"CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY,
BUCHAREST
DOCTORAL SCHOOL
MEDICINE

ABSTRACT OF DOCTORAL THESIS

ELECTROCHEMOTHERAPY OF GLANDULAR TUMORS - methods for optimizing 3D cell cultures to evaluate the immunogenic effect of ECT by IHC -

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CONTENT

Introduction

Electrochemotherapy (ECT) is a novel approach that combines chemotherapeutic drugs and electrical pulses to enhance drug delivery and efficacy in cancer treatment.

To optimize ECT applications, it's crucial to gain an extensive understanding of the underlying mechanisms at the tumor microenvironmental level such hypoxia gradients, cancer stem cells or immune surveillance. While various experimental models exist, our knowledge about these during ECT is still limited, especially when considering the glandular tissue. Therefore, a consensus on evaluating and assessing its impact has yet to be reached.

Furthermore, by incorporating ECT into 3D spheroids modeled for immune cellular response premises are created to better understand the immunogenic cell death is induced via electroporation. *i*-ECT addressing this issue is nowadays a hot scientific research domain that triggered ongoing clinical studies.

The goal of this study was to develop and characterize spheroids derived from various cell lines using optimized techniques such as hanging drop or agarose ultra-low adherent surfaces. The introduction of monocytes, electroporation (EP), and ECT in these spheroids alowed us to investigate their impact on the spheroid behavior and viability. By applying electrical pulses alongside chemotherapeutic agents, we aimed to evaluate drug uptake efficacy and immune cell-driven cytotoxic effects. Additionally, semiautomated morphometric analysis via brightfield imaging and molecular analysis techniques like immunohistochemistry (IHC) were optimized by proposing a procedure for embedding spheroids in agarose microwell arrays allowing for multiplexed batch-processing of spheroid samples. Furthermore, the thesis addresses key aspects of microphysiology and structure of tumoral microenvironment The feasibility of this method was tested using U87 glioblastoma, CaCo2 colon carcinoma and MCF7 adenocarcinoma cells, with several IHC tests performed on the 3D models while maintaining well location annotation. This research aimed to provide new insights into integrating ECT within 3D spheroid studies and bridge the gap between in vitro experiments and clinical applications motivated also by the new FDA adopted policy of the 3 “R” in scientific research (Replacement, Reduction, Refinement). By doing so, it may pave the way for both effective cancer research and treatment strategies.

I. GENERAL OVERVIEW

1. 3D cell cultures

Current 3D biological models include intact animals, organs, and in vitro 3D cells cultured within extracellular matrix-loaded gels. These models provide tissue-specific information at various levels of complexity. Reliable methods for culturing and analyzing these specimens, alongside advanced imaging techniques that can penetrate thick samples, are essential. In vitro 3D models promote higher levels of cell differentiation, tissue organization, and realistic responses to drugs or stimuli, surpassing the limitations of 2D cultures[1]. The introduction of 3D techniques has enhanced our ability to mimic the physical and biochemical conditions of tissues more accurately, contributing to better insights into cancer behavior, drug resistance, and therapeutic responses.

3D cell cultures create structures that closely mimic the conditions found in vivo, allowing for complex cell interactions and responses to environmental stimuli. These cultures are characterized by cellular aggregates that result in impaired diffusion of oxygen and nutrients, creating subpopulations of proliferating, quiescent, and necrotic cells (Fig. 1.1). This complexity has led to their widespread application in studies of metastasis, cell invasion, therapy resistance, and tissue engineering [2].

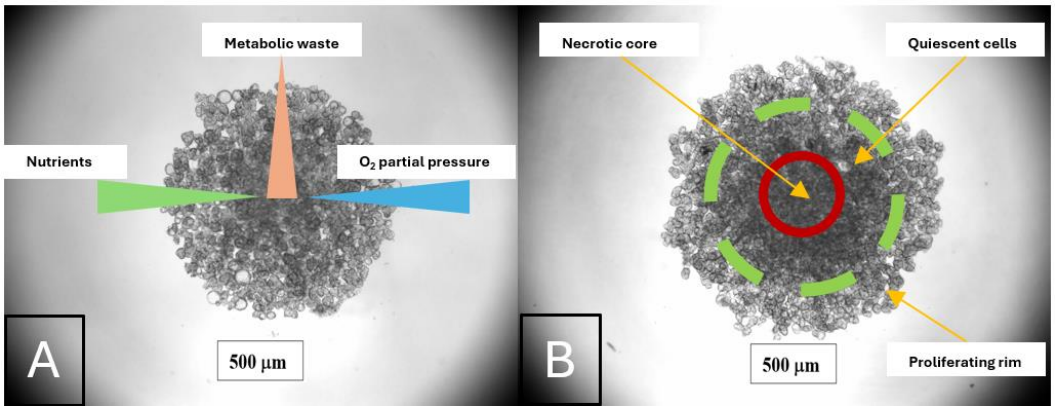

Fig. 1.1 MCF7 spheroids with schematics of various chemical gradients (A) and cell growth properties (B) (4× objective, scale bar 500 µm)(Adapted from [3, 4], with permission from Mary Ann Liebert, Inc. no. 5862260517385)

Several categories of 3D culture models exist, including tumor-derived spheroids, assembloids, and tumor-on-a-chip systems. Ex-vivo tumor tissue explants, spheroidal models, and tumorospheres provide varying degrees of complexity and insight into tumor behavior.

Techniques such as 3D bioprinting and body-on-a-chip platforms are advancing the field by allowing more accurate replication of tissue microenvironments and interactions. These techniques are especially useful for personalized medicine, drug testing, and preclinical studies, offering enhanced predictive accuracy and reducing the failure rate of drugs in clinical trials.

There are various methods for creating 3D cultures, categorized into scaffold-based, scaffold-free, and bioprinting techniques. Scaffold-based approaches use biocompatible materials to support cell growth, while scaffold-free methods promote self-attachment of cells without biomaterials. 3D bioprinting allows for the precise deposition of cells to construct tissue-like structures, providing an effective platform for drug screening and disease modeling. These models align with regulatory efforts to reduce animal testing and enhance preclinical drug development.

Imaging and analysis of 3D cultures are critical for understanding biological processes and material properties. Techniques like confocal microscopy, light sheet fluorescence microscopy, and magnetic resonance imaging are employed to analyze 3D spheroids. Automated image analysis has become essential for transforming qualitative data into quantifiable insights, improving reproducibility and efficiency. As imaging techniques advance, they allow for more accurate and detailed analysis of 3D cultures, which is crucial for biomedical research.

Currently 3D culture systems are being integrated into advanced therapeutic testing, including electroporation and electrochemotherapy. Despite progress, challenges remain, such as the need for improved systems that can rapidly produce and store large numbers of spheroids for analysis. Automation of these processes is crucial for enhancing reproducibility and reducing variability in experiments [5].

2. Electroporation and Electrochemotherapy (EP and ECT)

Electroporation is the process by which pulsed electric fields (PEF) induce structural changes in the cellular membrane, creating pathways for molecules to enter cells [4]. The plasma membrane acts as an insulating layer that selectively allows polar molecules to pass through. The interaction between electric fields and biological structures has applications in cancer treatment, gene therapy, wound healing, and bacterial disinfection. Mathematical modeling has helped define key parameters of PEF, such as amplitude, duration, shape, and repetition frequency, which allow for reversible or irreversible cell membrane permeabilization. Irreversible electroporation (IRE)

causes cell death and is used in non-thermal ablation techniques, particularly for treating lesions near critical structures [4].

In biomedical applications, reversible electroporation, commonly used in electrochemotherapy (ECT), enables the permeabilization of cell membranes, allowing anticancer drugs like bleomycin to enter and target tumor cells.

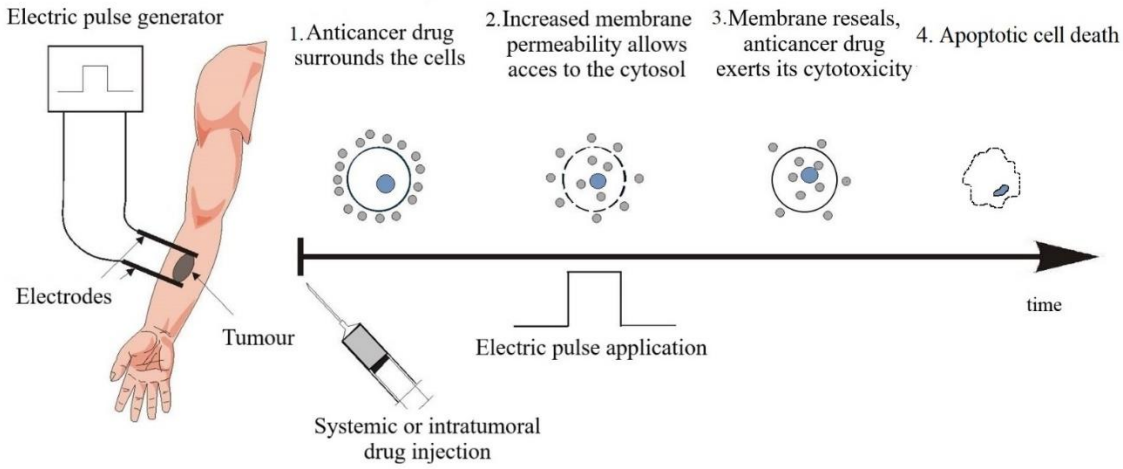

Fig. 2.1 Main steps of an ECT procedure are: i/administration by intravenous or local injection of the anticancer drug, ii/application of specific electrical pulses that facilitate drug penetration into the cell cytoplasm, iii/cell membrane resealing, iv/mitotic cell death of cancer cell (Published by G.Sersa, 2007[6], modified with permission from Elsevier, licence nr 5862110778627)

Electrochemotherapy (ECT) is a treatment combining low-dose chemotherapy and electric pulses to increase drug uptake in cancer cells. The technique relies on reversible electroporation to allow drugs to penetrate cell membranes, leading to the targeted death of cancer cells. ECT is increasingly used for treating inoperable tumors and metastases, with more than 150 hospitals worldwide implementing it in clinical practice. It offers benefits such as reduced chemotherapy dosages and minimal side effects.

ECT has also been proposed as an "in-situ vaccination" due to its ability to induce immunogenic cell death (ICD). This stimulates a systemic immune response, offering potential for combination therapies with immunotherapies such as immune checkpoint inhibitors (ICIs). Combining ECT with immunotherapy (referred to as i-ECT) is an emerging area of research, aiming to enhance local and systemic antitumor responses.

The perspectives for EP, ECT, and i-ECT in improving immunotherapy and personalized medicine are promising. The standardization of procedures for ECT continues to evolve, with a focus on minimizing invasiveness and optimizing treatment parameters. Investigating tumor characteristics and the tumor microenvironment can further improve ECT practice by identifying predictive biomarkers for treatment. Ongoing research is crucial for enhancing the effectiveness of ECT and its combination with immunotherapy, with the potential to revolutionize cancer treatment. Advances in understanding tumor biology and immune responses will support the development of personalized oncological treatments.

In conclusion, further exploration of biomarkers, tumor microenvironment, and immune responses in conjunction with ECT and immunotherapy will enhance treatment outcomes, offering new avenues for personalized cancer treatment and advancing clinical oncology.

II. PERSONAL CONTRIBUTIONS

1. Main hypothesis and general objectives

The doctoral studies consisted of five parts, with a progressive evolution as follows:

- Study 1: Obtaining viable monotypic and heterotypic spheroids from multiple cell lines for culturing with fresh human monocytes and testing the EP protocol (Ch. 5)
- Study 2: Obtaining MCF7 spheroids and test EP protocol (Ch. 6)
- Study 3: SHSY-5Y and U87 spheroids for EP and ECT protocol (Ch. 7)
- Study 4: TNF-α and IL-10 evaluations from U87 spheroids post ECT (Ch. 8)
- Study 5: Obtaining a low-cost optimized methodology for processing and testing spheroids from MCF7 and U87 cell lines (Ch. 9)

The main goal of this thesis was to develop an optimized approach for producing spheroids in ultra-low attachment environments using the liquid overlay technique. This method involved testing specific growth environments for obtaining specific types of 3D culture from several cell lines and evaluating the impact of the presence of monocytes in heterotypic 3D cultures, after exposure to electroporation (EP) and electrochemotherapy (ECT, - as applied in clinical conditions). The evaluation was conducted through semiautomated morphometric analysis of brightfield images and immunohistochemistry (IHC) analyses. Cell density, extracellular matrix

production, cytotoxic effects varied with the length of growth and type of EP and ECT exposure, thus these effects could be quantified over time and treatment responses could be defined.

Knowing that there is a cellular immune system response during ECT still under preclinical research we wished to determine any effect on spheroid growth in condition of monocyte co-culture, as determined by IL 10 and TNF alpha evaluations. Furthermore, we wished to provide an optimized low-cost protocol to batch process 48 samples in IHC thus providing an important tool in processing patient-derived samples and other cellular 3D cultures smaller than 1mm size. Firstly, we established several 3D culture personalized protocols such as handing-drop, collagen embedding and ultra-low attachment environment for several cell lines such as MCF7, U87, SHSY-5Y, A375, NIH 3T3, DC3F, CaCo2. The work is presented in Study 1: (Ch. 5)

Afterwards we defined proper growth conditions such as growth surfaces, growth medium types, time of growth and morphometric evaluations on brightfield images as we tested different electroporation and electrochemotherapy conditions known to facilitate a cellular immune response via monocyte activation. Monocyte activation was evaluated with ELISA-based testing of IL-10 and TNF-α. Finally, we developed a methodology for batch processing of multiple spheroids in immunohistochemistry.

2. General Methodology: Customized protocols for spheroids generation, image analysis and IHC processing

The study was conducted at the Center for Excellence in Biophysics and Cellular Biotechnology, Faculty of Medicine, Carol Davila University of Medicine and Pharmacy, Bucharest. All experimental procedures followed strict biohazard protocols under supervision, with no ethics approval required as the research used established cell lines from recognized cell banks like ATCC.

Cell Lines

Several cell lines were used, each with specific characteristics and growth conditions. These included non-malignant cell lines like NIH3T3 (mouse fibroblast), DC3F (hamster lung fibroblast), and Hs27 (human skin fibroblast). Malignant lines included B16F10 (mouse melanoma), A375 (human melanoma), CaCo-2 (human colon adenocarcinoma), MCF7 (human breast adenocarcinoma), SH-SY5Y (human neuroblastoma), and U87 MG (human glioblastoma). For 3D spheroid formation, two protocols were used: the hanging drop method and liquid overlay on ultra-low adherent surfaces coated with agarose.

Spheroid Growth Conditions

For the hanging drop technique, cells from 2D cultures were harvested, counted, and seeded in droplets on a petri dish for incubation up to 10 days. In the agarose coating method, 96-well plates were coated with a sterile 1% agarose solution to create a non-adherent surface for 3D culture. Cells were seeded into the wells, and the cultures were monitored for medium depletion. As for spheroid metabolic activity evaluation, comet DNA fragmentation assay, LDH metabolic assay and total protein dosage were evaluated in one spheroid culture type. At the end of the experiment, spheroids were fixed with paraformaldehyde.

Images of spheroids were captured using both upright and inverted microscopes, depending on the culturing method. ImageJ software was used for preliminary analysis, but OrganoSeg, a specialized software plugin, was employed for more accurate segmentation and analysis of spheroids over time. Various morphological and statistical parameters were computed, and data from segmented images were exported for further analysis.

Blood was collected in vacutainers, and the buffy coat was isolated and processed using gradient centrifugation and a MojoSort™ kit to isolate monocytes. These monocytes were used fresh in dedicated 3D co-culture protocols with spheroids.

Electroporation was performed using a B-Tech Cliniporator® with specific settings, including a pulse amplitude of 400V and a repetition frequency of 1 Hz. Spheroids were washed, transferred to electroporation cuvettes, and subjected to 8 rectangular bipolar pulses. After the procedure, spheroids were returned to culture plates for monitoring.

A custom mold was designed, and 3D printed to create a microwell array from agarose. This array was used to embed spheroids for batch immunohistochemistry (IHC) analysis. The microwell array was filled with spheroids, and paraformaldehyde was displaced with low-melting agarose to seal the spheroids before storage for IHC.

All data were organized using Microsoft Excel, with statistical analyses performed in Origin 8.0. Images and graphics were processed in PowerPoint for presentation and analysis.

3. Obtaining Personalized Protocols for Spheroids and Establishing Morphological Parameters for Growth Assessment

Culturing spheroids for various screening or pilot studies commonly relies on the hanging drop method or ultra-low attachment surfaces created by agarose coating. These two techniques are

cost-effective and easy to implement, promoting essential cell-to-cell and cell-to-matrix interactions. The key difference between the methods lies in spheroid viability: the hanging-drop technique limits growth to 4-6 days, while agarose coating offers longer viability.

The study's objectives were to:

- Test and optimize the hanging drop and agarose coating techniques for spheroid formation.
- Generate protocols for homotypic and heterotypic spheroids from different histological types and validate them for electroporation.
- Monitor spheroid integrity and growth using brightfield imaging and semi-automated morphometric analysis.
- Test the application of standardized metabolic and viability assays used in 2D cultures for spheroids.

Spheroid formation using the hanging drop method was tested on NIH3T3 and DC3F fibroblast cell lines, while the agarose-coated method was tested on NIH3T3, B16F10, and A375 melanoma cells. Electroporation was applied to hanging-drop spheroids of NIH3T3 and DC3F cells using a modified protocol, and effectiveness was verified by propidium iodide (PI) uptake. Several trials were conducted to compare spheroid formation and growth conditions, and metabolic activity was assessed using LDH assay.

Nine preliminary trials were conducted:

- Hanging drop spheroid formation on NIH3T3 and DC3F cells with varying droplet volumes.
- Comparison of 2% and 3% agarose-coated wells for NIH3T3 and B16F10 spheroids.
- Testing A375 spheroid formation in agarose-coated 96-well plates.
- Testing A375 spheroid formation with collagen coating and in medium containing collagen.
- Co-culturing NIH3T3 and A375 cells in various ratios to form heterotypic spheroids.
- Electroporation applied to DC3F spheroids grown in agarose-coated wells.
- Electroporation applied to MCF7 spheroids grown in agarose-coated wells.
- Viability assays, including Comet, LDH, and total protein assays, adapted for spheroid cultures.
- Testing different materials for microwell array production.

The hanging drop method yielded spheroids beyond the expected 6-day growth period, but clear apoptotic bodies were observed by day 10. The agarose coating method with 2% agarose performed better than 3% in producing spheroids that could be analyzed more easily, as 3% agarose created cracks and air bubbles. NIH3T3 spheroids formed robust structures, while

B16F10 developed looser aggregates. A375 spheroids required co-culturing with NIH3T3 fibroblasts to form stable heterotypic spheroids due to the melanoma line's inability to form extracellular matrix in low-adherent environments.

Electroporation did not significantly affect spheroid growth, and PI uptake confirmed partial permeabilization. The viability testing adapted from 2D protocols required adjustments for the 3D spheroid environment due to differences in nutrient diffusion and reagent uptake.

The hanging drop method allowed spheroid formation but offered a limited time window for growth and a laborious observation process. In contrast, the agarose coating method extended the growth period, enabling further experiments. Collagen surfaces provided compact spheroids but were more time-consuming to prepare. A minimum 5% ratio of NIH3T3 fibroblasts was necessary to form robust A375 spheroids. Electroporation protocols were optimized for spheroid cultures, and OrganoSeg software was preferred for image analysis due to its superior ability to delineate spheroids from background noise compared to ImageJ. Cell viability by LDH evaluation highlighted the inhomogeneous structure of spheroids and the presence of a necrotic area, only 54% of the cell population tested being viable, a fact also evidenced by DNA fragmentation images.

4. MCF7 Spheroids Production for Long-Term Monitoring, Electroporation, and Their Co-Culture with Monocytes

The aim of this study was to evaluate the impact of monocyte co-culture on electroporated spheroids formed from the MCF7 adenocarcinoma cell line. The specific objectives were:

- Formation of MCF7 spheroids following protocols established in Chapter 5.
- Co-culture of MCF7 spheroids with monocytes and morphological analysis of their interactions.
- Development of a protocol for electroporation (EP) of spheroids and assessing the impact of EP on the growth of monocyte-MCF7 heterotypic spheroids.

Monocytes were isolated according to the procedure described in Chapter 4.4 and maintained in sterile RPMI growth medium until seeding. MCF7 spheroids were formed using the liquid-overlay technique with low-adherent 96 U-shaped well plates coated with 1% melted agarose. MCF7 cells and monocytes were seeded at a ratio of 2:1 in 100 µL of culture medium, and medium was refreshed after 24 hours and every 5 days thereafter.

The plate was divided into four experimental groups based on the timing of monocyte addition:
MCF7 spheroids as control.
MCF7 spheroids with monocytes added simultaneously.
MCF7 spheroids with monocytes added at 24 hours.
MCF7 spheroids with monocytes added at 48 hours.
For electroporation experiments, MCF7 cells were seeded with monocytes in the same ratio, and electroporation was performed on day 21 of growth following the protocol from Chapter 4.4. Post-electroporation, the spheroids were transferred to new plates and monitored.

Protocols for forming both short-lived and long-lived heterotypic spheroids from MCF7 cells were successfully established. Monocytes maintained their characteristics throughout the experiment, with no significant morphological changes. The growth of heterotypic spheroids (MCF7+monocytes) exhibited variations depending on the timing of monocyte addition, with the most pronounced inhibitory effect observed when monocytes were added simultaneously with MCF7 cells.

When monocytes were introduced at different time points, spheroid growth dynamics differed. The simultaneous addition of monocytes led to an initial stabilization in spheroid size for the first four days, followed by a steady increase. Monocyte introduction at 24 and 48 hours resulted in spheroid growth similar to the control group until day four, after which a slower increase in size was observed.

A 30-day long-term culture of MCF7+monocytes spheroids was successfully maintained, and electroporation applied on day 21 caused a transient decrease in spheroid area and volume. However, growth kinetics post-EP were similar to control groups, suggesting that EP alone does not significantly alter long-term growth in the absence of chemotherapy.

Monocyte co-culture influenced the growth dynamics of MCF7 spheroids, with the timing of monocyte introduction playing a crucial role. Monocytes appeared to inhibit MCF7 cell proliferation, with the most pronounced effect observed when monocytes were added at the beginning of spheroid formation.

Electroporation caused a temporary decrease in spheroid size, likely due to the mechanical stress and membrane permeabilization induced by the electric pulses. Despite this transient effect, spheroid growth resumed similarly to the control group after EP. Future studies will explore the impact of EP in the presence of chemotherapeutic agents to better understand tumor-immune system interactions and enhance electrochemotherapy (ECT) efficacy.

This study successfully developed protocols for isolating monocytes, forming heterotypic MCF7 spheroids, and applying electroporation without concurrent chemotherapy. The results showed that monocyte presence influences spheroid growth, and EP induces a temporary inhibition of spheroid size.

5. Electrochemotherapy on Spheroids from SHSY5Y and U87 Co-Cultured with Monocytes

The aim of this study was to develop and validate a 3D spheroid/monocyte co-culture model to assess the effects of ECT on neuroblastoma (SHSY-5Y) and glioblastoma (U87) cell lines, and to explore whether ECT induces monocyte polarization into an anti-tumoral M1 phenotype.

SHSY-5Y spheroids were formed on agarose-coated 96-well plates and electroporated on day 15. ECT was performed with 8 bipolar rectangular pulses, using bleomycin, cisplatin, or temozolomide as cytotoxic agents. Monocytes were added after electroporation and spheroid growth was monitored by brightfield microscopy and analyzed with OrganoSeg software. The same process was followed for U87 spheroids, electroporated on day 9.

Spheroid growth was monitored across 10 time points over 16 days. Experiments were performed in triplicate, and volumetric analysis was conducted to evaluate growth rates post-EP and ECT.

A reliable co-culture model was established, and spheroid growth was significantly impacted by ECT. Bleomycin alone did not inhibit SHSY5Y spheroids, but its effect was strongly enhanced by EP. Temozolomide and cisplatin had strong inhibitory effects on their own. The presence of monocytes slightly enhanced the cytotoxic effect of bleomycin but had no significant effect on cisplatin or temozolomide treatment.

Electroporation increased bleomycin's efficacy by permeabilizing the cell membrane, confirming that EP enhances the effectiveness of low-permeability chemotherapeutics. Monocytes did not significantly impact spheroid growth after ECT with any of the three drugs, but there was a consistent trend of reduced growth in the presence of monocytes.

U87 spheroids responded similarly to SHSY5Y spheroids. EP alone had little effect on spheroid viability, confirming that the applied electrical pulses did not reach the threshold for irreversible electroporation. Co-culturing spheroids with monocytes also had minimal impact on growth. However, when EP and monocytes were combined, spheroid size decreased significantly, indicating enhanced cytotoxic effects.

For spheroids exposed only to chemotherapy, cisplatin had the greatest effect, followed by bleomycin and temozolomide. When chemotherapy was combined with monocytes, a slight enhancement in cell killing was observed for cisplatin and bleomycin, but not for temozolomide. The addition of monocytes had no significant effect when combined with ECT.

Overall, EP combined with bleomycin or cisplatin led to more pronounced cell death, with monocytes slightly enhancing this effect. Temozolomide, however, remained unaffected by the presence of monocytes.

This study demonstrated that EP could enhance the cytotoxicity of chemotherapeutics, particularly those with low permeability, such as bleomycin. Monocytes slightly enhanced the cytotoxic effects of ECT, but their impact was more evident when combined with EP alone. These results suggest that EP may trigger monocyte polarization into an anti-tumoral phenotype, augmenting the immunogenic response.

In U87 spheroids, EP combined with monocytes led to significant cell death, matching the efficacy of ECT protocols. This suggests that monocytes may contribute to an enhanced immune response post-EP, likely due to EP-induced release of damage-associated molecular patterns (DAMPs). Further research is needed to clarify how EP induces immunogenic cell death and whether these findings can be translated into clinical models.

This study successfully developed and validated two co-culture models for studying ECT and immune-cell interactions in SHSY5Y and U87 spheroids. EP enhanced the cytotoxicity of low-permeability drugs like bleomycin, and monocytes slightly augmented cell killing effects. However, when EP was combined with monocytes alone, it matched the efficacy of ECT protocols, suggesting a possible role for EP in monocyte polarization into an anti-tumoral phenotype.

These findings provide a foundation for further investigation into EP-based therapies, which could become a promising alternative for treating aggressive cancers by leveraging immune system interactions and enhancing drug delivery.

6. TNF – α and Interleukin 10 evaluation on U87 spheroids co-cultured with monocytes after ECT treatment

There is an increased interest in the evaluation of immune response involvement in the clinical outcome of ECT [46]. In the present study U87 glioblastoma spheroids were obtained, treated with ECT and further co-cultured with fresh human monocytes. We evaluated the IL-10 and TNF-

alpha levels as a response of naïve resident monocytes to the exposure of spheroids to ECT with either temozolomide (TMZ) or cisplatin (CIS).

U87 human glioblastoma spheroids were created on agarose-coated low adherent 96 well plates (see Ch. 4.2). The spheroids were electroporated on day 7 from seeding, with standard ECT pulses (see Ch. 4.5). Electroporated spheroids were pipetted on new wells, containing fresh human monocytes (see Ch.4.4).

Spheroids were exposed either to CIS or TMZ as follows: i) U87 spheroids in culture medium; ii) U87 spheroids with monocytes; iii) U87 spheroids exposed to EP alone; iv) U87 spheroids exposed to EP then cultured on monocyte layer

TNF-α evaluated from co-culture growth medium 5 hours after ECT and again at 7 days post treatment using quantitative sandwich ELISA (TNF alpha Abcam 181421).

The spheroids growth rate was computed as an area-based index using Organoseg software(4)

An optimized protocol of growth evaluation for U87 glioblastoma spheroids exposed to ECT and monocytes has been obtained (Fig. 8.1).

In the case of temozolomide (TMZ) treated samples, TNF-α level at 5h (Fig. 8.2) was similar in samples exposed only to TMZ, samples exposed to TMZ that grown afterwards with monocytes, and samples exposed to EP with TMZ. However, values of TNF-α at 5h increased significantly in the case of U87 spheroids exposed to their combination: TMZ +EP + monocytes. TNF-α at 7 days was also increased for the samples containing monocytes (with and without EP. The combination EP + monocytes did not further increase the levels of TNF-α with respect to monocytes only.

In the case of cisplatin (CIS) treated samples either EP or monocytes increased the levels of TNF-α for both 5h and 7days. When EP treated samples grown in the presence of monocytes, the TNF-α levels increased only at 7days (Fig. 8.2).

Interleukin-10 evaluation shown no significant production values in the experimental categories (data shown in Appendix 7).

TNF-α role in cancer progression is still a matter of debate as the physiological intratumor TNF-α levels are insufficient to induce cancer regression (as known from high TNF-α dose tumor treatments). There is growing evidence that TNF-□ increases the chance for immuno-avoidance and tumor angiogenesis, thus promoting cancer progression. Combinations of TNF-α blockers with current immunotherapies could improve the overall outcome [85].

The behavior of TNF-α increase was slightly different with respect to the drug used for ECT: more pronounced for CIS at both time measurements, and present only at 7 days for TMZ.
The lack of IL-10 detection allows to reasonably propose that the applied experimental conditions directed the initial naïve monocytes to an inflammatory status (M1, characterized by TNF-α production) and not towards an anti-inflammatory/tumor-tolerant status (M2, characterized by IL-10 production). These results could partially explain the results shown in Ch. 7, Fig. 7.11 regarding the EP-triggered cytotoxicity of monocytes in the U87 spheroids.
Monocytes addition in the U87 spheroids environment, after the spheroid exposure to EP and chemotherapy, determined and statistically significant increase in the levels of TNF-α at day 7 in both treatment groups, with the highest TNF-α values at day 7 found in the case of U87 spheroids exposed both to CIS treatment and monocyte co-culture after EP.

7. Cost-Effective Optimized Method to Process 3D Spheroids in Microwell Arrays for Immunohistochemistry Analysis

In the context of regulatory updates by the FDA, 3D cellular and computer models are prioritized as alternatives to animal testing for drug safety and effectiveness assessments, in line with the 3Rs of animal experiments (Replacement, Reduction, and Refinement). 3D models improve the predictive accuracy of preclinical testing, offering better insights into how cancer cells respond to treatments. Immunohistochemistry (IHC) remains the gold standard for histological evaluation, especially when analyzing specific areas within spheroids. However, there are challenges, such as antibody penetration and fluorophore spectrum overlap, in achieving accurate imaging. This paper proposes an optimized procedure for embedding spheroids in agarose microwell arrays for IHC analysis, tested on U87 glioblastoma and MCF7 adenocarcinoma spheroids. The process allows for efficient batch analysis of 3D cultured spheroids while maintaining sample traceability.
Spheroids from U87, MCF7, and CaCo2 cell lines were cultured following protocols described previously. After culturing, the spheroids were embedded in microwell arrays, processed using tissue processing machines, and cut into 3 μm sections for IHC staining. Standard staining procedures were used for various markers, including EMA, p53, Ki-67, IDH, and ARID1A for U87 spheroids, and ER, PR, HER2, and Ki-67 for MCF7 spheroids.

Hematoxylin and Eosin (H&E) staining confirmed the presence and integrity of the spheroids. The IHC staining was automated using BenchMark GX IHC/ISH.

The workflow for processing spheroids for IHC analysis is depicted in Fig. 9.1, showing the steps from 2D cell culture to IHC analysis. The growth rates, compactness, and stability of spheroids varied by cell type. Monitoring with OrganoSeg allowed precise timing for spheroid collection for IHC processing.

U87 spheroids were compact and stable, while MCF7 spheroids were less stable and required the addition of Hs27 fibroblasts to improve structural integrity. Volumetric analysis of U87 spheroids showed consistent growth, with spheroids remaining stable even after pipetting. In contrast, MCF7 spheroids were more fragile but became more stable when co-cultured with fibroblasts, as shown in Fig. 9.3.

H&E staining of U87 spheroids revealed anaplastic cells with pleomorphic nuclei and rare mitotic figures. Necrosis was observed in spheroids with diameters greater than 400 µm. IHC analysis showed that U87 spheroids were negative for EMA but positive for Ki-67 and ARID1A, confirming high proliferation rates and apoptotic areas. MCF7 spheroids co-cultured with Hs27 fibroblasts exhibited robust structural integrity, with strong ER and PR expression and Ki-67 positivity, confirming the tumor's luminal B-like Her2-negative classification.

CaCo2 spheroids were successfully processed for IHC, with positive Ki-67 staining but negative p53 results.

This study successfully established a method for embedding 3D spheroids in agarose microwell arrays for IHC analysis. U87 spheroids were compact and mechanically stable, allowing them to be processed without additional cell types. MCF7 spheroids required the addition of fibroblasts for structural stability during processing.

The method's advantages include the ability to process large batches of spheroids efficiently, ensuring traceability during IHC analysis. Spheroids larger than 400 µm developed necrotic cores, a well-known limitation due to oxygen diffusion constraints.

The agarose microwell arrays provided a convenient method for embedding and processing spheroids, allowing high-throughput analysis. However, challenges remain, including the need for automation to streamline spheroid transfer and image analysis. The method demonstrated here allows for flexible experimental setups, enabling histological and molecular studies across multiple experimental conditions.

The proposed method offers a simple, cost-effective, and efficient workflow for processing 3D spheroids for IHC analysis. It supports high-throughput production of homotypic and heterotypic spheroids from various cell lines, which can be sectioned and analyzed for protein expression while maintaining sample traceability. The method provides a valuable tool for cancer research and drug screening, particularly for histological and microenvironment-related studies.

8. Conclusion

During the doctoral studies, it was demonstrated that it is possible to optimize the generation of multiple types of 3D cell cultures on ultra-low adhesion surfaces. This allowed testing the effects of exposing spheroids to electrochemotherapy conditions and re-cultivating them in the presence of monocytes. The results obtained were evaluated through optical microscopy and semi-automated morphometric processing, using optimized metabolic viability methods tailored to the specific microenvironment conditions of 3D cell cultures, as well as immunohistochemistry evaluation. An innovative method was proposed for the simultaneous evaluation of 48 samples. The experiments conducted using the methods described in Chapter 3 led to the following results:

Study one (Ch. 5.5):

- Protocols for forming monotypic and heterotypic spheroids (NIH3T3, A375, B16F10, DC3F, MCF7) were successfully established.
- Agarose-coated wells were selected for their ability to extend spheroid growth times.
- Electroporation protocols were optimized for spheroids, and OrganoSeg software was chosen for superior image analysis over ImageJ.
- A protocol for embedding spheroids in agarose for H&E staining was successfully adapted.

2. **Study two (Ch. 6.5**):

- Protocols for monocyte isolation and co-culture with MCF7 spheroids were successfully developed.
- Electroporation led to a temporary growth inhibition in co-cultures.
- Future research will focus on how monocyte polarization (M1/M2) affects tumor growth and electrochemotherapy (ECT) efficacy.

3. In **study three (Ch. 7.4**), in order to characterize the effect of electrochemotherapy and the addition of monocytes in the culture medium, on the general growth rate, it was possible to demonstrate that:

- Electroporation and cytotoxic drug protocols significantly reduced tumor spheroid viability.
- Monocytes had a synergistic effect with bleomycin ECT but not with cisplatin or temozolomide.
- Electroporation may induce monocyte polarization into an anti-tumoral state (M1), increasing cell killing.
- Two co-culture models for studying immune cell interactions in ECT were successfully optimized, with further research needed on the mechanisms behind these effects.

4. **Study four (Ch. 8.5)**

- Monocytes added to U87 spheroids after electroporation and chemotherapy significantly increased TNF-α levels by day 7, particularly in the CIS-treated group.

5.**Study five (Ch. 9.5)** proposed an optimized, cost-effective method for immunohistochemistry (IHC) analysis of simultaneous spheroids that decreases both the time and costs associated with these evaluations, yielding essential scientific results more rapidly:

- A well-defined workflow was optimized for generating homotypic and heterotypic spheroids for immunohistochemistry (IHC) analysis.
- The spheroids were simultaneously embedded in an agarose microwell array and analyzed using semi-automated software, maintaining sample traceability during IHC staining and analysis.

Also in this study, a semi-automated protocol was developed for generating a morphometric profile of spheroids based on images obtained through transmission optical microscopy. This protocol can be used with any set of input images. Future improvements could allow for the direct calculation of the relevant parameters of the studied spheroids.

II.1. Personal contributions

Published articles:

- **Mircea Bogdan Matei**, et. al., FOCUSED REVIEW OF ELECTROCHEMOTHERAPY. Fiziologia - Physiology, 2024. 1(106): p. 63-70.

- **Mircea Bogdan Matei** et. al., PROSPECTS ON USING MONOCYTES ENRICHED MCF7 SPHEROIDS FOR ELECTROPORATION. Fiziologia - Physiology, 2024. 2(107).(accepted)
- **Mircea Bogdan Matei** et. al., Cost-effective optimized method to process 3D tumoral spheroids in microwell arrays for immunohistochemistry analysis. Journal of Medicine and Life, 2024. (accepted)
- Cucu, C. I.; **Matei, B. M**.; Giurcaneanu, C.; Popa, L. G.; Orzan, O. A.; Beiu, C.; Holban, A. M. Grumezescu, A. M.; Popescu, M. N.; Caruntu, C.; Mihai, M. M. - Electrochemotherapy and Other Clinical Applications of Electroporation for the Targeted Therapy of Metastatic Melanoma. Materials, 2021. 14(14).

Short communications:

- 3D Cellular Model for Evaluation of Electrochemotherapy Immune System Interactions: An Experimental Approach. **Mircea Bogdan Matei**, Christien Oktaviani Matei, Sibel Ali, Artsiom Klimko, Mihaela-Georgeta Moisescu, at 16th National Conference of Biophysics(CNB 2020);T5P5, 14 - 16 June 2020, Brasov, Romania
- Evaluation of Electrochemotherapy Efficacy on a 3D Spheroid Neuroblastoma/Monocyte Co-Culture Model. Artsiom Klimko, **Mircea Bogdan Matei**, Christien Oktaviani Matei, Tudor Savopol, Mihaela G. Moisescu. XXVIth International Symposium on Bioelectrochemistry and Bioenergetics of the Bioelectrochemical Society 10-15th of May 2021, Cluj-Napoca, Romania [abs210118].

Posters (international):

- 2022, 3rd World Congress on Electroporation– ISEBTT (International Society for Electroporation-Based Technologies and Treatments) 9-13 October 2022, Copenhagen, Denmark
  - Evaluation of TNF-α production due to electrochemotherapy applied on glioblastoma spheroids co-cultured with monocytes, **Mircea Bogdan Matei**, Mihaela G. Moisescu, Christien Oktaviani Matei, PO 18, page 18, ISBN 978-961-243-444-1
- 2019, 3rd World Congress on Electroporation– ISEBTT (International Society for Electroporation-Based Technologies and Treatments) 3-6 September 2019, Toulouse, France
  - Case report: First patient with skin melanoma metastases treated by bleomycin electrochemotherapy in Romania. Valentin Popescu, **Bogdan M. Matei**, Bogdan S.

Mastalier, Dan Andras, Alexandru Radulian, Valeriu Botea, Mihaela G. Moisescu, PO 059, page 204, ISBN 978-2-913923-38-6

- Teaching activity of subjects within the doctoral thesis:

since 2021 – teaching a seminar within the Master's Degree Biophysics and Cellular Biotechnology: "Immunomagnetic Cell Selection", in D3- Separation Methods (Year 1, sem II)

- Coordinator of 2 thesis in Medicine:
  - 2021, “In vitro evaluation of electrochemotherapy on a three-dimensional multicellular spheroid glioblastoma/monocyte co-culture model”, absolvent Artsiom Klimko
  - 2020, "Evaluation of the interaction of monocytes with 3D tumor structures - in vitro study", graduate Sibel Ali
- Coordinator of 3 dissertation theses within the Master's Degree Biophysics and Cellular Biotechnology:

- 2024, IHC Evaluation of 3D Caco2 and NIH3T3 Cell Cultures, graduate Vâlcu Claudia-Adelina
- 2022, Feasibility of the immunohistochemistry method in the characterization of three-dimensional cultures of U87 MG and MCF-7, graduate Marinescu Carmen Letitia
- 2022, Morphological and immunophenotypic changes in 3D glioblastoma cell cultures U87 MG, graduate Andreea-Cristina RĂDULESCU

- Project applications as Project coordinator:

- EANS RESEARCH FUND 2020 -In vitro evaluation of electroporation-based techniques effects on a three-dimensional spheroid glioblastoma/monocyte co-culture model (EP-3D-GM)
- EUROMEDEX 2022 - Interleukin evaluation of electrochemotherapy-induced response on a 3D culture of U87 glioblastoma-monocyte model

- Projects applications as project member:

- UEFISCDI PED application - IMUNECT (2021)
- UEFISCDI PED application - IMUNECT (2024)

8.2 Perspectives:

1. **Development of electroporation methods for 3D cultures:**

- creating an in-situ electroporation method (well-based) by printing a system of electrodes and wells and automating the generation of parametric results obtained from image acquisitions.

2. **Development of the co-culture system for spheroids with immune cells and evaluating a possible abscopal effect:**

- evaluating IL-10, TNF-α, and other cytokines under specific conditions for generating M1 and M2 monocyte types, as well as investigating the direct effect of electroporation on glioblastoma spheroids in the presence of monocytes.

3. **Expansion of the ECT field:**

-publishing a manuscript as the first author on the first patient treated with electrochemotherapy (ECT) in Romania and creating a national registry of patients treated with ECT (as an objective of a project submitted to UEFISCDI – "IMUNECT", currently under evaluation).

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2. **Mircea Bogdan Matei**, C.O.M., Ali Sibel, Leon Zăgrean, Mihaela Georgeta Moisescu, *PROSPECTS ON USING MONOCYTES ENRICHED MCF-7 SPHEROIDS FOR ELECTROPORATION.* Fiziologia - Physiology, 2024. **2**(107).
3. Ware, M.J., et al., *Generation of Homogenous Three-Dimensional Pancreatic Cancer Cell Spheroids Using an Improved Hanging Drop Technique.* Tissue Eng Part C Methods, 2016. **22**(4): p. 312-21.
4. **Mircea Bogdan Matei**, C.O.M., Ali Sibel, Leon Zăgrean, Mihaela Georgeta Moisescu, *FOCUSED REVIEW OF ELECTROCHEMOTHERAPY.* Fiziologia - Physiology, 2024. **1**(106): p. 63-70.
5. **Mircea Bogdan Matei**, C.L.M., Christien Oktaviani Matei, Alex-Sebastian Pînzariu, Leon Zăgrean, Mihaela Georgeta Moisescu, *Cost-effective optimized method to process 3D tumoral spheroids in microwell arrays for immunohistochemistry analysis.* J Med Life, 2024(6 (Accepted)).
6. Sersa, G., et al., *Electrochemotherapy in treatment of tumours.* Eur J Surg Oncol, 2008. **34**(2): p. 232-40.