BABEȘ-BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

Summary

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Scientific co-supervisor:
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"Iuliu Hațieganu"

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Therapeutic Approaches of Osteoarthritis Based on Oligonucleotide Delivery Systems

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TABLE OF CONTENTS OF THESIS

KEYWORDS

Osteoarthritis, cartilage degeneration, inflammation, oxidative stress, supramolecular systems, quality-by-design, design of experiments, hyaluronic acid hydrogels, disulfide crosslinking, polyamidoamine nanoparticles, *in vitro* evaluation, cationic liposomes.

OUTLINE OF THE THESIS

1. Osteoarthritis Pathophysiology

Osteoarthritis (OA) is a degenerative joint disease characterized by the breakdown of cartilage, leading to pain, stiffness, and impaired movement. Traditional treatments often focus on symptom management rather than addressing the underlying cartilage damage. Recent advancements in biomedical engineering and regenerative medicine have introduced novel methods to repair chondrocytes, the cells responsible for maintaining cartilage. One promising approach involves substituting damaged cartilage with engineered tissues or biomaterials. These substitutes can provide a supportive environment for chondrocytes, promoting their repair and regeneration. This approach not only aims to restore the structural integrity of the cartilage but also to enhance its functional properties, offering a more effective long-term solution for OA patients.

In addition to tissue substitution, these innovative methods can facilitate the targeted delivery of therapeutic agents directly to the damaged cartilage. Utilizing advanced drug delivery systems, such as hydrogels and nanoparticles, allows for the controlled release of drugs over an extended period. This approach ensures a higher concentration of the therapeutic agent at the site of injury while minimizing systemic side effects. Thus, hyaluronic acid (HA) hydrogels are used not only for their lubricating properties but also as carriers for cells, anti-inflammatory drugs and growth factors. This dual functionality can simultaneously alleviate pain and stimulate the repair processes within the joint, addressing both symptoms and underlying causes of OA.

Furthermore, the integration of genetic material delivery, such as messenger RNA (mRNA) and small interfering RNA (siRNA), into these delivery systems hold significant potential for cartilage repair in OA. This potential depends on several key properties of the genetic material and their stability, hence it is critical to prevent their degradation by nucleases, requiring protective delivery systems. Efficient delivery to cartilage tissue is essential, requiring systems capable of penetrating the extracellular matrix. Targeting specificity ensures that the genetic material reaches the appropriate cells while minimizing off-target effects. Additionally, low immunogenicity is vital to avoid unwanted immune responses. Controlled release mechanisms can provide sustained

therapeutic effects, and the genetic material must exhibit effective gene silencing or expression to modulate the molecular pathways involved in cartilage repair. Among all genetic material delivery systems, polyamidoamine (PAA) nanoparticles and liposomes are two advanced delivery vehicles being explored for this purpose. PAA nanoparticles can encapsulate genetic material like the mRNA and ensure its stability and targeted release within chondrocytes. Cationic liposomes can efficiently deliver genetic material like the siRNA by merging with the cell membrane and releasing their payload into the cell. By delivering these genetic materials directly to the chondrocytes, it is possible to modulate their behavior at the molecular level, enhancing their capacity to repair and maintain healthy cartilage. This targeted genetic therapy represents a cutting-edge approach, potentially transforming the management of OA and paving the way for personalized and highly effective treatments. Together, these novel methods offer hope for mitigating the symptoms of Osteoarthritis and fundamentally altering the progression of the disease. Therefore, the research proposed in this doctoral thesis aimed to:

i. Develop and optimize different delivery systems through the Quality-by-design (QbD) approach to efficiently transport drugs and genetic material to chondrocytes.

ii. Assess the therapeutic efficacy and biocompatibility of these delivery systems in *in vitro* experimental models.

The present thesis consists of 5 chapters that are presented briefly below.

Chapter I: Introduction and Overview

This chapter provides an overview of the pathophysiology of Osteoarthritis, factors inducing Osteoarthritis, the limitations of current treatment options, and the potential of supramolecular systems for drug and gene delivery in Osteoarthritis. It sets the stage for the subsequent chapters by highlighting the importance of innovative delivery systems for effective OA treatment.

Chapter II: Development and Optimization of Hyaluronic Acid-Derived Hydrogels as supplementation for the damaged cartilage

This chapter discusses the development and optimization of hyaluronic acid-derived hydrogels using the QbD approach. It focuses on enhancing the rheological properties of hydrogels to adequate lubrication and shock absorption, thereby reducing pain and increasing joint function. It is biocompatible and could be used for efficient drug or gene delivery within the joint for prolonged residence time. The chapter details the materials used, crosslinking procedure, optimization, and characterization techniques employed to achieve the desired hydrogel properties suitable for tissue engineering.

Chapter III: Development and Optimization of Polyamidoamine-Based Nanoparticles for mRNA delivery

This chapter presents the formulation and optimization of polyamidoamine-based nanoparticles for the efficient delivery of mRNA to chondrocyte and Jurkat cell lines. It emphasizes achieving better cell viability and transfection efficiency for the proprietary polyamidoamine nanoparticles by employing the QbD approach. The chapter covers the selection of polyamidoamine type, the ratio of polymer to genetic material, the concentration of nanoparticles, and *in vitro* evaluations of cell viability and transfection levels. This study shows the employment of the QbD approach for the efficient screening of formulation variables and maximizing the therapeutic responses.

Chapter IV: Development and Optimization of Co-Loaded Lipoplexes with siRNA and curcumin

This chapter explores the development and optimization of lipoplexes co-loaded with therapeutic small molecules like curcumin along with genetic material like siRNA for repairing chondrocytes in osteoarthritic conditions through the QbD approach. It includes the in-detail QbD approach, as in previous chapters, along with preparation methods of liposomes and lipoplexes, physicochemical characterization, *in vitro* cell testing, and optimization. The optimum formulation of liposomes underwent characterization and was also tested for stability and release profile.

This chapter assesses the therapeutic efficacy of co-loaded lipoplexes in *in vitro* models of inflamed and oxidative-stressed chondrocytes. It examines the anti-inflammatory and antioxidative effects of the optimal formulations, as well as their ability to promote chondrocyte survival and function under pathological conditions.

Chapter V: Conclusions and Future Directions

This chapter summarizes the key findings from the thesis, highlighting the different approaches explored to repair chondrocytes and prevent the progression of Osteoarthritis. It discusses the implications of the research for future therapeutic strategies and suggests potential avenues for further investigation.

CHAPTER I
INTRODUCTION

Osteoarthritis (OA) is a chronic and progressive joint disorder that significantly impacts the quality of life for over 250 million people worldwide. It primarily affects older adults, but its incidence is rising among younger individuals due to factors such as obesity, sports injuries, and genetic predispositions. OA is characterized by the gradual degradation of articular cartilage, which covers the ends of bones in joints. This cartilage is essential for providing a smooth, lubricated surface for joint movement and load transmission with minimal friction. In OA, this balance is disrupted, leading to increased degradation of extracellular matrix (ECM) components like type II collagen and proteoglycans.

The pathophysiology of OA involves a complex interplay of mechanical, biochemical, and genetic factors. Mechanically, abnormal joint loading due to malalignment or trauma can initiate cartilage breakdown. Biochemically, chondrocytes in OA-affected cartilage produce enzymes such as matrix metalloproteinases (MMPs) that degrade collagen and aggrecan, essential components of the ECM. Inflammatory cytokines like interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) exacerbate this degradation. Genetically, specific polymorphisms increase susceptibility to OA by affecting ECM components and inflammatory pathways.

Several risk factors contribute to OA development. Age is one of the most significant risk factors, with the prevalence of OA increasing as individuals grow older due to cumulative wear and tear on joints and a decline in cartilage regeneration capacity. Genetic predisposition also plays a crucial role in OA susceptibility, with certain genes affecting cartilage structure and metabolism, increasing OA risk. Mechanical stress from joint injuries or repetitive use can damage cartilage, while obesity increases stress on weight-bearing joints like the knees and hips. Anatomic abnormalities such as joint malalignment or dysplasia lead to uneven stress distribution on cartilage, accelerating degeneration. Metabolic factors like diabetes contribute to systemic inflammation that accelerates cartilage breakdown.

Current treatments for OA focus on symptom relief rather than disease modification. Medications like NSAIDs provide temporary pain relief but carry risks such as gastrointestinal or cardiovascular complications. Physical therapy and weight management help maintain joint function but do not reverse cartilage loss. Surgical interventions like joint replacement can provide significant pain relief but involve risks such as infection or prosthesis failure. These limitations highlight the need for treatments targeting the underlying causes of OA.

Emerging therapies aim to address these limitations by focusing on regenerative medicine and advanced drug delivery systems. Supramolecular systems are at the forefront of gene therapy,

offering potential solutions for OA treatment by enabling targeted delivery and sustained release of therapeutic agents. These systems particularly, nanoparticles, liposomes, and hydrogels are designed for the precise delivery of genetic material or drugs and provide several advantages in gene therapy and cartilage repair. They protect genetic material from degradation in biological environments and can be functionalized with targeting ligands to enhance specificity for affected tissues. They can also be engineered for controlled or stimuli-responsive release, ensuring delivery in response to biological signals.

Hydrogels mimic natural cartilage properties and can deliver drugs or stem cells directly to joints, promoting tissue regeneration. Polymeric nanoparticles deliver drugs or genes to specific joint tissues, reducing inflammation and stimulating repair. Liposomes provide a biocompatible platform for delivering both drugs and genetic material, enhancing their effectiveness while minimizing systemic exposure.

Quality-by-Design (QbD) principles are applied to ensure these systems meet predefined quality standards through systematic design, optimization, and control of critical quality attributes (CQAs). This approach ensures that gene therapy delivery systems are designed and produced to meet high standards of efficacy, safety, and stability.

In conclusion, the integration of advanced supramolecular systems into OA treatment represents a significant advancement in overcoming current therapy limitations. These innovative systems facilitate targeted delivery, sustained release, and enhanced regenerative effects, offering a comprehensive approach to OA management. As research continues, these technologies hold the potential to transform OA treatment by improving patient outcomes through more effective therapies that address the disease's underlying causes. By integrating QbD principles into their development, these therapies are poised to offer more reliable and effective solutions tailored to individual patient needs.

THE AIMS AND OBJECTIVES OF THE THESIS

AIM OF THE THESIS

The primary aim of this thesis is the development and optimization of complementary supramolecular delivery systems for the treatment of Osteoarthritis (OA). The research focuses on the systematic design, optimization, and evaluation of hyaluronic acid-based hydrogel matrices, polymeric nanoparticles, and liposomal formulations to enhance the efficacy and safety of therapeutic agents for the treatment of OA.

OBJECTIVES

1. To optimize delivery systems via Quality-by-design (QbD):

This objective involves the systematic optimization of hydrogels, polymeric nanoparticles, and liposomal formulations using QbD strategies, ensuring the safety and efficacy of the delivered therapeutic agents.

2. To evaluate the biological efficacy of the optimized delivery systems through *in vitro* studies:

This objective focuses on testing the biological performance of the optimized hydrogels, nanoparticles, and liposomes *in vitro* to assess their therapeutic potential for OA.

CHAPTER II

A quality-by-design approach to optimize disulfide-linked hyaluronic acid (HA) hydrogels

This study explores the development of disulfide-linked hyaluronic acid (HA) hydrogels, aiming to provide an innovative scaffold for tissue engineering, particularly for the treatment of joint diseases such as Osteoarthritis (OA). OA, characterized by cartilage degradation and chronic inflammation, remains a significant medical challenge, with current therapies offering limited regenerative benefits. In this context, HA hydrogels are promising due to their biocompatibility, water retention capacity, and ability to mimic the extracellular matrix (ECM). However, uncrosslinked HA suffers from poor mechanical stability and is prone to enzymatic degradation. To address these challenges, the present work utilized a Quality by Design (QbD) approach to systematically optimize the synthesis and properties of disulfide-linked HA hydrogels. By focusing on the critical factors influencing the gel's stability, mechanical performance, and biocompatibility, the study demonstrated the effectiveness of QbD in engineering biomaterials with tailored properties for biomedical applications.

The initial phase of the research involved modifying HA by integrating cysteine moieties into its molecular backbone. This modification enabled the formation of disulfide crosslinks at physiological pH, ensuring in situ gelation under minimally invasive conditions. The inclusion of these thiol groups addressed the inherent limitations of uncrosslinked HA, enhancing the hydrogel's structural integrity and resistance to enzymatic degradation. A QbD framework was implemented to achieve precise control over the hydrogel's properties. The development process began with defining a Quality Target Product Profile (QTPP), which outlined key performance parameters such as gelation time, mechanical properties, and cytocompatibility. For intra-articular

applications, the target attributes included a storage modulus (G′) above 2000 Pa, ensuring sufficient elasticity to cushion joints and cell viability exceeding 80%, reflecting the biocompatibility essential for tissue regeneration.

Critical Quality Attributes (CQAs) were identified to ensure the hydrogel's optimal performance. These included the degree of modification (DM), which directly influenced crosslinking density and rheological properties such as storage and loss moduli (G′ and G″). Critical Material Attributes (CMAs), such as the molecular weight of HA, and Critical Process Parameters (CPPs), including the molar ratio of coupling reagents, were also considered. A Failure Modes and Effects Analysis (FMEA) was employed to assess risks during synthesis, identifying variables with the highest potential to impact the hydrogel's quality. This systematic evaluation guided the optimization of synthesis conditions, ensuring reproducibility and robustness in the final product. To refine these variables, a Design of Experiments (DoE) approach was adopted, focusing on the interaction between HA molecular weight, coupling reagent concentration, and their combined effects on the hydrogel's CQAs.

The optimization process revealed that higher molecular weight HA (200 kDa) and a controlled coupling reagent ratio (0.2 mmol equivalent to HA) were critical for achieving desirable hydrogel characteristics. The final formulation exhibited a degree of modification of 12%, a storage modulus of 2321 Pa, and a loss modulus of 15 Pa. These mechanical properties are crucial for load-bearing applications, as they ensure elasticity without compromising structural stability. Rheological testing also highlighted the hydrogel's ability to withstand physiological stresses while maintaining its functional integrity. Notably, the hydrogel exhibited in situ gelation under physiological conditions within 50-60 minutes, facilitated by the dynamic covalent disulfide bonds. This feature makes the hydrogel particularly suitable for minimally invasive applications, as it allows for localized gelation following intra-articular injection.

The biocompatibility of the hydrogel was thoroughly evaluated using a chondrocyte cell line (C28/I2), which served as a model for cartilage regeneration. Encapsulating these cells within the hydrogel matrix, the study was conducted by live/dead staining and lactate dehydrogenase (LDH) assays to assess cell viability over seven days. Results demonstrated over 85% viability, confirming the hydrogel's non-toxic nature and its compatibility with living tissues. This level of biocompatibility highlights the hydrogel's potential as a scaffold for delivering cells and promoting tissue repair. Additionally, the study emphasized the hydrogel's adaptability in maintaining structural integrity under varying physiological conditions, enhancing its suitability for long-term therapeutic applications. Further degradation studies demonstrated the hydrogel's strength, with controlled degradation rates in the presence of enzymes or reducing environments, making it ideal for the sustained delivery of therapeutic agents.

The research provides a comprehensive understanding of how QbD principles can be applied to design biomaterials with specific properties, reducing variability and enhancing reproducibility. The use of a systematic experimental design ensured that the optimized formulation met all targeted performance criteria. The hydrogel's ability to combine mechanical strength, biocompatibility, and ease of administration positions it as a promising candidate for tissue engineering and regenerative medicine. The findings not only advance the development of HA-based hydrogels but also establish a scalable methodology for engineering biomaterials, paving the way for their clinical translation. This study represents a significant step toward addressing the unmet needs in osteoarthritis treatment, offering a novel scaffold that can support cartilage repair and regeneration.

CHAPTER III

A quality-by-design approach to optimize the transfection efficiency of poly(amidoamine)-based nanoparticles (PAA NPs) with mRNA

The study explores the optimization of poly(amidoamine)-based nanoparticles (PAA-NPs) for mRNA delivery using Quality by Design (QbD) principles, a framework aimed at enhancing pharmaceutical processes and ensuring product quality. These disulfide-linked nanoparticles are noted for their cationic and bio-reducible properties, which allow them to protect genetic material during delivery and release it efficiently within cells under physiological conditions. The study focuses on optimizing key parameters such as the polymer-to-genetic material ratio (P: G ratio) and RNA dosage to achieve maximal transfection efficiency while maintaining cell viability (CV), a critical aspect of their applicability in gene therapy.

The PAA nanoparticles are highly tunable, allowing precise control over size, charge, and stability. The redox-sensitive disulfide bonds ensure targeted delivery and intracellular release of mRNA, enhancing therapeutic outcomes. Despite these advantages, challenges such as toxicity at higher doses and inefficient delivery to hard-to-transfect cells, like Jurkat cells, necessitated a systematic optimization approach. To address this, we employed a QbD methodology comprising Failure Mode and Effects Analysis (FMEA) and Design of Experiments (DoE) to evaluate and refine critical quality attributes (CQAs) systematically. The QbD approach offered a more efficient alternative to traditional "one variable at a time" methods, reducing experimental variability and enabling a deeper understanding of interdependent factors.

The study began by defining the Quality Target Product Profile (QTPP), specifying desired nanoparticle attributes such as particle size, polydispersity index (PDI), zeta potential, transfection

efficiency, and cell viability. Particle size, ideally between 10–100 nm, was essential for cellular uptake and tissue penetration, while a narrow PDI (<0.2) ensured homogeneity. A zeta potential above +30 mV was critical for colloidal stability and interaction with cell membranes. High transfection efficiency (>75%) and CV (>80%) were prioritized to achieve therapeutic efficacy while ensuring safety.

Three PAA polymers (A, B, and C) were screened for performance across different P: G ratios and RNA dosages. Polymer B emerged as the optimal candidate, achieving superior transfection efficiency and stability. Using dynamic light scattering (DLS), the nanoparticles were characterized, showing a particle size below 100 nm, a PDI < 0.2, and a zeta potential ranging from +24 to +33 mV, which met QTPP criteria. Importantly, Polymer B exhibited the best balance between cell viability and cell transfection while also having a compact particle size and sufficient charge density to enable efficient genetic material encapsulation and delivery.

To evaluate the performance of the nanoparticles, three cell lines, HEK293, C28/I2, and Jurkat, were selected, each representing distinct cellular environments. HEK293 cells, commonly used for transient gene expression studies, served as a benchmark due to their ease of transfection. C28/I2 cells, a chondrocyte model relevant to osteoarthritis therapy, provided insights into transfection in specialized cell types. Jurkat cells, known for their low membrane permeability, posed a significant challenge, making them an ideal model for testing the robustness of the nanoparticles.

The results highlighted the critical role of the P: G ratio and RNA dosage in determining transfection outcomes. For HEK293 cells, the optimized conditions achieved a transfection efficiency of up to 5541.59 μunits/μg of β-galactosidase mRNA, with CV above 90%. In C28/I2 cells, transfection efficiency reached 2961.94 μunits/μg, demonstrating the potential of the nanoparticles for therapeutic applications in joint diseases. Jurkat cells, despite their challenging nature, achieved a transfection efficiency of 74.98 μunits/μg under optimal conditions. These findings highlight the flexibility of Polymer B in adapting to different cellular environments.

The optimization process revealed several key insights. Smaller particle sizes resulting from appropriate P: G ratios enhanced cellular uptake and reduced aggregation-related cytotoxicity. The redox-sensitive nature of the nanoparticles facilitated the intracellular release of mRNA, enhancing therapeutic efficacy. However, excessive polymer concentrations or high P: G ratios led to reduced CV, likely due to nanoparticle-induced membrane disruption and mitochondrial stress. The response surface plots and regression equations provided a clear understanding of the interplay between P: G ratio, RNA dosage, and cellular responses, enabling precise optimization.

The design space established through QbD defined the optimal P: G ratio (10:1 to 18:1) and RNA dosage (408–480 ng) to achieve high transfection efficiency with minimal toxicity.

Validation experiments confirmed the accuracy of the predictive models, with observed values aligning closely with predictions. For HEK293 and C28/I2 cells, transfection efficiency exceeded predictions, validating the robustness of the nanoparticles. Although Jurkat cells exhibited reduced CV alongside improved transfection, the results highlighted the potential of the nanoparticles to overcome barriers in hard-to-transfect cell lines.

In conclusion, the study demonstrates the efficacy of QbD principles in optimizing PAA nanoparticles for mRNA delivery. The integration of statistical tools and bioanalytical assays enabled the identification of critical parameters and their optimal ranges, resulting in a scalable and reproducible formulation. These findings contribute to the development of safer and more effective gene delivery systems, advancing the field of nanomedicine and offering new possibilities for personalized therapies. The study highlights the transformative potential of QbD in addressing challenges in nanoparticle-based therapeutics, paving the way for their clinical translation.

CHAPTER IV

A quality-by-design strategy to develop curcumin and siRNA co-loaded lipoplexes to combat osteoarthritis-related inflammation and oxidative stress

Inflammation and oxidative stress are central to OA pathogenesis, driven by cytokines such as IL-6, IL-8, and IL-1β, which upregulate matrix-degrading enzymes like matrix metalloproteinases (MMPs) and aggrecanases. These processes result in the breakdown of cartilage, compromising joint function. Additionally, oxidative stress exacerbates damage by producing reactive oxygen species (ROS) that activate cellular signaling pathways, including NF-κB and MAPK. This study presents an innovative dual therapeutic strategy to combat OA using lipoplexes co-loaded with curcumin and small interfering RNA (siRNA). Curcumin, known for its anti-inflammatory and antioxidant properties, inhibits NF-κB and ROS-mediated damage, while siRNA silences genes encoding inflammatory cytokines, targeting OA at the molecular level.

This research also utilized a Quality by Design (QbD) approach to develop and optimize curcumin-loaded cationic liposomes (CLCL) for siRNA delivery. Liposomes were selected as the nanocarrier due to their ability to encapsulate hydrophilic and hydrophobic molecules, protect siRNA from degradation, and facilitate cellular uptake. QbD principles ensured robust, reproducible formulations by systematically optimizing critical quality attributes (CQAs), including particle size, zeta potential, encapsulation efficiency, and siRNA complexation capacity. A Quality Target Product Profile (QTPP) was established, specifying the requirements for intra-

articular delivery, including particle sizes of 100–200 nm for efficient cartilage penetration, a positive zeta potential (~30 mV) to enhance cellular uptake and high encapsulation efficiency (>80%) for curcumin. Risk assessment through Ishikawa diagrams and Failure Mode and Effects Analysis (FMEA) identified critical material attributes (CMAs) and process parameters (CPPs), such as lipid composition and hydration conditions, influencing liposome properties.

Design of Experiments (DoE) was employed to evaluate the impact of various factors, including the choice of helper lipids (DOPE, DOPC, DPPC), cationic lipid concentration, cholesterol content, and pegylated phospholipids, on liposome characteristics. DOPE emerged as the optimal helper lipid, producing formulations with smaller particle sizes (139–184 nm), uniform distribution (PDI < 0.2), and a zeta potential of +33 to +52 mV. The liposomes demonstrated an encapsulation efficiency of over 85% for curcumin and siRNA complexation capacity exceeding 90%, ensuring high therapeutic payloads. The results were statistically analyzed, and a design space was established. The optimal formulation with a lipid composition of 8 mM DOPE, 2 mM MPEG-DSPE, 5 mM DOTAP, and 5 mM cholesterol was prepared and evaluated further. Quality attributes of the optimal formulation such as particle size (<200 nm), PDI (<0.2), zeta potential (+30 mV), encapsulation efficiency (>80%), cell viability and transfection were found to be close to the predicted values and fulfilled the QTPP. Transfection studies using luciferase siRNA as a model system showed over 70% gene silencing efficiency, validating the efficacy of the lipoplexes in delivering functional siRNA to chondrocytes.

Additional evaluation for the stability, *in vitro* cellular uptake and release profile of the optimal formulations were performed. Stability studies confirmed that the optimized liposomes (Opt-CLCL) retained their physicochemical properties during six-month storage at 4°C. Lyophilization with cryoprotectants preserved their integrity and therapeutic efficacy, facilitating long-term storage and transport. Confocal microscopy using the Cy5 labelled optimal liposome formulation confirmed efficient cellular internalization of the optimal formulation lipoplexes. *In vitro,* release studies revealed that curcumin was released from the liposomes in a sustained manner under conditions mimicking inflamed joints, with the release profile influenced by environmental pH and liposomal composition. This sustained release ensured prolonged therapeutic effects, which is critical for addressing the chronic nature of OA.

The biological evaluation of lipoplexes was conducted using the human chondrocyte cell line C28/I2 and primary chondrocytes isolated from osteoarthritic patients. Under inflammatory conditions induced by IL-1β, the co-loaded lipoplexes effectively reduced cytokine levels (IL-6 and IL-8) at both the mRNA and protein levels. Curcumin's anti-inflammatory effects were attributed to its ability to inhibit NF-κB activation and reduce ROS production, while siRNA-mediated gene silencing amplified the therapeutic effects by targeting cytokines at the

transcriptional level. These findings highlight the synergistic potential of combining small-molecule drugs with gene-silencing technology.

Oxidative stress was induced in chondrocytes using hydrogen peroxide, and the lipoplexes were evaluated for their antioxidant effects. Total oxidant status (TOS), total antioxidant capacity (TAC), and malondialdehyde (MDA) levels were measured to quantify oxidative stress. The lipoplexes significantly reduced oxidative markers, with curcumin enhancing endogenous antioxidant enzyme activity and siRNA further mitigating oxidative damage by suppressing inflammatory pathways.

This study demonstrates the potential of co-loaded lipoplexes as a novel therapeutic strategy for OA by addressing its multifactorial pathogenesis. The combination of curcumin's anti-inflammatory and antioxidant properties with siRNA's gene-silencing capabilities offers a targeted, multifunctional approach to combat OA. The QbD-driven optimization ensures reproducibility and scalability, making the formulation suitable for further preclinical and clinical development. Beyond OA, this platform technology could be adapted for treating other inflammatory and degenerative diseases, marking a significant advancement in nanomedicine and personalized therapy. Future research should focus on *in vivo* validation, exploring pharmacokinetics, biodistribution, and long-term efficacy in animal models to pave the way for clinical applications.

CHAPTER V

General Conclusions

Osteoarthritis (OA) is a prevalent and debilitating condition that continues to affect millions globally, with current treatments offering only symptomatic relief rather than addressing the underlying disease mechanisms. This doctoral thesis focused on developing and optimizing nanomedicine-based delivery systems aimed at improving OA treatment. Specifically, the research explored the use of supramolecular systems, including hyaluronic acid (HA)-derived hydrogels, polyamidoamine (PAA)-based nanoparticles, and lipoplexes, using a Quality-by-Design (QbD) approach to enhance cartilage repair and regeneration by efficiently delivering therapeutic drugs and genetic material to chondrocytes.

The first objective was to optimize HA-derived hydrogels for intra-articular delivery, focusing on rheological properties, mechanical strength, and biocompatibility. The QbD approach allowed precise control over formulation parameters, resulting in hydrogels that supported cartilage repair while enabling effective drug and cell delivery. Similarly, PAA-based nanoparticles were

optimized for mRNA delivery to chondrocytes, demonstrating high transfection efficiency and minimal cytotoxicity, making them a promising platform for gene therapy in OA treatment. In addition, curcumin-loaded liposomes were co-loaded with siRNA to target inflammation and oxidative stress, offering a dual therapeutic approach to address the complex pathophysiology of OA.

Therapeutic efficacy and biocompatibility were assessed in *in vitro* models of inflamed and oxidative-stressed chondrocytes. The HA hydrogels exhibited excellent biocompatibility and effective cell delivery, while the PAA nanoparticles demonstrated high transfection efficiency and safe gene delivery. The co-loaded lipoplexes showed remarkable anti-inflammatory and antioxidant effects, significantly reducing oxidative stress markers and promoting chondrocyte survival in OA conditions.

The research presents several novel contributions, including the application of QbD to optimize HA-derived hydrogels, the development of PAA-based nanoparticles for gene delivery, and the formulation of co-loaded lipoplexes for combined anti-inflammatory and regenerative effects. These advancements provide new insights into nanomedicine's potential for OA treatment and other inflammatory conditions.

Looking ahead, these delivery systems have broad potential for treating other diseases and tissues, including bone regeneration and other degenerative diseases. Further optimization and preclinical studies will be necessary to translate these findings into clinical applications, with future research exploring genetic modifications to enhance nanoparticle efficacy. This research lays a solid foundation for future advancements in OA treatment and regenerative medicine through targeted drug delivery and tissue regeneration strategies.

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

Chapter I

Ranamalla, S.R., Porfire, A.S., Tomuță, I. and Banciu, M., (2022). An Overview of the Supramolecular Systems for Gene and Drug Delivery in Tissue Regeneration. *Pharmaceutics, 14(8)*, p.1733.

https://doi.org/10.3390/pharmaceutics14081733

Chapter II

Ranamalla, S.R., Tavakoli, S., Porfire, A.S., Tefas, L.R., Banciu, M., Tomuța, I. and Varghese, O.P., (2024). A quality-by-design approach to optimize disulfide-linked hyaluronic acid hydrogels. *Carbohydrate Polymers, 339*, p.122251.

https://doi.org/10.1016/j.carbpol.2024.122251

Chapter III

Ranamalla, S.R., Porfire, A.S., Banciu, M. And Tomuța, I., (2024). A Quality-by-design Approach To Optimise The Transfection Efficiency Of Poly (Amidoamine)-Based Nanoparticles With mRNA. *Farmacia, 72 (3).*

https://doi.org/10.31925/farmacia.2024.3.14

Chapter IV

Ranamalla, S.R., Porfire, A.S., Licarete, E., Tefas, L., Parvathaneni, R.P., Varghese, O.P., Sesarman, A., Focsan, M., Tudoran, L.B., Tomuța, I., Banciu, M., (2024). Curcumin and siRNA co-loaded lipoplexes: A QbD strategy to combat osteoarthritis-related inflammation and oxidative stress. *BioRxiv.*

https://doi.org/10.1101/2024.11.25.625050

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

Pontes, A.P., van der Wal, S., Ranamalla, S.R., Roelofs, K., Tomuta, I., Creemers, L.B. and Rip, J., 2023. Cell uptake and intracellular trafficking of bioreducible poly (amidoamine) nanoparticles for efficient mRNA translation in chondrocytes. Frontiers in bioengineering and biotechnology, 11, p.1290871.

https://doi.org/10.3389/fbioe.2023.1290871