National University of Science and Technology POLITEHNICA Bucharest

Doctoral School of Chemical Engineering and Biotechnologies

Summary Ph.D. Thesis

Polymeric biomaterials with special applications

PhD candidate

Eng. ION (căs. MIRICĂ) Andreea-Cristina

PhD supervisor

Prof. Dr. Eng. Horia IOVU

Bucharest
2025

Acknowledgements

I would like to express my deepest gratitude to my advisor, Prof. Horia Iovu, for his unwavering guidance, encouragement, and invaluable insights throughout my PhD studies. Also, I would like to thank for granting me the privilege to carry out my research within the Advanced Polymeric Materials Group (APMG).

I extend my gratitude to my thesis committee members Prof. Sorina Gârea and Prof. Paul Stănescu for their constructive feedback and expertise with a special acknowledgment to Prof. Cătălin Zaharia. His unwavering support, patience, inspiration, and valuable advice were instrumental not only during my Master's but also throughout my doctoral studies.

My gratitude to all my colleagues, and members of the Advanced Polymer Materials Group for their collaboration and valuable contributions, which significantly enhanced the depth and breadth of this work. I would like to express special thanks to Ionuț Radu whose mentorship was instrumental in shaping the trajectory of my research and academic development.

I am also thankful to Lecturer Iuliana Biru for the RAMAN measurements, Dr. Minodora Marin for circular dichroism analysis, Dr. Bianca Gălățeanu and Dr. Ariana Hudiță from the Faculty of Biology, University of Bucharest for their kind help in performing the biological assays.

I would like to thank to the members of the research group in which I currently work for the trust and the scientific guidance given, especially Dr. Dana Stan and Dr. Lorena Bocancia-Mateescu because they offered me the chance to continue my research activity. Thus, I had the opportunity to expand my area of knowledge, studying and using the infrastructure available within the DDS Diagnostic, which were useful in obtaining the results of the last chapter of original contributions in this thesis.

In deep appreciation, I extend my sincere gratitude to my cherished family and friends. Your steadfast encouragement and unwavering support have been the cornerstone of my academic journey. I sincerely thank Adrian, my husband for his support, understanding and love offered during this period and for being the constant pillar in my life.

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Keywords: controlled release, fibroin, nanoparticles, electrochemical detection, biosensors, antineoplastic drugs

CHAPTER 1. Literature data

1.1 Drug delivery systems

Drug delivery is the process of transporting pharmaceutical compounds to specific sites in the body to achieve targeted therapeutic effects while minimizing side effects and to accomplish targeted distribution and/or controlled release of therapeutic ingredients. Drug delivery systems (DDSs) have been intensively studied throughout many years in order to increase therapeutic effectiveness by considerably improving how drugs interact with the body through pharmacokinetic-pharmacodynamic (PK-PD) relationship. To improve the limitations regarding the drug solubility, bio-disposition, circulation duration, immune response potential and to accomplish selective targeting, DDSs often use vehicles to carry the medicines. The optimal drug delivery vehicles should be safe for biological systems, biodegradable, customizable and capable of targeting specific illnesses [1]. The progress of therapeutic delivery mechanisms has been implemented to mitigate a variety of critical issues conferred by traditional methods. Key concerns such as poor drug solubility, precise control of drug release, and the extending of circulation period must be addressed. Furthermore, efforts are being undertaken to decrease toxicity and diminish immunogenicity [2-4], which is directly related to non-selective or non-specific harm to healthy tissues [5, 6]. Achieving a good drug recovery response is dependent on deploying suitable medication delivery techniques.

1.2 Materials

The fast advancements in nano-scale engineering in the last decades have actually presented a transformative aspect to the treatment of chronic conditions, especially cancer, utilizing nanostructures referred to as nanoparticles. This conceptual framework holds substantial promise for revolutionizing drug delivery techniques.

Just recently, there has been a proposal to optimize healing efficacy with the controlled and multiple drug administration, contrasting with conventional treatments. Both artificial and all-natural polymers find application in medicine distribution [40, 41]. Within the scientific community, nanotechnology is commonly considered the crucial science of the brand-new millennium. Running at nanoscale dimensions (1-100 nm), nanotechnology entails the controlled control of products. This dimensional variety plays host to various fundamental biological elements for different living microorganism processes. Designated vehicles within this size range have actually been created, including nanoparticles, dendrimers, and nanotubes [42]. Nanoparticles, appearing as nanocapsules or nanospheres, serve a crucial duty in the targeting and controlled release of energetic principles such as medicines, hormonal agents, and vitamins.

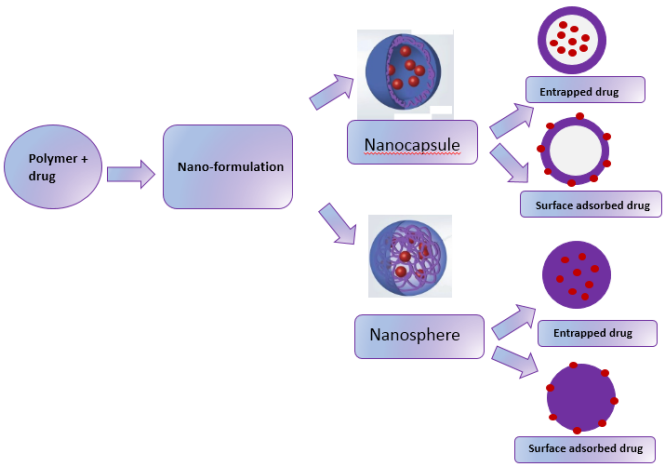


Fig. 1 Types of biodegradable nanoparticles: nanocapsules or nanospheres

Silk stands apart as an impressive natural fiber, identified by its one-of-a-kind top qualities as a polymeric biomaterial. With distinct architectural properties, self-assembling capacities, structural integrity and good mechanical properties, manufacturing adaptability, biological degradation and biocompatibility, silk is a multifaceted product [60].

The silk obtained from *Bombyx mori*, the native silkworm, consists mainly of these two known proteins: fibroin and sericin. Fibroin is the structural protein responsible for the strength and elasticity of silk and is the best-studied silk protein. It consists of two fundamental parts: the light-chain fibroin (about 25 kDa) and the heavy-chain fibroin (about 325 kDa) [64]. The heavy chain contains a core sequence with alanine-glycine repeats. In the structural part of silkworm cocoons, the silk fibroin is surrounded by a sericin coat, a adhesive protein that contributes to the formation of composite fibers within the cocoon. Sericin acts as an adhesive that binds the different layers of the continuous silk fiber together. Silk fibroin's physical and chemical attributes, encompassing exceptional durability, chemical resistance, and antimicrobial properties as well as its constrained flexibility and elongation, are primarily due to its crystalline structure.

Screen-printed electrodes (SPEs) are devices that measure chemical reactions through the electrical properties and they are made by printing conductive inks onto a range of different base materials (plastic, aluminum oxide or ceramic substrates). They enable quick real-time analysis characterized by high parameters used to evaluate the performance of assays. The performance parameters of the electrode depends on the composition of the inks used in its manufacture, with variants such as carbon, silver, gold and platinum influencing these properties [93].

1.3 Cancer Pathogenesis and Therapeutic Approaches

Cancer ranks among the foremost causes of death globally, with an estimated 10 million fatalities attributed to it in 2020 [113]. As reported by the World Health Organization (WHO), the most frequently diagnosed cancers are breast cancer, with 2.26 million diagnoses; lung cancer, with 2.21 million cases; colon and rectal cancer, totaling 1.93 million cases; prostate cancer, at 1.41 million cases; non-melanoma skin cancer, which has 1.20 million cases; and stomach cancer, with 1.09 million cases. [114].

There is no universal treatment strategy that is applicable for all cancer patients. For each and every individual diagnosed with cancer, it is essential to create a personalized treatment customized to make the most of outcomes and minimize adverse effects. This method might include various options such as chemotherapy, immunotherapy, hormonal treatment, radiation therapy, drugs administration, surgery and participation in medical trials, according to the type and progression of cancer.

The fluoropyrimidine 5-fluorouracil it is an antineoplastic drug which shows two different mechanisms of action against cancer cells by hindering necessary biosynthetic processes and conflicting with the synthesis of DNA and RNA, thereby interrupting typical cell division and development procedures [152]. This antimetabolite chemotherapeutic agent discovers extensive use in treating different malignant tumors, consisting of breast, colon, pancreas, stomach, skin and cancers of the head and neck. Regardless of improvements in novel cancer therapies, 5-FU stays a keystone in colorectal (CRC) therapy, standing apart as one of

one of the significant effective and frequently employed drugs used in chemotherapy mix regimens [153].

Drugs which have in their chemical structure platinum, such as cisplatin, are recognized as agents that inhibit cell proliferation and are employed as medication of various cancer types (testicular, ovarian, lung and neck cancer and others). Cisplatin (cis-diammine-dichloro-platinum II) proposed mechanism of action involves the losing one chloride ligand and the creation of complexes with nucleic acids, with approximately 60-65% being intra-strand GG adducts, 25-30% AG adducts, 5% GNG (where N can be any nucleotide), and only 1-3% inter-strand GG adducts [182, 183]. Cisplatin enters cells via diffusion of copper transporters Ctr1 (SLC31A1) and Ctr2 (SLC31A2) [184].

Bleomycin is an antineoplastic antibiotic typically utilized in chemotherapeutic treatment to care for specific types of cancers, such as Hodgkin's lymphoma, testicular cancer, squamous cell carcinomas and some types of ovarian cancers. The action procedure of bleomycin, a glycopeptide antibiotic exhibiting DNA cleavage upon interaction with transition metal ions, is believed to interact with DNA through its terminal peptide moiety. In spite of this, the exact mechanism continues to be incompletely recognized. One of the most considerable and possibly serious negative effects of bleomycin is lung poisoning. Bleomycin can trigger damages to lung cells, causing pulmonary fibrosis [190].

Experimental study

Thesis aims and original contributions

Thesis aims

The aim of this doctoral thesis focuses on the development of new drug delivery systems utilizing biopolymers with medical applications. These systems consist of vehicles such as nanoparticles to transport therapeutics with the aim of enhancing drug solubility, minimizing toxicity, extending circulation time, restricting bio-distribution, attaining specific targeting, and reducing immunogenicity. The newly developed pharmaceutical delivery platforms aim to address the challenges associated with conventional drug delivery in the field of cancer therapy. This thesis delineates the methodologies employed in the development of drug delivery systems, characterization methodologies and the resultant findings. Additionally, the thesis includes strategies detailing the functionalization of electrochemical sensors tailored for detecting drugs employed in cancer therapy.

The thesis is structured in four chapters: the literature study (chapter 1), the development of PEGylated silk fibroin nanoparticles for drug delivery systems (chapter 2), gastrointestinal tract target delivery system based on surface-modified silk fibroin nanoparticles (chapter 3) and the developments of electrochemical biosensors for the detection and quantification of anti-neoplastic drugs (chapter 4).

The literature research study includes knowledge relating to drug delivery systems, their relevance in mitigating the toxic results of conventional treatments, along with the

interpretation of controlled release mechanisms. Drug delivery systems have actually seen significant advancements and diversities, employing numerous nanoparticle formulations to improve the controlled release and targeted delivery of therapeutic agents. Throughout the study of drug delivery systems, a diverse range of nanoparticles, consisting of silk fibroin nanoparticles, has been examined for their potential in controlled drug release. Particularly, the biocompatibility of silk fibroin was highlighted as a vital element demonstrating its potential in the advancemed of nanocarriers for the adequate release of 5-FU for colon cancer treatment. To accomplish a personalized treatment method tailored to the details demands of each patient and to fine-tune the recommended dose of anticancer medicines, drug concentration evaluation is important and essential. Consequently, the literature chapter includes a section on electrochemical sensors for the detection of cancer medication. Generally, this chapter gives useful understandings into the present landscape of cancer therapy and detection of antineoplastic drugs and lays out potential approaches for improvement through ingenious nanomedical techniques.

The second chapter of the thesis focuses on the development of polymeric nanocarriers based on silk fibroin (SF) chemically modified with polyethylene glycol (PEG) as a drug delivery approach. The enhancement of therapeutic efficacy for encapsulated anti-cancer drug 5-fluorouracil (5-FU) was pursued through the modification of particle size, chemistry, drug release and biological properties via PEG functionalization of the SF nanoparticles. The developed PEGylated silk fibroin nanoparticles are investigated by complex biological assessment to reveal the efficiency over colorectal cancer cell lines.

In the third chapter are described the methods for obtaining surface-modified silk fibroin nanoparticles for protection of anticancer drugs in the gastrointestinal tract. More precisely, this investigation led to the creation of a controlled drug delivery mechanism for orally administering anticancer drugs. This system involved surface-modified SF loaded with 5-fluorouracil and enclosed within a chitosan/polyvinyl alcohol outer capsule, serving to shield the drug from degradation while traversing the gastrointestinal tract. The SF platforms aim to be synthetized by several chemical coupling reaction pathways by bridging nanoparticles in agglomerates such as cross-linked architectures. Therefore, the platform has the task of keeping the nanoparticles together to protect and control the release of 5-FU. The examination focuses on the drug release kinetics of an innovative drug delivery system employing surface-modified silk fibroin nanoparticles across distinct pH environments. This investigation aims to closely mimic the *in vivo* release conditions. To protect the 5-FU from degradation by the multiple physiological barriers en route to the target site (colon), the platform is enclosed in a capsule made of chitosan coated with polyvinyl alcohol.

The final chapter is about the detection and quantification of anti-neoplastic drugs by electrochemical measurements. In this chapter are established the protocols for the functionalization of the electrochemical sensors to enable the precise detection of cisplatin and bleomycin. Additionally, characterization methodologies and the resulting calibration curves derived from these techniques are documented.

CHAPTER 2. Development of PEGylated silk fibroin nanoparticles for drug delivery systems and the *in vitro* studies of the nanocarriers for 5-fluorouracil chemotherapy in colorectal cancer

This chapter presents the development of drug delivery formulations based on silk fibroin and polyethylene glycol nanoparticles and the validation of this system in terms of morphology, drug release and cytotoxic potential. The modified SF nanoparticles were loaded with 5-fluorouracil for colorectal cancer therapy and were tested both on colorectal adenocarcinoma cells (HT-29) and blood cells.

2.1 Development of PEGylated silk fibroin nanoparticles

This investigation is based on the development of polymeric nanoparticles obtained from silk fibroin, which underwent chemical modification using PEG for the drug delivery purposes. The morphological investigation and size distribution profile of the nanoparticles were performed by scanning electron microscopy (SEM) and dynamic light scattering (DLS). Drug release behavior for 5-fluorouracil-loaded PEGylated silk fibroin nanoparticles was investigated.

Figure 2 highlights the pathway and system causing the development of nanoparticles from acrylic acid, silk fibroin, and PEG. Initially, the first step was the activation of the carboxy groups (-COOH) in acrylic acid with NHS. Secondly, the hydroxy groups (-OH) from SF structure (mainly containing serine 11.9% and threonine 1%) are reacting with the activated acrylic [204]. This interaction causes the obtaining of esters. Ultimately, the polymerization reaction occurs by the reaction of the C=C from PEG/TEG diacrylates chemical structures with the double covalent bonds between carbon atoms from the modified silk fibroin-acrylic acid compound. At the end of the process, acetone is added to generate nanoparticle formation, and subsequent heating at 60 °C for the crosslinking of PEGylated silk fibroin nanoparticles.

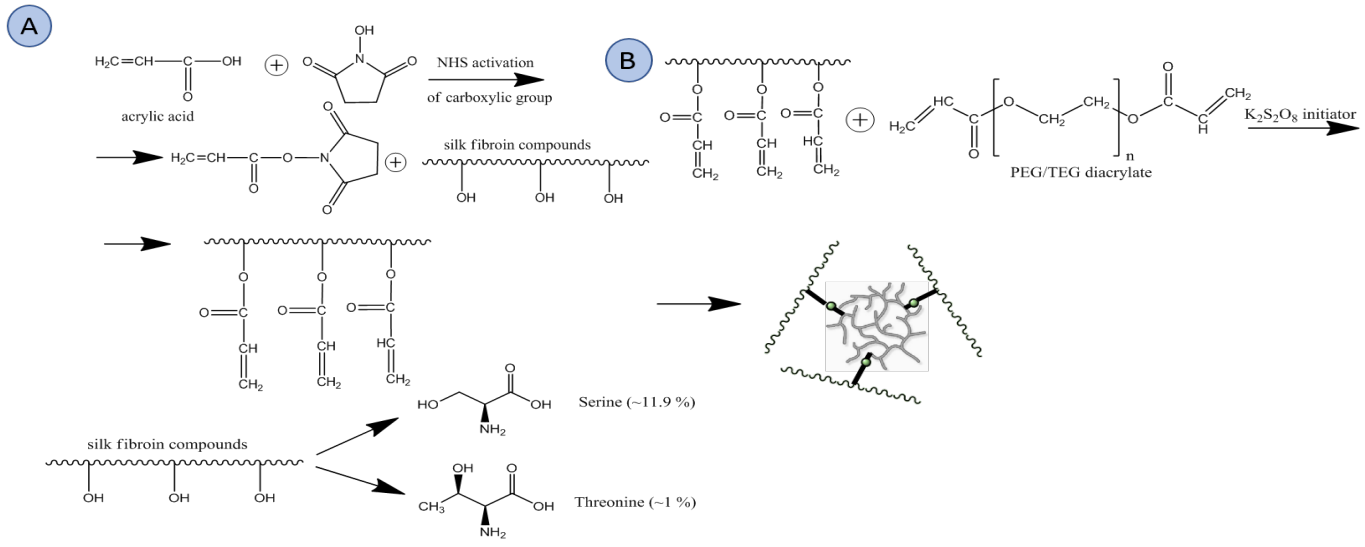


Fig. 2. The chemical functionalization technique of SF-PEG nanoparticles formation: A) silk fibroin-acrylic acid; B) silk fibroin-acrylic acid and polyethylene glycol

FTIR spectroscopy

The characteristic peaks of silk fibroin can also be found in the PEGylated samples. Figure 3 shows the FTIR spectra of PEG diacrylate (11), TEG diacrylate (22) and the SF-PEG/TEG nanoparticles. During the chemical modification process, the C-N stretching vibration (which is part of the amide III band) breaks into two separate peaks in the spectrum. The positions of these two peaks are related to the concentration of the PEG (polyethylene glycol) formulations.

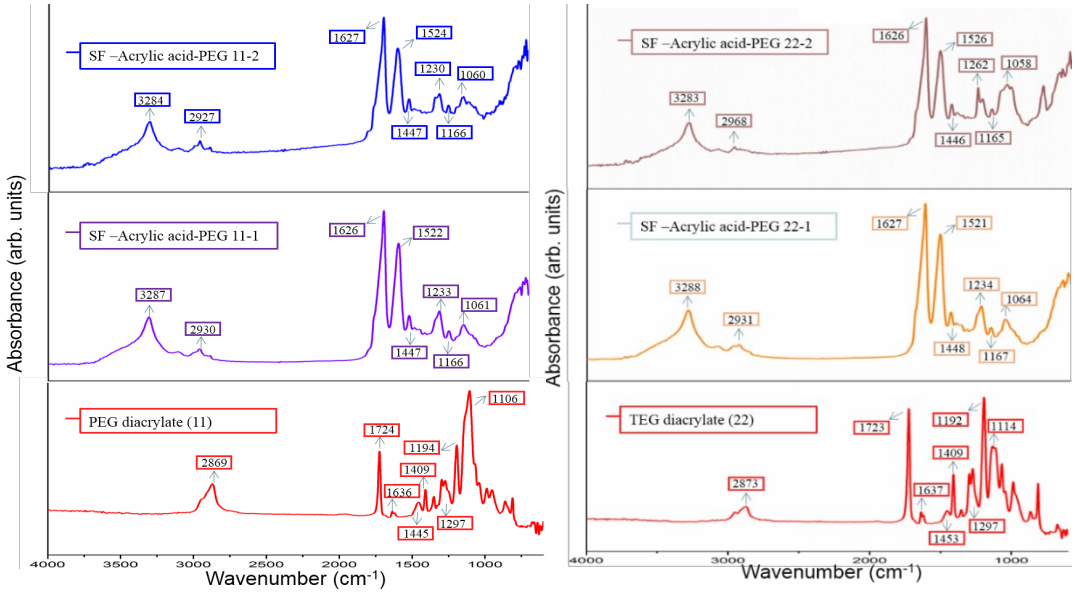


Fig. 3. ATR-FTIR spectra of PEGylated SF nanoparticles

RAMAN spectrometry

The physicochemical examination was additionally performed out using RAMAN analysis. In figure 4, the peaks of silk fibroin and SF modified with acrylic acid are displayed. The SF spectrum showed four main peaks: one around 2937 cm^{-1}, associated with the stretching of carbon-hydrogen (C-H) bonds, another at 1667 cm^{-1}, which is linked to the carbonyl group (C=O), and two peaks in the 1453/1388 cm^{-1} range, which are related to the bending vibrations of carbon-hydrogen bonds. The new peak at 1234 cm^{-1} in the SF-Acrylic acid spectrum is likely related to the amide III band, specifically the C-N stretching vibration from the native fibroin.

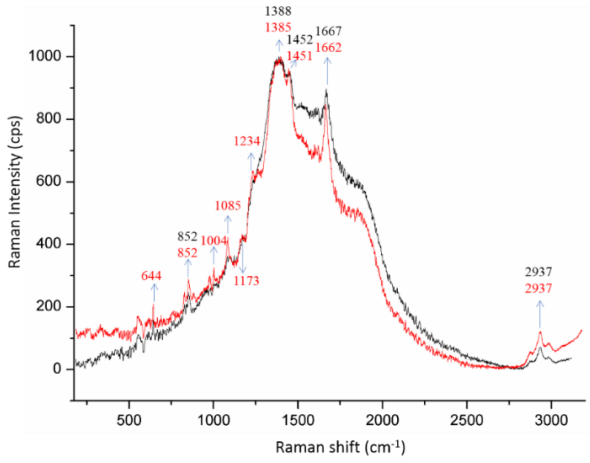


Fig. 4. RAMAN spectra of silk fibroin - color black and silk fibroin modified with acrylic acid - color red

Drug release behavior

The release profile of the PEGylated SF nanoparticles loaded with the neoplastic drug, 5-fluorouracil is illustrated in figure 5. The release of 5-FU was assessed at 15-minute intervals during the first hour, followed by assessments at 30-minute intervals during the following two hours. Finally, a final assessment was made 240 minutes after the start of the experiment.

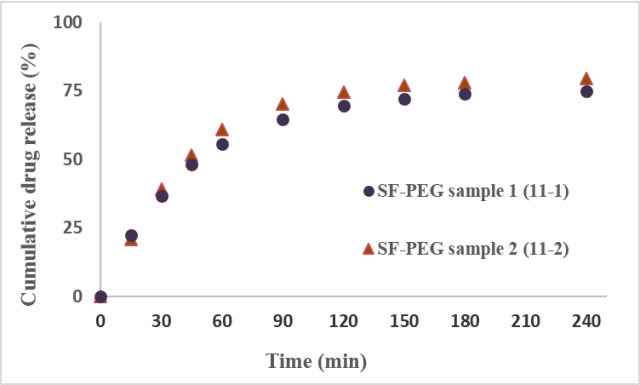


Fig. 5. Release profile of 5-FU from SF-PEG nanoparticles 11-1 and 11-2

2.2 The *in vitro* studies of the nanocarriers for 5-fluorouracil chemotherapy in colorectal cancer

The last section of Chapter 2 is dedicated to more extensive investigations on the validation of the drug delivery system of PEGylated silk fibroin nanoparticles created and described in the first chapter sections. This work synthesized silk fibroin modified with PEG nanoparticles both pristine and loaded with antineoplastic drug 5-FU and evaluated their morphology, dimensions and drug release properties. Moreover, the effective *in vitro* dose for HT-29 adenocarcinoma cells was determined, confirming the nanocarriers capacity to be absorbed, resulting in prolonged cytotoxicity. Furthermore, the cytotoxic capability of these nanocarriers on colorectal cancer and blood cells was studied. Finally, the suggested 5-fluorouracil drug release method was assessed for its effect on cancer cell migration and invasiveness.

Evaluation of the Half-Maximal Inhibitory Concentration (IC_{50})

The cytotoxic effect of silk fibroin nanoparticles modified with PEG and loaded with 5-FU was performed by using MTT viability assay. The viability of colorectal adenocarcinoma cells showed a concertation dependence decrease according to the dose of 5-FU loaded nanocarrier administrated. Concentrations in the range of 2–6 mg/mL does not influence the viability of tumoral cells. From 8 mg/mL 5-FU loaded NPs, however, a notable reduction in size of viable HT-29 cells occurred. Evaluation of the data enabled the determination of the IC_{50}. After 24 hours of treatment with 12 mg/mL 5-FU concentration of the loaded NPs, HT-29 cell viability showed a 50% decline. Thus, the concentration of 12 mg/mL was established as the IC_{50}, which is the dose applied in all subsequent biological investigations.

***In Vitro* Cytotoxicity Assessment**

Cells treated with 5-FU-loaded nanoparticles exhibited a significant change that lowered cell survival of HT–29 cells lower than 10% after 72 hours of treatment (Figure 6A). Additionally, tumoral HT–29 cells were subjected to MitoTracker dye staining, independent of mitochondrial potential, to label live mitochondria within cultures subjected to unmodified state and nanoparticles modified to carry the drug 5-FU for targeted delivery for 24 hours and 72 hours (figure 6B). The impact of the tested nanocarrier ± 5-FU NPs on the integrity of HT–

29 cell membranes was assessed by measuring LDH activity in the extracellular medium after 24 and 72 hours of treatment, as shown in Figure 6C. The introduction of 5-FU loaded nanoparticles lead to an increase in leaked LDH, indicating induced structural lesions in HT–29 cells.

The investigation of the effects of treatment with nanocarriers ± 5-FU on the viability and proliferation of colorectal cancer cells was validated using the live/dead assay (Figure 6D). Microscopic analysis of the HT–29 cell cultures showed robust cell proliferation in both the untreated samples and those treated with the pure SF/PEG NPs. In our examination of the results of nanoparticles ± 5-FU on nitric oxide production, nitrite levels were measured in tumoral cell subjected to pristine and 5-FU encapsulated nanoparticles for 24 h and 72 h (Figure 6E). Untreated PEGylated silk fibroin system generated no changes in NO production, without any noticeable differences in nitrite levels observed in between the treated sample and controls at either time point. Nevertheless, the treatment with drug loaded nanocarriers caused a considerable boost in NO production.

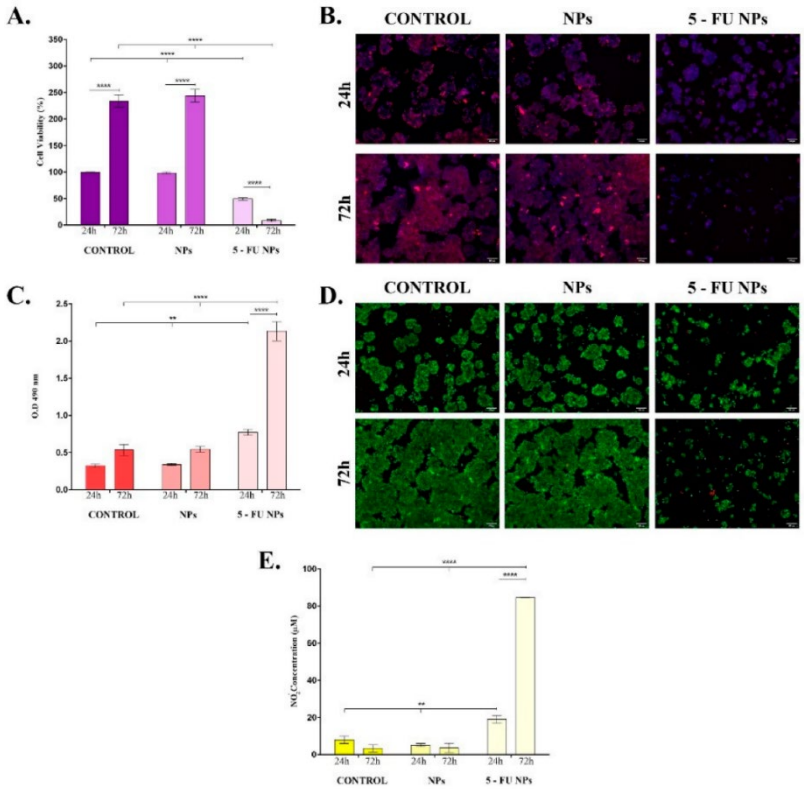


Fig. 6. *In vitro* cytotoxicity evaluation: (A). Cell viability; (B). Mitochondrial integrity; (C). LDH release; (D). Cell viability and proliferative potential; (E). NO release

In vitro Cell Migration and Invasion Assays

When the tumoral cells were exposed to the unloaded nanocarriers for 48 hours, they showed unaltered movement, with the ratio of migrating cells matching that of untreated cells. Nonetheless, the addition of NPs loaded with 5-FU resulted in a considerable reduction in the migratory capability of the tumoral cells. This problem results to a 75% decrease in cell migration in comparison to control. The invasion assay confirmed this pattern and revealed a significant adjustment in the invasive potential of tumor cells upon direct exposure to 5-FU-loaded nanoparticles.

General conclusions of Chapter 2

The significant objective of this chapter in the development of PEGylated silk fibroin nanoparticles was to build an adequate drug delivery carrier by chemically modifying PEG and

improving drug effectiveness. Integrating PEG into protein nanoparticles can decrease opsonization and enhance tumor cell delivery.

The experiment involved a sequence of careful steps, starting with the purification of silk fibroin and customization via a complex process using acrylic acid and PEG. The resulting nanoparticles were subjected to various analytical tests, including FTIR, Raman spectroscopy, dynamic light scattering (DLS), scanning electron microscopy (SEM) and drug release assessments. Fourier transform infrared (FT-IR) assessment confirmed the effective chemical modification of silk fibroin. In addition, Raman spectroscopy showed peaks attributed to the modified silk fibroin, highlighting the addition of acrylic acid. Dynamic light scattering (DLS) provided information on the size distribution of the nanoparticles in the solutions. SEM imaging provided a detailed morphology of the nanoparticles, revealing differences between the solutions of poly(ethylene glycol) diacrylate and tetra(ethylene glycol) diacrylate.

The PEGylated silk fibroin nanoparticles were successfully synthesized and characterized, the extensive analyses offered insights into structural modifications, nanoparticle size, and drug release profiles, laying a solid foundation for potential advancements in cancer therapy. The biological evaluations focusing on specific tumor cell lines were imperative to validate these nanoparticles for effective cancer treatment.

Pristine SF/PEG NPs exhibit excellent biologic compatibility with colorectal tumor cells (HT-29) and human blood cells. This indicates that they possess the potential for secure applications in biological systems. The loaded nanoparticles with 5-FU demonstrated a substantial decrease in tumoral cell viability. The treatment also resulted in enhanced expression of TNF-α that could add to therapeutic feedbacks versus cancer cells. Also, the treatment with 5-FU loaded nanocarriers has minimized the migration and invasion abilities of tumoral cells. This recommends their capacity in preventing cancer cells metastasis, an essential element in cancer progression.

CHAPTER 3. Gastrointestinal tract target delivery system based on surface-modified Silk Fibroin nanoparticles

In this chapter is presented the development of a controlled drug delivery system for oral administration of anticancer drugs using surface-modified silk fibroin (SF) loaded with 5-fluorouracil (5-FU) enclosed in a chitosan/PVA outer capsule to safeguard the drug from enzymatic and chemical degradation within the gastrointestinal environment. Silk fibroin nanoparticles (NPs) were obtained by nanoprecipitation, and the whole complex delivery system (platform) was prepared by coupling the chemistry of silk fibroin with Boc-NH-(PEG)-COOH.DCHA. The modified NPs were loaded with 5-fluorouracil and embedded in a chitosan/polyvinyl alcohol capsule. The chemical modification of silk fibroin was evaluated by FTIR-ATR, XPS and Circular dichroism. The morphological characteristics of the silk fibroin nanoparticles and their architectures were investigated using SEM. More, all the individual components of the platform, as well as the assembled system were tested for *in vitro* cytotoxicity on HT-29 colorectal adenocarcinoma cells. Drug release tests at neutral and acidic pH revealed the need to protect the polymeric capsule. Drug release in the gastrointestinal tract

of capsules loaded with the SF platforms showed that only 10-11% of the drug was released in the stomach, 23-25 % in the small intestine and 58-62 % at the target site (colon). *In vitro* indirect studies showed effective cytotoxic effects of the platforms on the HT-29 adenocarcinoma cells.

Physicochemical characterization by FT-IR

The clear peaks related to silk fibroin (figure 7 right) consist of amide I at 1648 cm^{-1}, amide II at 1534 cm^{-1} and amide III at 1239 cm^{-1}, which are designated to the stretching C =O vibration, the bent N-H resonance and the stretching C-N resonance, specifically. After the initial modification with the coupling agent BOC, exceptional shifts to reduced values took place for all major amides: Amide I at 1625 cm^{-1}, Amide II at 1522 cm^{-1} and Amide III at 1233 cm^{-1}. These shifts are because of the binding of BOC within the lysine amino groups, bring about the development of new amide bonds. In enhancement, the form of amide III (stretching C-N vibration) started to change and showed a split into two heights.

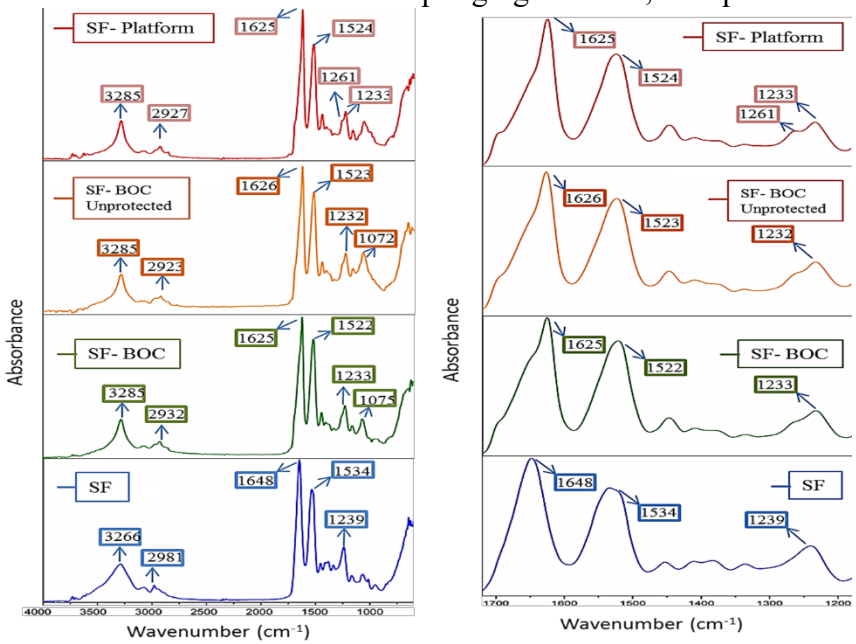


Fig. 7 FT-IR spectra of silk fibroin (SF), modified SF-BOC, unprotected SF-BOC and nanoparticles SF platform

Physico-chemical characterization by X-ray photoelectron spectroscopy (XPS)

XPS investigation of material surfaces is a key physicochemical characterization that supplements the platform development insights gained from FT-IR results. The modified BOC-SF showed four second C 1s peaks, suggesting the reliable adjustment: the peak centered at 283.70 eV, connected with the C-C bond, shifted to a lower value with decreased intensity; the peak centered at 285.25 eV, associated with the C-H bond, had an increased value due to the coupling agent's structure containing of hydrocarbon species. Additionally, the C-C bond to C-H bond ratio decreased significantly from 1.21 in fibroin to 0.64 in BOC-SF. This drop can be clarified by the coupling agent's contribution of many C-H groups relative to the C-C bond.

Morphological characterization (SEM)

The overview image of the 2% platform shows agglomerates of nanoparticles with a tuft-like structure. On closer inspection of the high-resolution image (figure 8), the nanoparticles have a size of around 100 nm and a smooth surface. In contrast, the overview image of the 5% platform also shows nanoparticle agglomerates. For the 5% platform, the constitutive nanoparticles are larger, while the modified SF-BOC nanoparticles appear smaller compared to the 2% silk fibroin concentration.

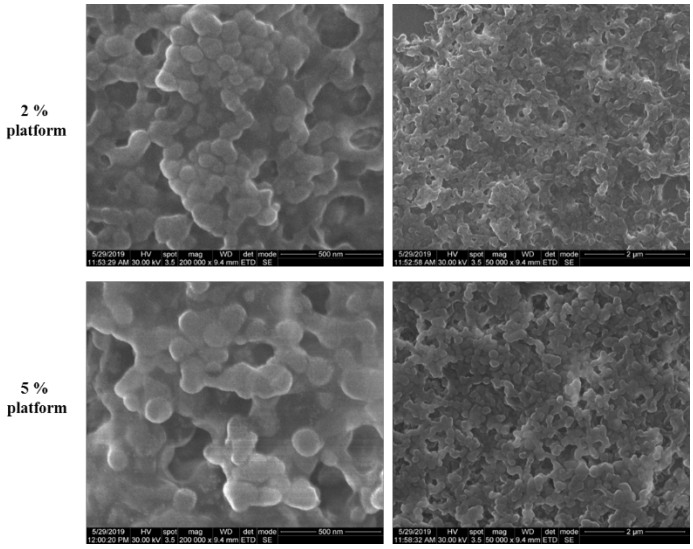


Fig. 8 SEM images with constitutive nanoparticles of SF platforms

Drug release measurements

The evaluation of the *in vitro* drug release profiles for the two SF platforms, which differ in their 5-FU concentrations (2%, 4%, 6% and 8% w/w), showed similar behavior under both neutral and acidic pH conditions. Remarkably, the 2% SF platform exhibited a significantly higher release efficiency than the 5% platform in both scenarios.

To protect 5-FU from degradation while it passes through several physiological barriers on its way to its destination (colon), a chitosan coated with and APV capsule was established. Release studies carried out in the gastrointestinal tract utilizing these capsules encapsulated with SF platforms revealed that just slight amount of 5-FU was liberated during the stomach stage (10%), adhered to by a small release of 25% during the flow through the small intestine, and a substantial increase in release in the designed location - colon (around 60%).

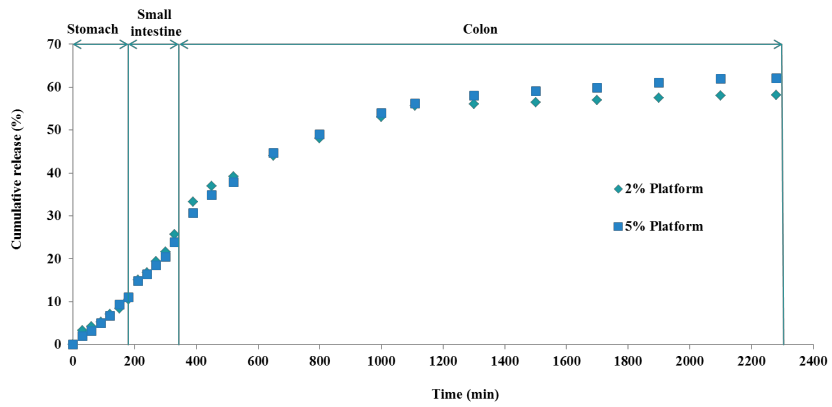


Fig. 9 Drug release test in the gastrointestinal tract for platforms with both 2% platform and 5% platform

Platforms indirect biocompatibility

When HT-29 cells were subjected to the P + 5-FU therapy, their viability considerably lowered, especially after 72 hours. This suggests that the treatment has a substantial cytotoxic effect on these cancer cells. The in vitro study highly suggests the possibility of the P + 5-FU therapy to be cytotoxic against HT-29 colorectal adenocarcinoma cells, revealing potential for further research in cancer therapy. Nonetheless, prior to the substance can be used clinically, comprehensive study is still necessary to demonstrate its safety and security and effectiveness.

General conclusions of Chapter 3

The study's approach to platform creation, full characterization, and strategic assessment of drug release along the GI tract establishes a solid foundation for prospective applications in controlled oral drug delivery systems, notably cancer therapy. The results demonstrated effective chemical alterations to silk fibroin, which were confirmed using a variety of characterization techniques including as FT-IR, XPS, CD, and morphological analysis. The morphological characterization revealed changes in platforms based on various silk fibroin concentrations, which influenced drug release behaviors.

The drug release studies, carried out at both neutral and acidic pH to mimic the gastrointestinal system, offered crucial insights. The systems showed particular release profiles, with different efficiencies and rates in the stomach, small intestine and large intestine. the results of the study show that the created platforms, especially the 5% variant, have excellent potential for targeted medication delivery to the colon while avoiding early release in the stomach. The managed and targeted release of medicines suggests that they can be used to maximize colorectal cancer cells treatment.

CHAPTER 4. Development of electrochemical biosensors for the detection and quantification of anti-neoplastic drugs

Sensors manufactured by using screen-printed electrodes are extremely precise and selective in detecting diverse analytes. However, obstacles like as uneven replication, surface imperfections, and manufacturing complications make it difficult to produce a high-quality sensor. The cleaning procedure is a crucial step in achieving consistent results. The first subchapter focuses on cleaning screen-printed electrodes to enhance the surface quality of working electrodes made by gold and platinum.

The second section describes how to obtain biosensors for the particular detection of two anti-neoplastic medicines, cisplatin and bleomycin. Cancer is among the foremost worldwide mortality drivers, and it is frequently treated with extremely toxic anticancer drugs. As a result, individualized monitoring of therapy with these medications is a goal, bringing into foreground the ideas of personalized and precision medicine into special attention.

4.1 The optimal method for cleaning screen-printed electrodes

This study assessed the effectiveness of various chemical agents and cleaning techniques through those two electrochemical characterization methods: cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Also, the potential of the cleaning method to enhance the surface quality of the working electrodes was observed.

Chemical Cleaning of Screen-Printed Electrodes

The distinction in the magnitude of the oxidation/reduction peak current observed prior to and following the degreasing step with acetone over Au electrode was located to be inconsequential (0.01 μA). On the platinum electrode, the measurements revealed a significant difference of 7.88 μA. As a result, a significant boost in the amplitude of the oxidation peak was detected, confirming the importance and performance of the degreasing phase for platinum work electrodes.

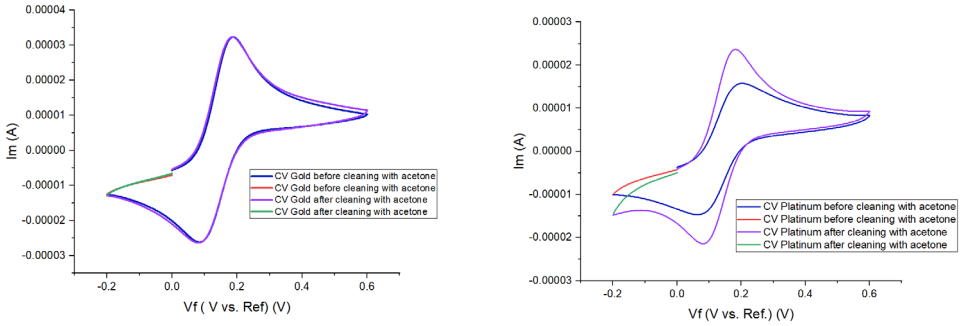


Fig. 10. CV of screen-printed electrodes with gold and platinum working electrodes prior to and following the degreasing step with acetone

The SPE featuring Au and Pt work electrodes underwent electrochemical cleaning in a PBS buffer solution containing 10 mM hydrogen peroxide. This method not only effectively removed contaminants but also had the potential to increase electrode sensitivity through the introduction of new C-O groups, as outlined in previous studies [255].

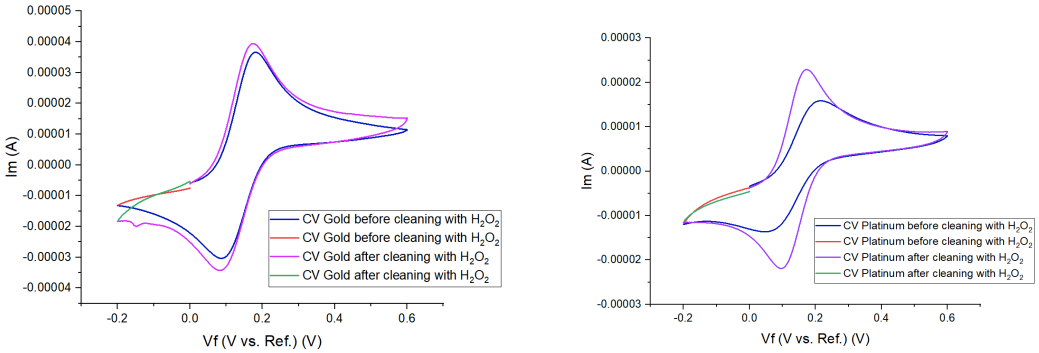


Fig. 11 CV of the gold and platinum electrode prior and following electrochemical cleaning

Electrochemical cleaning of SPE using negative potential

The impedance data support the effectiveness of the cleansing procedure. Upon comparing the EIS plots of the Au WE prior and following electrochemical cleaning, a modest enhancement in the redox diffusion process is noted. This improvement can be seen in the reduction of the Rp value by 10.3 ohms, as can be seen in figure 12

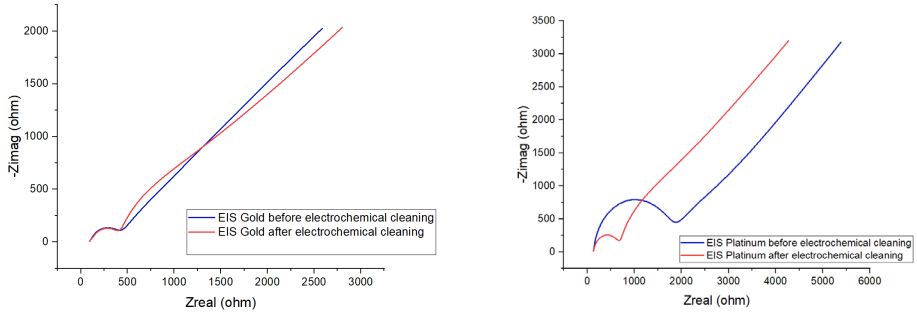


Fig. 12 Nyquist plots of the WE prior and following electrochemical cleaning using negative potential

4.2 Screen-printed electrode-based sensors for the electrochemical detection of cisplatin and bleomycin in serum samples

This research study aims to develop two prototype sensors for detecting two typically used antitumor agents, cisplatin and bleomycin, in cancer therapy within biological samples. The prototypes are intended for electrochemical detection of different drugs, with the utmost objective of monitoring and customizing treatment actions. Monitoring requires measuring antineoplastic concentrations in the blood plasma of patients getting treatment. This kind of biosensor can carry out real-time, quick, and sensitive research studies, providing info on the concentration of antineoplastics in the plasma.

Electrochemical detection and quantification of cisplatin from synthetic serum

In principle, cisplatin forms an adduct with HSA via specific amino acid residues, including His 105, Met 298, Met 329, and others. Cisplatin was detected by submerging the electrode in a 50 mM hydrogen peroxide solution, which causes an oxidation process from Pt(II) to Pt(IV) that was monitored using CV measurements. Figure 13 illustrates the schematic that outlines the sensor functional modification process and the operational principle behind cisplatin quantification.

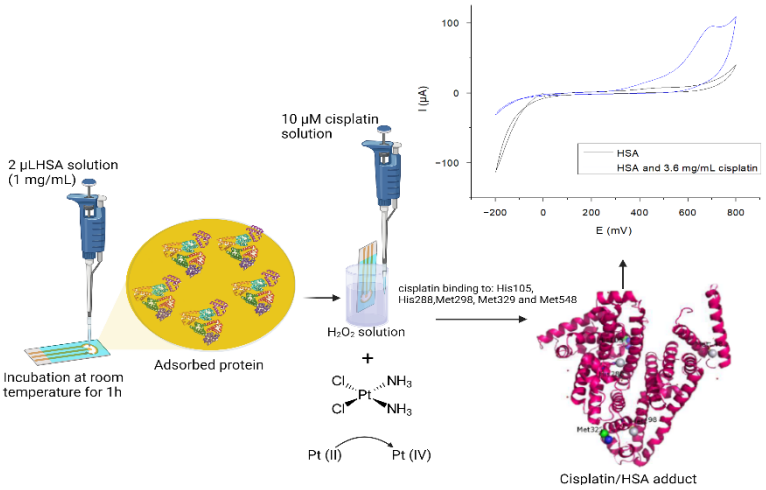
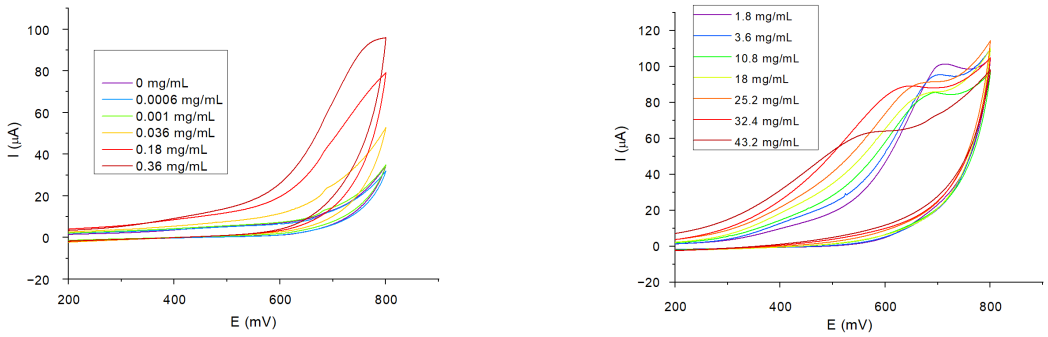


Fig. 13 The representative scheme of cisplatin biosensor development

Through this method, 12 different concentrations of cisplatin from 0.6 x 10^3 mg/mL to 43.2 mg/mL were tested in 6 mL of H_2O_2 (five microliters of serum by adding the correct volume of cisplatin stock solution) as shown in the figure 14.



(**A**) (**B**)

Fig. 14 (**A**): CV plots of cisplatin between 0 mg/mL (HSA functionalized electrode) and 0.36 mg/mL (**B**): CV plots of cisplatin from 1.8 mg/mL to 43.2 mg/mL

The correlation coefficient value for the low concentrations was $R^2 = 0.9936$ and for the high concentrations was $R^2 = 0.9905$.

According to published research, the interaction process of the bleomycin resistance protein to bleomycin (BLE) is not entirely understood. Several possibilities suggest that the interaction of BRP with BLE is similar to the bonding of bleomycin with DNA [265]. Figure 15 presents the voltammograms obtained for bleomycin (BLE) detection in human serum over a concentration range of 0.23 µg/mL to 7.56 µg/mL. As the concentration of BLE rises, a clear rise in the differential current is observed, from 3.93 µA at 0.23 µg/mL to 6.8 µA at 7.56 µg/mL. This progressive increase in current demonstrates the capability of the proposed electrochemical protocol to quantitatively detect BLE in complex biological matrices such as serum. The results suggest a strong correlation between BLE concentration and current response, indicative of reliable sensor performance.

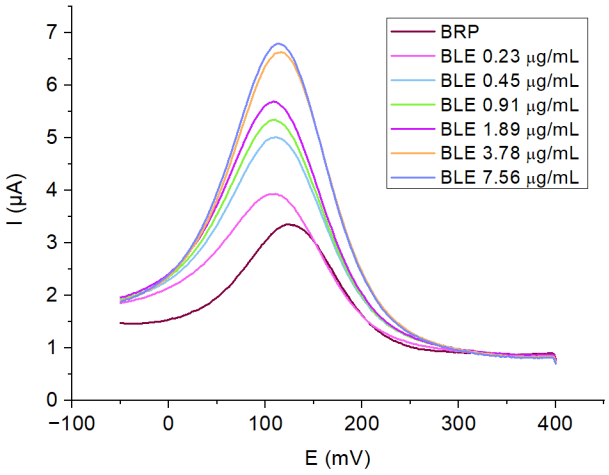


Fig. 15 Voltammogram plots of BLE levels of 0.23 µg/mL to 7.56 µg/mL

General conclusions of Chapter 4

The research study on cleaning methods for screen-printed electrodes reveals numerous necessary results such as: the comparative analyzes with different solvents and electrochemical approaches show distinctions in the cleansing results; for the platinum working electrodes the most efficient cleaning method was by applying ethanol directly on the subphase of the electrode, while for the gold working electrodes, the acetone cleaning treatment was more effective. The H_2O_2/CV method was highlighted by the SEM and elemental analysis by smoother and more uniform subphases and for the gold electrodes a better distribution on the electrode surface.

The cleaning study paves the way for future research into combined cleaning approaches and emphasizes the importance of comprehensive device cleaning processes. To acquire very precise electrochemical performance specifications such as sensitivity, selectivity, and accuracy, it is necessary to establish a tailored cleaning procedure.

The second part of the Chapter 4 study highlighted the importance of developing highly sensitive biosensors for the detection of antineoplastic drugs: cisplatin and bleomycin from biological specimens. These sensors are designed to meet the special requirements of monitoring drugs concentration at any time of chemotherapy treatment such as very high sensitivity. The cisplatin biosensor had a quantification limit of 0.6 µg/mL, whereas the bleomycin biosensor had a quantification limit of 0.23 µg/mL. The both biosensors need performance improvement and a future perspective is to develop SPEs for continuous monitoring of drugs concentration within biological samples to minimize the potential for side effects. Another application is the development of portable electrochemical biosensors by facilitating immediate treatment adjustments through on-site measurements.

CHAPTER 5. General conclusions

The results presented in this thesis are characterized by a high degree of originality and highlight two research directions in the field of cancer, which include new treatment methods and advances in early diagnosis and personalized medicine. This work describes the methodologies employed in the development of novel DDSs, the characterization techniques and the resulting outcomes. In addition, strategies for adapting electrochemical sensors for drug detection of drugs used in cancer therapy are described.

In the second chapter, the experimental investigations provided numerous insights into the development of silk fibroin nanoparticles modified with PEG diacrylates for therapeutic delivery and their *in vitro* evaluation for encapsulating 5-fluorouracil and release behavior in the treatment of cancer. The comprehensive research of the drug delivery system included its chemical, morphological and biological characterization.

The successful synthesis and assessment of PEGylated SF NPs enabled considerable examinations that revealed structural adjustments, nanoparticle dimension, and drug release features. This lays a solid system for future advances in cancer therapy. The fundamental biological evaluations, with a concentrate on certain tumoral cell lines, were critical in validation the efficiency of these nanoparticles for cancer cells therapy. The drug loading effectiveness was fantastic, and the release pattern revealed an extended and sustained drug release from the modified nanoparticles. This reveals that these nanoparticles have the ability as DDSs for the encapsulation and release of 5-FU.

This innovative drug delivry system, derived from the bonding of silk fibroin with PEG, showed strong biocompatibility with adenocarcinoma cells (HT-29) and biocompatibility with human blood cells. This underlines its potential for risk-free application in biological conditions. PEGylated modified nanoparticles encapsulated with antineoplastic drug 5-FU brought substantial decrease in the survival of tumoral cells. Additionally, the treatment caused an upregulation of TNF-α, showing a potential therapeutic response versus cancer cells. The motility and metastatic behavior of the tumoral cells HT-29 also decreased by applying of SF/PEG NPs loaded with 5-FU, suggesting that they might avoid cancer cells cell metastasis- a vital aspects in cancer cells progression. The potential of SF/PEG nanoparticles to became a drug delivery system for intravenous administration is demonstrated by the good analysis results. Nevertheless, the in vivo studies are warranted to understand the nanoparticles interactions, cytotoxicity and bioavailability.

The research in phase three focused on the advancement and characterization of silk fibroin (SF) nanoparticle systems for dental delivery of 5-fluorouracil. The development of the platform entailed chemical modification and encapsulation strategies to specifically control the release of the medicine along the gastrointestinal (GI) tract. This intricate advancement process caused the obtaning of two SF-based platforms (2% and 5% silk fibroin), each tailored to a specific release behavior of the drug. Physicochemical characterization methods, including FT-IR, XPS and CD spectroscopy, were made use of to confirm the success of the modification steps and structural adjustments throughout the coupling reactions. Morphological evaluation by SEM exposed the different densities of the nanoparticles within the different platforms.

Drug release profiles in neutral pH media revealed various patterns for the 2% and 5% SF platforms. The 2% platform showed higher efficiency and faster initial release while the 5% platform had a slower and a lot more sustained release profile. the platforms simulated the passage of the gastrointestinal system and showed very little 5-FU release in the stomach, a little bit higher release in the small intestine and significantly greater release in the colon. This controlled release behavior is consistent with the expected habits that is critical for a targeted drug delivery system.

Biocompatibility results showed a considerable impact on HT-29 cells after exposure to the SF platform and 5-FU therapy. There was a significant decline in cell viability and a rise in LDH release, recommending cytotoxic effects, and the boosted nitrite levels showed the induction of oxidative stress in the treated HT-29 cells, potentially causing cellular apoptosis.

These findings suggest that the developed platforms, particularly the 5% concentration variant, offer promising potential for targeted drug delivery to the colon while mitigating premature release in the stomach. Nevertheless, further *in vivo* studies are recommended to validate these findings and ascertain the practical applicability of these silk fibroin-based platforms in clinical trials, opening avenues for more efficient and patient-centric cancer treatments in the future.

Screen-printed electrode-based biosensors demonstrate excellent accuracy and selectivity in detecting numerous analytes. Nonetheless, the look for an extraordinary sensing unit is interfered with by barriers such as irregular replication, surface impurities and the complexity of fabrication. A critical action to make sure consistent results is the purification process. Chapter 4.1 defined the performance of various chemical compounds and cleaning strategies, concentrating on gold and platinum electrodes. The effectiveness of cleansing was assessed by polarization resistance (Rp), surface area morphology evaluation by SEM imaging elemental structure analysis by EDX.

The study not only sheds light on the effectiveness of different cleaning methods, but also underlines the importance of customized cleaning approaches for screen-printed electrodes. The study opens avenues for further research into mixed cleaning methods and encourages researchers to consider the effects of solvent treatments on whole electrode devices. These findings are crucial for optimizing cleaning methods and as a result optimizing the electrochemical properties.

In the final section of original contributions within this PhD thesis, dedicated to the electrochemical detection of antineoplastic drugs, the functionalization methods for the development of cisplatin and bleomycin biosensors on gold screen-printed electrodes were investigated. The cisplatin biosensor was developed by strategically chemically modify the working electrode with HSA, taking advantage of platinum medicines' well-established affinity for plasma proteins. Furthermore, the BRP, which has been shown to be efficient at attaching to bleomycin analogs, was used in the creation of the bleomycin detection biosensor. Electrochemical methods such as cyclic voltammetry for the detection of cisplatin and square wave voltammetry for the quantification of bleomycin offer a sensitive and effective approach for monitoring changes in the concentration of these drugs, with advantages such as rapid analysis, low detection limits and the potential for point-of-care applications.

Dissemination

I) List of ISI-quoted papers

1) **Andreea-Cristina Ion**, Ionut-Cristian Radu, Eugeniu Vasile, Elena Iuliana Biru, Ariana Hudita, Bianca Galateanu, Horia Iovu, Catalin Zaharia, DEVELOPMENT OF PEGylated SILK FIBROIN NANOPARTICLES FOR DRUG DELIVERY SYSTEMS, **U.P.B. Sci. Bull.**, Series B, Vol. 83, Iss. 4, 2021, ISSN 1454-2331, **IF 2021: 0.164**
2) Hudiță, Ariana, Ionuț Cristian Radu, Cătălin Zaharia, **Andreea Cristina Ion**, Octav Ginghină, Bianca Gălățeanu, Luminița Măruțescu, Florin Grama, Aristidis Tsatsakis, Leonid Gurevich, and et al. 2021. "Bio- and Hemo-Compatible Silk Fibroin PEGylated Nanocarriers for 5-Fluorouracil Chemotherapy in Colorectal Cancer: In Vitro Studies" Pharmaceutics 13, no. 5: 755, **IF 2021: 6.321**
3) Dana STAN, **Andreea-Cristina MIRICĂ (ION)**, Rodica IOSUB, Diana STAN, Nicolae-Bogdan MINCU, Marin GHEORGHE, Marioara AVRAM, Bianca ADIACONIȚĂ, Gabriel CRĂCIUN, Andreea-Lorena BOCANCIA-MATEESCU, What Is the Optimal Method for Cleaning Screen-Printed Electrodes? Processes 2022, 10, 723. https://doi.org/10.3390/pr10040723, **IF 2022: 3.5**
4) Radu, Ionut-Cristian, **Andreea-Cristina Ion Mirica**, Ariana Hudita, Eugenia Tanasa, Horia Iovu, Catalin Zaharia, and Bianca Galateanu. 2023. "Thermosensitive Behavior Defines the Features of Poly(N-isopropylacrylamide)/Magnetite Nanoparticles for Cancer Management" Applied Sciences 13, no. 8: 4870. **IF 2023: 2.7**
5) Radu, Ionut-Cristian, Iuliana-Elena Biru, Celina-Maria Damian, **Andreea-Cristina Ion**, Horia Iovu, Eugenia Tanasa, Catalin Zaharia, and Bianca Galateanu. 2019. "Grafting versus Crosslinking of Silk Fibroin-g-PNIPAM via Tyrosine-NIPAM Bridges" Molecules 24, no. 22: 4096. **IF 2019: 3.267**
6) **Andreea-Cristina Mirica (Ion)**, Dana Stan, Dragos-Cosmin Zaharia, Horia Iovu, Sorin Mocanu, Marioara Avram, and Lorena-Andreea Bocancia-Mateescu. "Screen-Printed Electrodes—A Promising Tool for Antineoplastic Drug Detection (Cisplatin and Bleomycin) in Biological Samples" International Journal of Molecular Sciences 25, 2024, no. 15: 8030. https://doi.org/10.3390/ijms25158030, **IF 2024: 4.9**

Article sent to publication

1) **Andreea-Cristina Mirică**, Ionut-Cristian Radu, Eugenia Tanasa, Horia Iovu, Catalin Zaharia, Rafael Luque, Ariana Hudita, Bianca Galateanu, CROSSLINKED SILK FIBROIN NANOPARTICLE ARCHITECTURES EMBEDDED IN CHITOSAN CAPSULES FOR ORAL DELIVERY IN CANCER MANAGEMENT. International Journal of Biological Macromolecules. **IF 2024: 8.2**

II) Participation to national and international conferences
Oral presentations

1) **Andreea-Cristina Ion**, Ionut-Cristian Radu, Catalin Zaharia, Ariana Hudita, Bianca Galateanu, Eugenia Tanasa, Horia Iovu, "*Nanohydrogels platforms for drug delivery systems by self-assembling of hydrophilic polymers*", 21st Romanian International Conference on Chemistry and Chemical Engineering, September 4-7 2019, Constanta, Romania.
2) **Andreea-Cristina Ion**, Ionut-Cristian Radu, Catalin Zaharia, Bianca Galateanu, Eugenia Tanasa, Horia Iovu, "*Silk Fibroin Self-Assembled Architectures Loaded With Antineoplastic Drug for Cancer Therapy*", BPC 21 (Bucharest Polymer Conference), June 9-11 2021, Bucharest, Romania

Poster presentations

1) **Andreea Cristina Mirică (ION)**, Lorena Andreea Bocancia Mateescu, Sorin Mocanu, Dana Stan, Electrochemical biosensors for monitoring treatment with antineoplastic drugs, A-XIV-A Conferință Națională a asociației de medicină de laborator din România (cu participare internațională), 14-16 Iunie 2023, Timișoara

III) Patent application

1) Patent application number A2023 00283/07.06.2023. Dana Stan, Lorena Andreea Mateescu, **Andreea-Cristina Mirică (ION)**. PROCEDEU DE OBȚINERE BIOSENZOR PENTRU DETECȚIA ELECTROCHIMICĂ A CISPLATINEI DIN PROBE DE PLASMĂ ARTIFICIALĂ

List of abbreviations

3D - three-dimensional
5-FU - 5-Fluorouracil
Ala – Alanine
ATR - Attenuated Total Reflectance
BOC - Boc-NH-(PEG)-COOH.DCHA
CB - celestine blue
CE - counter electrode
CFU - colony forming units
CNF - carbon nanofibers
CRC - Colorectal cancer
CTN - carbon nanotubes
CV - Cyclic Voltammetry
DAPI - 4′,6-diamidino-2-phenylindole
DDSs - Drug Delivery Systems
DLS - Dynamic Light Scattering
DMEM - Dulbecco's modified Eagle medium
DNA - Deoxyribonucleic acid
DOX – doxorubicin
DPD - dihydropyrimidine dehydrogenase
EDTA - Ethylenediaminetetraacetic acid
EDX - Energy Dispersive X-Ray Analysis
EIS - Electrochemical impedance spectroscopy
ELISA - enzyme-linked immunosorbent assay
EPR - Enhanced Permeability and Retention
FDA - Food and Drug Administration
FT-IR - Fourier-transform infrared
Gly – Glycine
GSH – Glutathione
HCV - hepatitis C virus
HIV - Human Immunodeficiency Virus
HPLC - High-performance liquid chromatography
ICPMS - Inductively coupled plasma mass spectrometry
IR - immediate release
LC-MS - Liquid chromatography–mass spectrometry
LOD – limit of detection
mAb- monoclonal antibodies
MDR - multidrug resistance
MNPs - magnetic nanoparticles
MR - modified release
MTT - 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide

MWCNT - multi-walled carbon nanotubes
NED - N-1-naphthyl ethylenediamine dihydrochloride
NHS - N-hydroxysuccinimide
NIPAM - N-isopropyl acrylamide
NPs – nanoparticles
PBS - Phosphate-buffered saline
PCA - poly(alkylcyano acrylates)
PEG – Polyethylene glycol
PGA - poly(glycolic acid)
PLA - Poly(lactic acid)
PLGA - poly(lactic-co-glycolic acid)
PNIPAM - Poly(N-isopropyl acrylamide)
PTX - paclitaxel
PVA - Polyvinyl alcohol
PVP - polyvinylpyrrolidone
RE - reference electrode
RESOLV - Rapid Expansion of Supercritical Solution into Liquid Solvent
RESS - Rapid Expansion of Supercritical Solution (RESS)
SAMs - self-assembling monolayers
$scCO_2$ – supercritical carbon dioxide
SEM - scanning electron microscopy
Ser – Serine
SERS - surface-enhanced Raman spectroscopy
SF - Silk fibroin
SPEs - Screen-printed electrodes
TFA - Trifluoroacetic acid
Thr - threonine
TNF-α - Tumor necrosis factor-α
UHPLC - Ultra-high performance liquid chromatography
UV-VIS – Ultraviolet-Visible
Val - valine
WE - working electrode
WHO - World Health Organization

Selective bibliography

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