"GHEORGHE ASACHI" , TECHNICAL UNIVERSITY FROM IASI
"CRISTOFOR SIMIONESCU" FACULTY OF CHEMICAL ENGINEERING AND ENVIRONMENTAL PROTECTION
The title of the research project: *Overcoming brain-blood barrier with new functionalized nanoparticles based on biopolymers containing two antitumoral drugs co-encapsulate*
Project code: PN-III-P1-1.1-PD-2021-0553
Financing contract: 61/2022
Project manager: Dr. Ing. Camelia Elena Tincu

Summary of the results obtained for the project PN-III-P1-1.1-PD-2021-0553

• *Objectives foreseen/achieved;*
The following objectives were foreseen in the funding request:

The main objective of this project is to develop a delivery system that can effectively overcome the blood-brain barrier to treat human glioblastoma. The delivery system consists of nanoparticles made of human serum albumin that are modified with a peptide (low molecular weight protamine) and cross-linked with carbonyl groups derived from oxidized gellan or oxidized pectin. Additionally, two drugs - temozolomide and curcumin - are co-encapsulated within the nanoparticles. In order to achieve this goal, several specific objectives must be achieved.

1. Physico-chemical characterization of precursors and functionalized and cross-linked nanoparticles, with or without drugs encapsulated- completed 100%.

2. Kinetic studies of in vitro drug release from nanoparticles, ex vivo testing of nanoparticulate systems, evaluation of biomaterial characteristics of nanoparticles (cytotoxicity, hemocompatibility, microbiological activity), and evaluation of drug encapsulated in nanoparticles at temperature, pH, and light- completed 100%.

3. Highlighting the protective nature of the polymer matrix for the incorporated drugs when complexed with metal ions - 100% completed

As part of stage 1 (2022) of the project "Obtaining and Physico-Chemical Characterization of Precursors and Functionalized and Cross-Linked Nanoparticles Containing or Not Encapsulated Drugs," 7 scientific research activities were planned. All activities were successfully completed, and the objectives for 2022 and the project indicators were achieved. Below is a brief description of the main activities during this stage.

In the **Act 1.1**. we focus on modifying human serum albumin (HSA) by using a peptide such as low molecular weight protamine (LMWP). The LMWP peptide helps improve the diffusion coefficient of albumin to the brain, which can aid drug delivery into cancer tumors. This strategy employs HSA-mediated targeting and LMWP-mediated crossing of the blood-brain barrier. To obtain LMWP, we proteolytically cleave salmon protamine sulfate in the presence of thermolysin. The proteolysis process is confirmed by checking FT-IR and 1H-NMR spectra.. The method for obtaining modified albumin with LMWP is also presented. In order to modify HSA with the LMWP peptide, the SH-group of HSA was first activated with succimidyl-4-(N-maleidomethyl)-cyclohexane-1-carboxylate (SMCC), and then the activated HSA was functionalized with LMWP. The compounds obtained were characterized by Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR).

The number of free amine groups in HSA, protamine sulfate, and LMWP were determined using the ninhydrin assay. The ninhydrin test was used to determine the number of free amino groups in albumin, which was found to be 6.31±0.7 x 10-4. The test was also conducted on the modified protamine fractions collected from the chromatographic column. It was observed that the number of moles of free amine groups increased from one fraction to another, indicating that LMWP was obtained. Within Act.1.2. information is presented on the synthesis and characterization of oxidized gellan (GO) and oxidized pectin (PO) in the presence of sodium periodate to obtain aldehyde groups. The kinetics of the oxidation reaction were determined, and the concentration of aldehyde groups in the products obtained following oxidation was determined quantitatively by indirect titration with $Na_2S_2O_3$. The oxidation degree of the oxidized polysaccharides increases over time up to 72 h for oxidized gellan and 24 h for oxidized pectin; its value remains constant after these oxidation periods. The molecular weight of GO and PO was determined by the viscosimetric method, and the structure of the synthesized compounds was analyzed by FT-IR and NMR spectroscopy, thus demonstrating that the oxidation reaction occurred with the formation of aldehydic groups. The molecular weight of gellan decreases with increasing oxidation degree and oxidation time. In **Act 1.3**, the synthesis of nanoparticles based on HSA and HSA modified with LMWP cross-linked with aldehyde groups originating from oxidized polysaccharides (using different molar ratios) containing temozolomide (TMZ) and curcumin co-encapsulated by a method based on ultrasonication and precipitation. In order to obtain the nanoparticles, different molar ratios were used between the amine groups in HSA and the aldehyde groups in oxidized polysaccharides (gellan or pectin) **Act. 1.4.** presents another method for obtaining nanoparticles based on HSA and HSA modified with LMWP cross-linked with aldehyde groups derived from oxidized polysaccharides by self-assembly containing two drugs, co-encapsulated curcumin, and temozolomide.

In Act.1.5, the obtained nanoparticles were characterized by evaluating the average diameter (laser beam diffractometry), morphologically (scanning electron microscopy), and structurally by FT-IR spectroscopy. The size of the nanoparticles in most cases is up to 200 nm, thus having the possibility to overcome the blood-brain barrier. In **Act 1.6**, the ability of nanoparticles to encapsulate drugs was evaluated. The nanoparticles obtained can include temozolomide and curcumin, and it was observed that the immobilization efficiency is higher for curcumin. However, there is a possibility that it is a consequence of the fact that temozolomide can form polymer-drug conjugates through the interaction of the amine group in its structure with the aldehyde groups in oxidized polysaccharides In activity 1.7, some preliminary studies on obtaining nanoparticles are presented. It was considered necessary before obtaining HSA nanoparticles cross-linked with oxidized polysaccharides first to carry out a systematic study on obtaining hydrogels of this type in order to determine the degree of swelling and the index of conversion of amino groups into Shiff bases. The degree of swelling of the hydrogels was observed to depend on the hydrophilicity of the samples and the pH. The degree of swelling increases with the increase of the amount of oxidized gellan in the polymer matrix, and maximum values of the swelling degree were obtained at pH=7.4. It was also observed that the value of the conversion degree of amino groups into Schiff bases increases with the increase in the amount of oxidized gellan in the samples. During the **project's second stage in 2023**, 11 scientific research activities were planned. All of these activities were completed successfully, resulting in achieving 100% of the proposed objectives and project indicators for that year. One of the activities, **Act. 2.1** involved the characterization of two types of human serum albumin (HSA) nanoparticles (NPs) that were obtained by using nanoprecipitation and self-assembly techniques. The nanoparticles were thoroughly analyzed using various methods such as laser diffractometry, FT-IR spectroscopy, scanning electron microscopy, degree of conversion, immobilization efficiency, and swelling degree. Within the Act 2.2. the release kinetics of drugs co-encapsulated in nanoparticles was studied, and this study was carried out at pH=7.4 and pH=4. The amount of drug released was determined spectrophotometrically, and it was shown that the drug release occurs in a controlled and sustained manner. The release efficiency was higher at pH=7.4 for curcumin and at pH=4 for TMZ and increased when the cross-linking degree decreased. In Act **2.3,** a study was conducted to determine cell apoptosis induced by drugs co-encapsulated in nanoparticles. Flow cytometry was used to test two types of nanoparticles with encapsulated drugs on the glioma tumor line C6 and the

normal cell line V79-4. The study found that the nanoparticles obtained by nanoprecipitation induced a strong apoptotic mechanism in C6 cells, while those obtained by self-assembly showed a potential synergistic effect**. I**n **Act 2.4**, the ability of functionalized albumin nanoparticles to overcome the blood-brain barrier was evaluated using the artificial membrane permeability test (PAMPA), which showed promising results for drugs co-encapsulated in such nanoparticles. During **Act 2.5**., the antioxidant activity of curcumin was assessed using the DPPH assay. Both free and immobilized curcumin in NPs were evaluated using the DPPH assay. The results showed that the immobilization of curcumin did not affect its antioxidant activity. Moreover, it was observed that the IC50 value for curcumin encapsulated in HSA nanoparticles exposed to UVA light at 365 nm was lower than that of free curcumin that was not UV-irradiated. These results demonstrate that the polymer matrix in which the curcumin was encapsulated had a protective effect. During **Act 2.6**, we evaluated the cytotoxicity of nanoparticles on two different cell lines - the C6 glioma tumor line and the V79-4 normal cell line. To determine the cytotoxicity, we used the MTT assay. We treated both tumor and normal cells with HSA nanoparticles obtained through self-assembly or nanoprecipitation. The treatment of tumor or normal cells with HSA nanoparticles, obtained by self-assembly or nanoprecipitation, cross-linked with oxidized pectin or oxidized gellan, with or without co-encapsulated active principles, determined a cytotoxic effect differentiated in intensity by using the MTT test. We found that the cytotoxic impact was much more pronounced in C6 tumor cells compared to normal V79-4 cells. This proves that these vectors can induce a selective response, which is advantageous for anticancer therapy. In Act 2.7, the microbiological activity that was performed on bacterial cultures of Staphylococcus aureus ATCC 25293 and Escherichia coli ATCC 25922 was evaluated. The results showed that drugs encapsulated in nanoparticles (50µg/ml) have no antibacterial activity due to the too-low concentration of the encapsulated drug. In **Act 2.8**, the hemolysis test was performed for functionalized HSA nanoparticles without encapsulated drugs, and the results showed that all nanoparticles had good hemocompatibility. In **Act 2.9**, we evaluated the temperature stability of nanoparticles. We used a TA Instrument Q600 analyzer and performed thermogravimetric analysis to do this. As per the results, we found that the nanoparticles' stability decreases as the temperature increases. We also observed that the nanoparticles prepared through nanoprecipitation are more stable than those obtained through self-assembly. Within the **Act 2.10**. the stability of free and encapsulated curcumin to degradation in solutions of different pH (3, 6.8, 7.4, and 9) was evaluated, and in **Act 2.11,** the degradation at light and air over a period of 30 days was evaluated spectrophotometrically. The results showed that the polymer matrix protects curcumin from degradative factors, and $t_{1/2}$ for degradation is greater than 15 h regardless of the pH value, and the light degradation percentage of encapsulated curcumin was lower than that of free curcumin.

In stage III (2024) of the project entitled "Highlighting the protective character of the polymer matrix for drugs incorporated when complexed with metal ions", two activities were foreseen. All activities were completed so that the objectives proposed for the year 2024 and the project indicators were achieved with a percentage of 100%. In **Act 3.1,** a stability study was conducted on drugs that were encapsulated and complexed with metal ions. Copper (Cu^{2+}) and Zinc (Zn^{2+}) ions were used from copper sulfate and zinc sulfate, respectively. The maximum absorption of curcumin/metal ion complexes dissolved in DMSO occurred at lower wavelengths, specifically 429 nm and 433 nm for those with Cu^{2+} ions and Zn^{2+} ions, respectively. The absorption peak of free curcumin varied, and shoulders appeared in different complexes depending on the involvement of the metal ion. Attempts were made to obtain metal complexes with TMZ (temozolomide), but it was observed that TMZ does not form metal complexes with Zn^{2+} ions. The absorption band at 331 nm, specific to temozolomide, for metal complexes with Cu^{2+} ions decreased significantly in intensity, making it impossible to have conclusive results. The results showed that nanoparticles cross-linked with Cu^{2+} ions do not form metal complexes, and the maximum absorbance wavelength was 435 nm. However, a shoulder appears at the wavelength of 457 nm in all samples analyzed, indicating some interactions with Cu^{2+} ions.

On the other hand, in the samples cross-linked with Zn2+ ions, a shift of the wavelength where the absorbance is maximum occurred at 428 nm. This may be due to the interaction of the metal ion with

the carbonyl groups of the oxidized polysaccharides that have not reacted with the amine groups of albumin. Statistical data analysis was used throughout the project (Act.3.2.).

Dissemination Of Results Obtained During The Project

April 1, 2022 - March 31, 2024

• **Indicators to achieve:**

-Publication of 3 articles in ISI journals with a high impact factor, in open access regime - achieved 100%
-Participation with scientific papers at 4 international conferences - achieved 100%
-Writing the annual research reports and the final research report 100%
-1 web page of the project - 100% done

• **Realized indicators:**

- Publication of 3 articles in ISI journals with a high impact factor, in open access mode
- Participation with scientific papers at 5 International Conferences
- Drafting of annual research reports and the final research report
-1 web page of the project-100% done

- **Participation in conferences:**

• *Oral communication:*

1. ***Camelia Elena Iurciuc (Tincu),*** *Christine Jérôme, Marcel Popa, Carmen Gafițanu, Eliza Grațiela Popa, Lăcrămioara Ochiuz, Biocompatible hydrogels films with the inclusion complex of β-cyclodextrin/curcumin immobilized for biomedical applications,* International Conference on Natural Products in Drug Discovery and Development – Advances and Perspectives, PSE Meeting 2022, September 19 – 22, 2022, Iași, Romania
2. Camelia Elena Tincu (Iurciuc), Oana-Maria Darabă, Leonard Ionuț Atanase, Marcel Popa, Lăcrămioara Ochiuz, *β-cyclodextrin/curcumin complex immobilized in the albumin-based hydrogel films cross-linked with oxidized gellan for biomedical applications*, International Congress of "Apollonia" University in Iași, Preparing the future by promoting excellence, XXXIIIth Edition, March 2 - 5, 2023, IASI, ROMANIA

• *Poster presentations:*

1. Camelia Elena Tincu (Iurciuc), Silvia Vasiliu, Ștefania Racoviță, Leonard Ionuț Atanase, Marcel Popa, Lăcrămioara Ochiuz, *New drug release system based on functionalized albumin cross-linked with oxidized polysaccharides for the potential treatment of brain tumors,* International Congress of "Apollonia" University in Iași, Preparing the future by promoting excellence, XXXIIIth Edition, March 2 - 5, 2023, IASI, ROMANIA
2. Camelia Elena Tincu (Iurciuc), Silvia Vasiliu, Ștefania Racoviță, Marcel Popa, Lăcrămioara Ochiuz, *Nanoparticles based on Functionalized Albumin Cross-linked with Oxidized Polysaccharides Used for Drug Delivery in Brain Tumors Therapy,* 3rd International Conference on Bioengineering and Polymer Science, Bucharest, ROMANIA - June 7-10, 2023
3. Camelia Elena Tincu (Iurciuc), Silvia Vasiliu, Ștefania Racoviță, Gabriela Vochița, Daniela Gherghel, Leonard Ionuț Atanase, Marcel Popa, Lăcrămioara Ochiuz, *Development of a drug delivery nanosystem composed of functionalized human serum albumin cross-linked with oxidized polysaccharides for treating brain tumors*, International Congress of "Apollonia" University of Iași, Preparing the future by promoting excellence, XXXIV Edition, February 29 - March 3, 2024, IAțI, ROMANIA

-Published articles:

1. **Tincu (Iurciuc) Camelia Elena,** Brahim Bouhadiba, Leonard Ionut Atanase, Corneliu Sergiu Stan, Marcel Popa, and Lăcrămioara Ochiuz. 2023. “An Accessible Method to Improve the

Stability and Reusability of Porcine Pancreatic α-Amylase via Immobilization in Gellan-Based Hydrogel Particles Obtained by Ionic Cross-Linking with Mg^{2+} Ions" *Molecules* 28, no. 12: 4695. https://doi.org/10.3390/molecules28124695

2. **Tincu (Iurciuc), Camelia-Elena**, Călin Vasile Andrițoiu, Marcel Popa, and Lăcrămioara Ochiuz. 2023. "Recent Advancements and Strategies for Overcoming the Blood–Brain Barrier Using Albumin-Based Drug Delivery Systems to Treat Brain Cancer, with a Focus on Glioblastoma" *Polymers* 15, no. 19: 3969. https://doi.org/10.3390/polym15193969
3. Submission for publication of the article entitled "Albumin-Based Hydrogel Films Covalently Cross-Linked With Oxidized Gellan With Encapsulated Curcumin For Biomedical Applications", Authors: Camelia Elena Tincu (Iurciuc), Oana-Maria Darabă, Christine Jerome, Marcel Popa, Lăcrămioara Ochiuz at Polymers magazine. (Manuscript ID: polymers-2956947)