journal of Biological Chemistry*, vol. 286, no. 26, pp. 23 044–23 053,

Jul. 2011, ISSN: 00219258. DOI: 10 . 1074 / jbc . M110 . 210195. [Online]. Available: <https://linkinghub.elsevier.com/retrieve/pii/S002192581948842X> (visited on 09/11/2023).

- [70] M. T. Masucci, M. Minopoli, G. Di Carluccio, M. L. Motti, and M. V. Carriero, “Therapeutic Strategies Targeting Urokinase and Its Receptor in Cancer,” en, *Cancers*, vol. 14, no. 3, p. 498, Jan. 2022, ISSN: 2072-6694. DOI: 10.3390/cancers14030498. [Online]. Available: <https://www.mdpi.com/2072-6694/14/3/498> (visited on 09/11/2023).

Appendix A

Supplementary Data

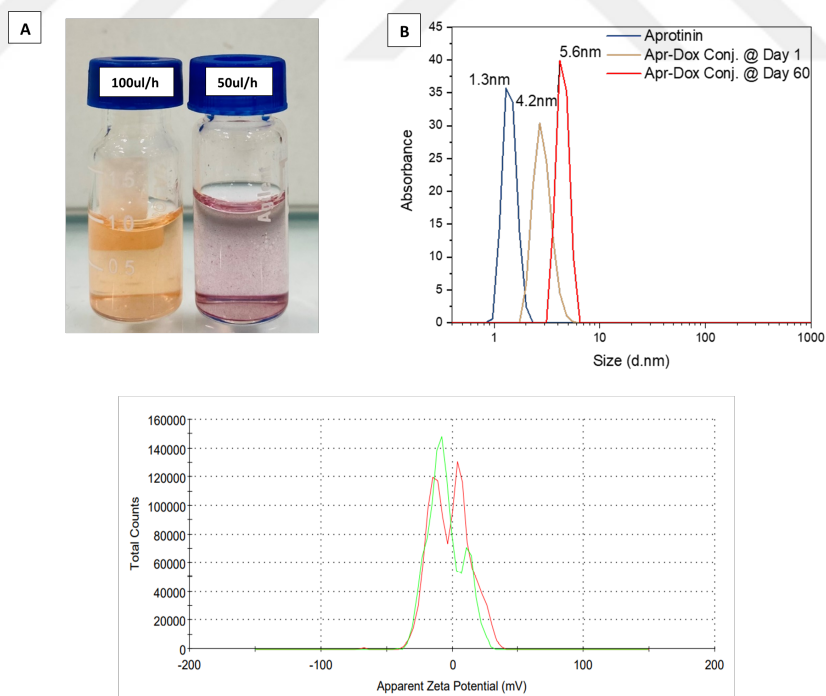


Figure A.1: (A) The stability analysis of drug formulation after 8 weeks of formulation at different flow rates. The figure shows that the formulation was more stable at a 100 µl/h flow rate than 50 µl/h. (B) Stability analysis of the same formulation using DLS and (C) Reproducibility of zeta potential results to ensure the reliability of the obtained data

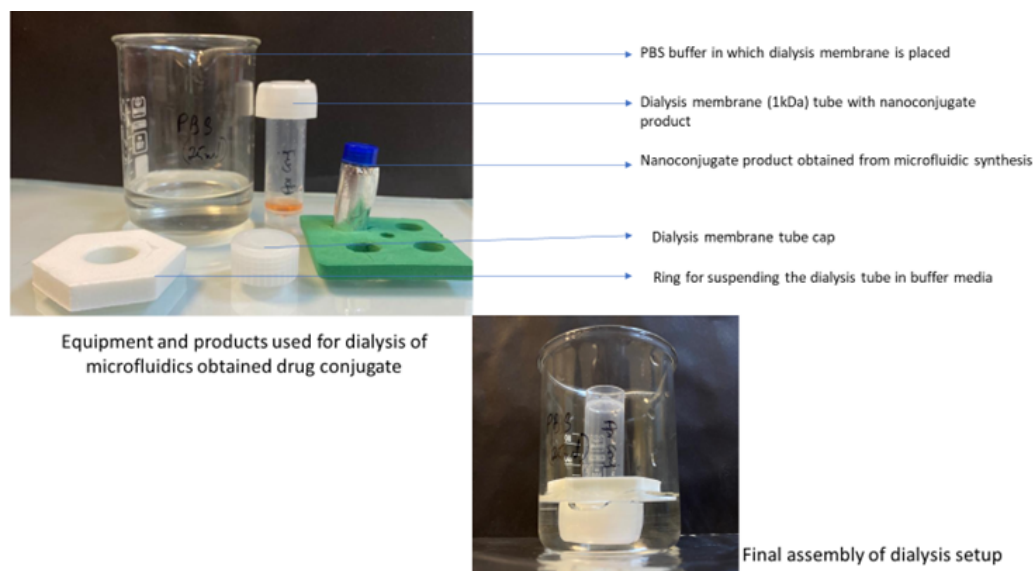


Figure A.2: The removal of unbound drug from synthesized bioconjugate



Figure A.3: The assembly utilized for drug release at different pH

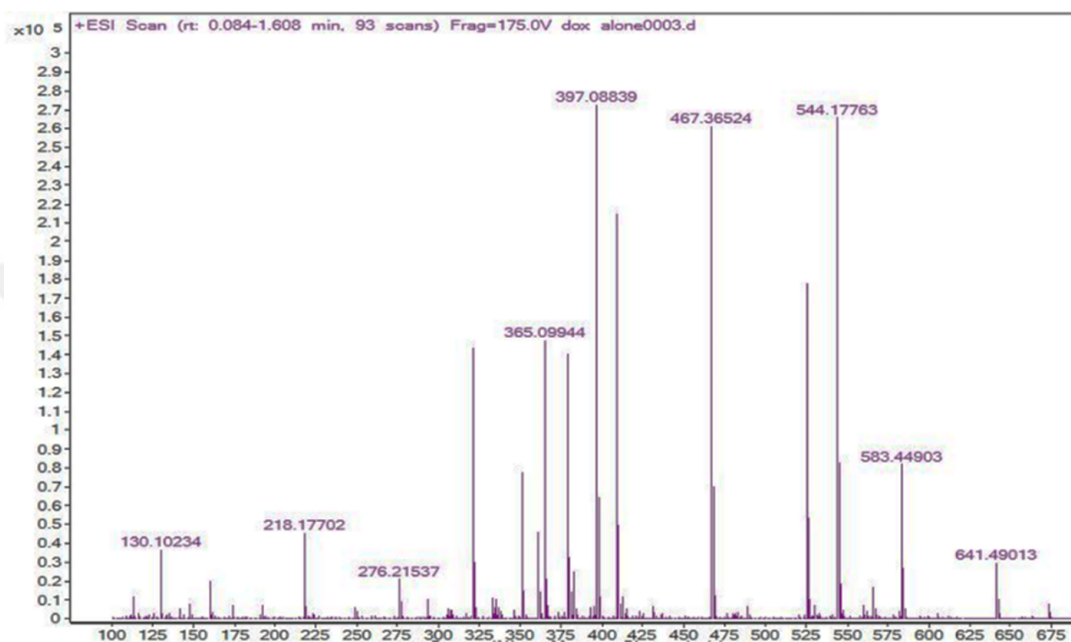


Figure A.4: QTOF data of Dox solution showing peak at 397nm and 544nm

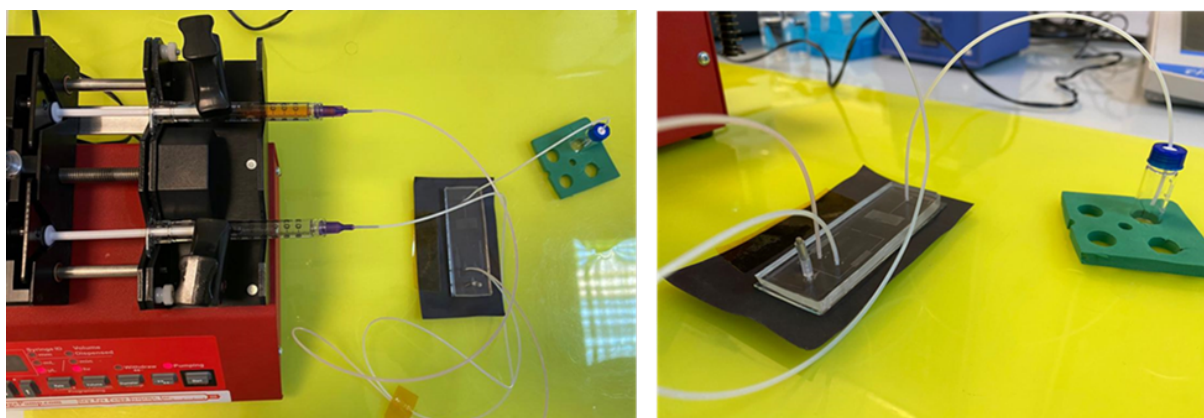


Figure A.5: The microfluidic setup for nano-bioconjugate synthesis

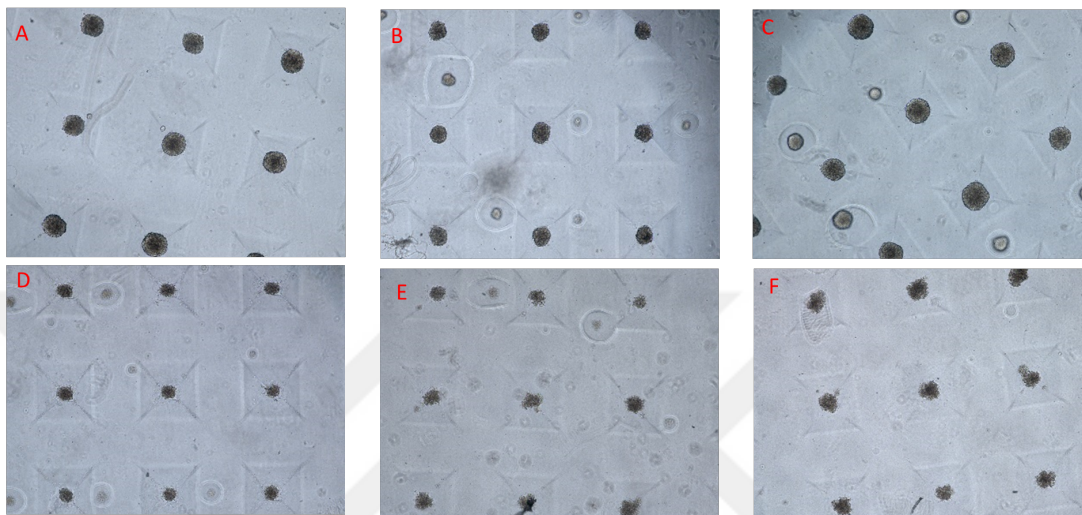


Figure A.6: Brightfield images of C4-2 spheroids (A) control without treatment (B) Dox 2.5 μ M treated (C) Aprotinin 1.5 TIU treated (D) Apr-Dox 2 μ M treated (E) Apr-Dox 6 μ M treated (F) Apr-Dox 10 μ M treated

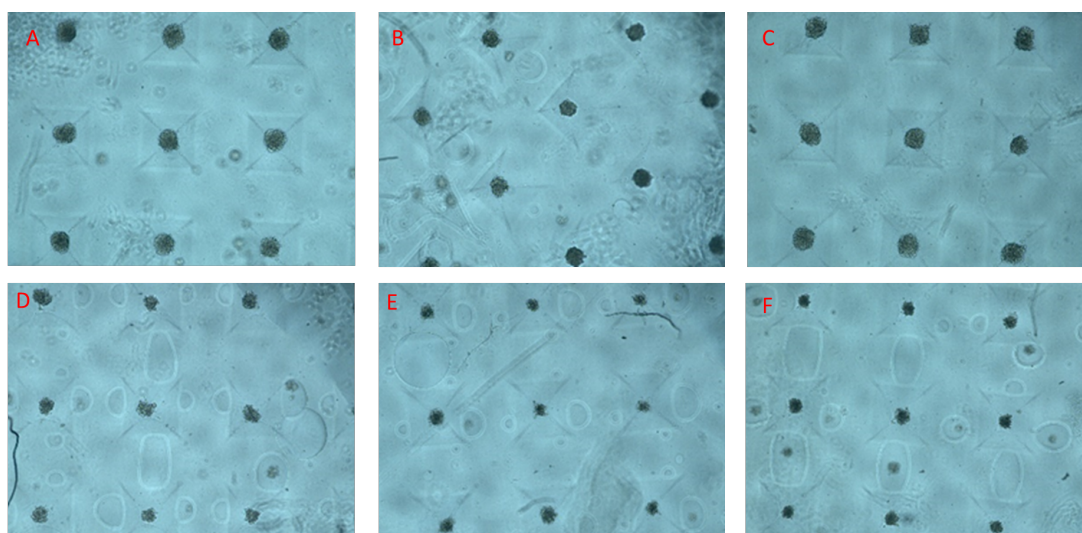


Figure A.7: Brightfield images of H-1299 spheroids (A) control without treatment (B) Dox 2.5 μ M treated (C) Aprotinin 1.5 TIU treated (D) Apr-Dox 2 μ M treated (E) Apr-Dox 6 μ M treated (F) Apr-Dox 10 μ M treated

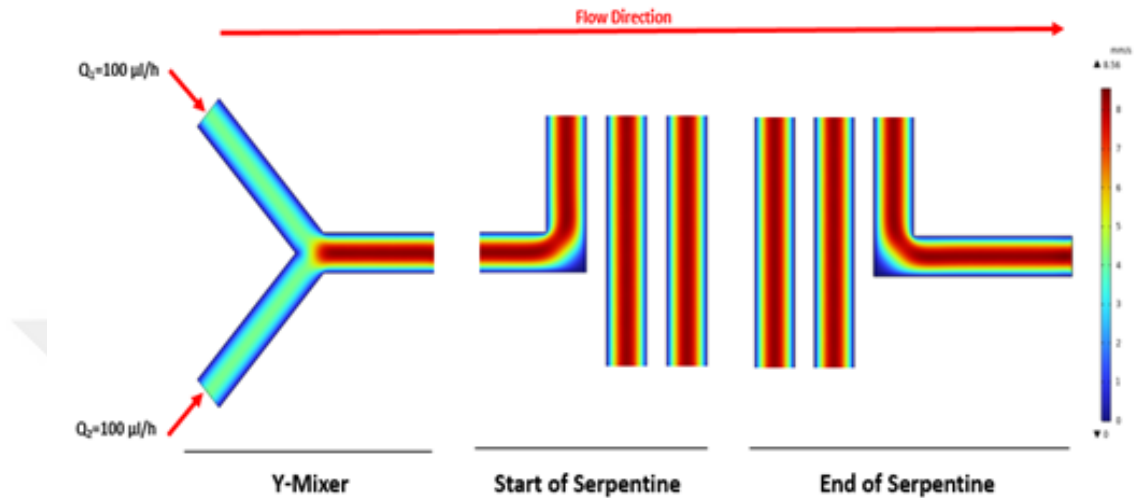


Figure A.8: Simulation to elucidate the velocity profile of Y-mixer for synthesizing nano-bioconjugates using microfluidic chip

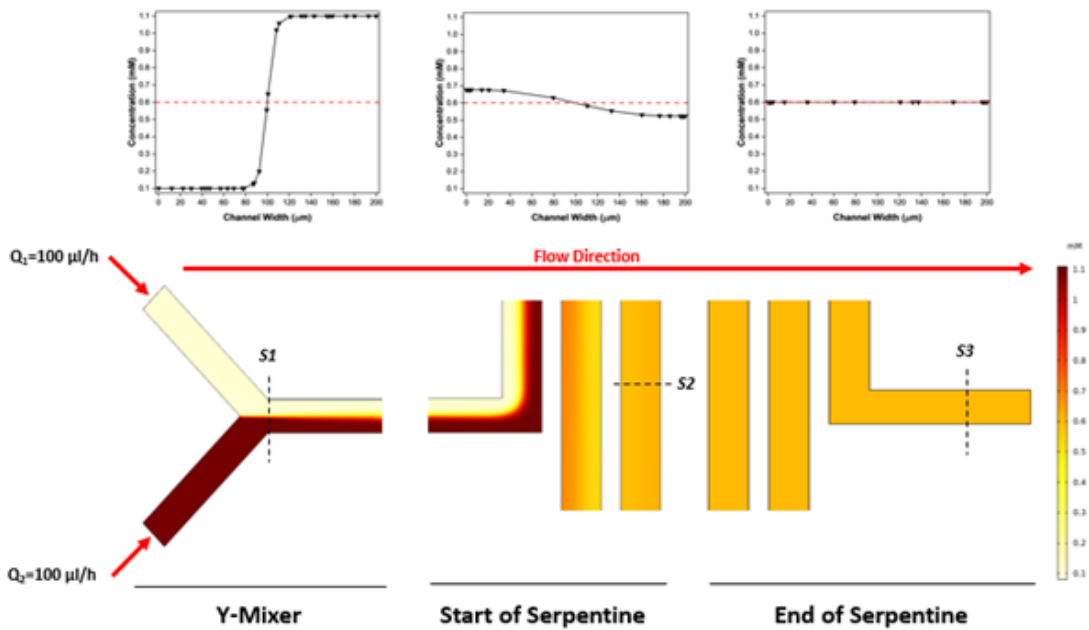


Figure A.9: Numerical simulations showing mixing performance along the Y-mixer at optimal flow rate (100µl/h) for synthesizing nano-bioconjugates using microfluidic chips

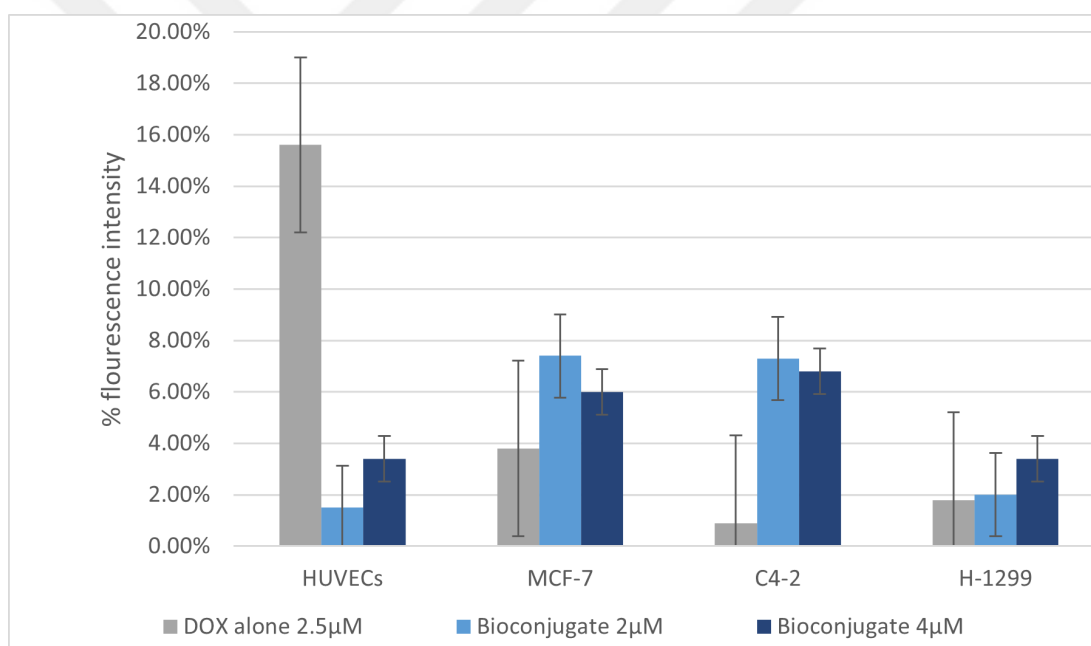


Figure A.10: Combined graph for dox internalization and fluorescence emission determined via flow cytometry

Sample	Absorbance	Hemolysis
PBS (-)	0.324	0
Deionized water (+)	2.912	91.8
Triton X-100 (+)	3.141	100
DOX-APR bioconjugate 10ul	0.411	3.0
DOX-APR bioconjugate 20ul	0.237	3.0
DOX-APR bioconjugate 30ul	0.257	2.3
DOX-APR bioconjugate 40ul	0.233	3.2

Table A.1: Drug conjugates' hemolysis activity and absorbance values compared to the positive and negative control.

Release Model	x-axis	y-axis	Equation
Zero Order	t	% Drug Release	$C_o + K_o t$
First Order	t	Log % Drug Release	$\text{Log } C_t = \log C_0 + K_t t / 2.303$
Higuchi	$\sqrt{t}$	% Drug Release	$C_t = A\sqrt{D}(C_o - C_s)C_s t$

Table A.2: Details of various drug release models. where C_t = amount of drug dissolved in time t , C_0 = the initial amount of drug in the solution, K_0 = zero order release constant, K_t = first order release constant, C_s = drug solubility in the matrix media, D = diffusivity of drug molecules in the matrix, M_t / M = fraction of drug released at time t , K_p = release rate constant, n = release exponent