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Engineering Nanotherapeutic Strategies for Osteoarthritis Treatment

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ABSTRACT

Osteoarthritis (OA), the most prevalent joint disorder, is characterized by the degeneration of cartilage tissue leading to pain, stiffness, and impaired mobility. Primarily affecting the elderly, OA can also impact athletes, postmenopausal women, and individuals with conditions like diabetes. Despite it being a predominant contributor to physical disability, the absence of disease-modifying treatments for OA highlights the critical demand for novel therapies that preserve the well-being of individuals who are at risk of developing the disease.

As conventional treatment options offer limited relief and are incapable of halting OA progression, the field of nanomedicine has emerged as a promising frontier in this pursuit. Nanoscale materials such as nanoparticles (NPs) can be designed to carry a variety of therapeutic agents directly to the affected areas of the joint enabling precise and controlled therapies. In particular, NPs can circumvent the challenges faced by traditional medicines and are able to enter the cells embedded within the dense and charged cartilage extracellular matrix. Nonetheless, the limited knowledge of their interactions with complex biological environments impedes their clinical applications.

The foundational principle of the nanocarrier systems explored in this thesis is based on the use of cationic NPs. By leveraging electrostatic interactions with negatively charged components within the joint, these NPs serve as optimal tools for addressing the overlooked aspects of OA drug delivery, such as a protein-rich synovial fluid (SF) and an active catabolic environment. The findings in this work cover the SF-induced protein corona formation and its substantial effects on the NP uptake into cartilage and joint-associated cells. An enzymatically active cartilage milieu was also found to hinder the NP uptake and dictate the immunological responses, thereby influencing their therapeutic potential.

By illustrating the complexity of the dynamic OA environment, the investigations of the nanomaterial-cartilage interface serve as the fundamental framework for developing optimal cartilage drug delivery strategies. Accurate disease models and extensive NP characterization in a physiologically relevant environment are necessary for paving the way toward personalized approaches in medical practice.

Keywords: nanomedicine, osteoarthritis, nanoparticles, cartilage

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Synovial fluid profile dictates nanoparticle uptake into cartilage - implications of the protein corona for novel arthritis treatments
Ula von Mentzer, Tilia Selldén, Loise Råberg, Gizem Erensoy, Anna-Karin Hultgård Ekwall, Alexandra Stubelius
Osteoarthritis and Cartilage. 2022. 30(10), 1356-1364.

II. Cationic Nanoparticle Interactions with Catabolic Cartilage Modifies Macrophage Cytokine Production
Ula von Mentzer, Fritjof Havemeister, Loise Råberg, Gizem Erensoy, Elin K Esbjörner, Alexandra Stubelius
Manuscript

III. Functionalizing nanoparticles: dual strategy for probing cartilage degradation
Ula von Mentzer, Gizem Erensoy, Philipp Sonntag, Mohamed Ashraf Mostafa Kama, Sangeun Lee, Anna-Karin Hultgård-Ekwall, Alexandra Stubelius
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IV. Engineering Dendrimers for Improved Zonal Distribution in Catabolic Cartilage
Ula von Mentzer, Elin Svensson, Ruslan Ryskulov, Anna-Karin Hultgård Ekwall, Aldo Jesorka, Alexandra Stubelius
Manuscript

V. Physiological levels of estradiol limit murine osteoarthritis progression
Carmen Corciulo, Julia M. Scheffler, Piotr Humeniuk, Alicia Del Carpio Pons, Alexandra Stubelius, **Ula von Mentzer**, Christina Drevinge, Aidan Barrett, Sofia Wüstenhagen, Matti Poutanen, Claes Ohlsson, Marie K Lagerquist, Ulrika Islander
J Endocrinol. 2022. 255(2):39-51.

Additional papers originating from Ph.D. work not included in this thesis:

VI. Biomaterial Integration in the Joint: Pathological Considerations, Immunomodulation, and the Extracellular Matrix
Ula von Mentzer, Carmen Corciulo, Alexandra Stubelius
Macromol Biosci. 2020. Jul.22(7):e2200037.

VII. Achieving Precision Healthcare through Nanomedicine and Enhanced Model Systems
Elin Svensson, **Ula von Mentzer**, Alexandra Stubelius
ACS Materials Au. 2023.3c00073

CONTRIBUTION REPORT

Below follows a description of my (UvM) contributions to the papers, where the initials are from authors in the list of publications:

I. UvM designed the studies along with AS. UvM conducted material synthesis and characterization, in vitro and ex vivo experiments, performed data analysis, and wrote the paper together with AS.

II. UvM designed the studies with AS. UvM conducted material synthesis, in vitro, and ex vivo experiments, performed data interpretation, analyzed the data, and wrote the paper together with AS.

III. UvM designed the studies with AS. UvM conducted partial material synthesis, in vitro and ex vivo experiments, performed data interpretation, analyzed the data, and wrote the paper together with AS.

IV. UvM designed the studies with AS. UvM conducted material synthesis, in vitro, and ex vivo experiments, performed data interpretation, analyzed the data, and wrote the paper together with AS.

V. UvM assisted in histological study interpretation and analysis.

PREFACE

This dissertation was submitted for the partial fulfillment of the degree of Doctor of Philosophy. The original work presented in this dissertation was carried out between October 2019 and March 2024 at the Department of Life Sciences at Chalmers University of Technology, under the supervision of Associate Professor Alexandra Stubelius. The research was funded by the Chalmers University of Technology Nano Area of Advance.

Ula von Menzter
January 2024

ABBREVIATIONS

3D	three-dimensional
ACAN	aggrecan
ADA	ADAMTS5
ADAMTS5	disintegrin and metalloproteinase with thrombospondin motifs 5
AFM	atomic force microscopy
ANOVA	analysis of variance
API	active pharmaceutical ingredient
CD	circular dichroism
COL2	collagen type II
DAMP	damage-associated molecular pattern
DDS	drug delivery system
DLS	dynamic light scattering
DMMB	dimethyl methylene blue (assay)
E2	17β-estradiol
ECM	extracellular matrix
EDX	energy-dispersive X-ray spectroscopy
EM	electron microscopy
EMA	European Medicines Agency
FC	flow cytometry
FCS	fetal calf serum
FDA	U.S. Food and Drug Administration
FITC	fluorescein isothiocyanate
FLS	fibroblast-like synoviocytes
GAGs	glycosaminoglycans
H&E	hematoxylin/eosin
HA	hyaluronic acid
HYA	hyaluronidase
IA	Intra-articular
IL	interleukin
IV	intravenous
M-CSF	macrophage colony-stimulating factor
MDMs	monocyte-derived macrophages
MMP	matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NP	nanoparticle
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
PAMAM	polyamidoamine
PC	protein corona
PDI	polydispersity index
PEG	Polyethylene glycol
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PRP	platelet-rich plasma
RA	rheumatoid arthritis
ROS	reactive oxygen species
SEM	scanning electron microscopy
SF	synovial fluid
TEM	transmission electron microscopy
UV-Vis	ultraviolet-Visible (spectroscopy)

Table of Contents

1. Introduction 1
2. Joint, Cartilage and Osteoarthritis 3
2.1. Anatomy of Joints and Cartilage 3
2.2. Systemic Risk Factors 5
2.3. Symptoms 6
2.4. Diagnosis 6
2.5. Current Treatments 7
2.6. Pathogenesis and Progression of OA 8
2.7. OA Model Systems 9
3. Materials in Nanomedicine 11
3.1. Dendrimers 12
3.2. Polymeric NPs 12
3.3. Lipid NPs 12
3.4. Silica NPs 12
3.5. Protein NPs 13
3.6. NPs for OA 13
3.7. Clinical Translation 13
4. Drug Delivery Considerations 15
4.1. Route of Administration 15
4.2. Biological Barriers 16
4.3. Intrinsic Properties 17
4.3.1. Size 17
4.3.2. Shape 17
4.3.3. Hydrophilicity and Hydrophobicity 18
4.3.4. Surface charge 18
4.4. Surface Modifications 19
4.4.1. Tracking 19
4.4.2. Targeting 20
4.4.3. Stability 20
4.4.4. Controlled release 21
5. Experimental Methods 23
5.1. Nanoparticle Characterization 23
5.1.1. Nuclear Magnetic Resonance 23
5.1.2. Electron Microscopy 24
5.1.3. Ultraviolet-Visible Spectroscopy 24
5.1.4. Dynamic Light Scattering 25

5.2. Biological models 26
5.2.1. Cell Culture 26
5.2.2. Cytotoxicity 27
5.2.3. Explant Extraction and Culture 27
5.2.4. Tissue Histology 28
5.3. Biological Interactions with Nanoparticles 28
5.3.1. Fluorescence Spectroscopy 28
5.3.2. Confocal Fluorescence Microscopy 29
5.3.3. Flow Cytometry 29
5.4. Biomolecular Interactions with Nanoparticles 29
5.4.1. Biochemical Assays 29
5.4.2. Atomic Force Microscopy 30
5.4.3. Circular Dichroism spectroscopy 30
5.4.4. Proteomics 31
5.5. Statistics 31
6. Original Work 33
6.1. The Impact of Patient Synovial Fluid on NP Uptake 34
6.2. NP Interactions with Catabolic Cartilage 37
6.3. NPs as Cartilage Degradation Probes 40
6.4. Functionalized NP Distribution in Catabolic Cartilage 44
6.5. Estradiol Effects on Murine OA Progression 47
6.6. Concluding Remarks 49
7. Future Outlook 51
8. Acknowledgments 53
9. References 54

1. Introduction

Throughout history, the connotation of ageing varied from embodying wisdom and respect to being synonymous with decline and irrelevance. Nowadays, technological and medical advances have shifted the typical population paradigm towards a steadily growing elderly demographic.[1,2] This unprecedented global growth of the ageing population has presented new socioeconomic and scientific challenges.[3]

Efforts to improve the well-being and health span of current and future generations focus on aging-associated illnesses such as arthritis, cancer, and cardiovascular diseases.[4–8] Arthritis and its most common form, osteoarthritis (OA), have experienced a rapid increase in cases, with a 113% rise over the past 30 years, and are anticipated to continue to grow globally.[9] OA is primarily characterized by joint deterioration, where the degeneration of cartilage tissue, found at the ends of the bones, compromises overall mobility and ability to withstand compressional loads. OA has been deemed as one of the oldest forms of disability, with evidence of its existence tracing back to ancient civilizations[10–12], thus it is not surprising that for a long time, it was viewed as a simple "wear and tear" condition arising over time. This view, however, was first challenged by a research team from Stanford back in 2011 by demonstrating that initial damage to the joint triggers a variety of molecular cues leading to the activation of the complement system.[13] Currently, OA is viewed as an active biochemical and inflammatory process that involves multifactorial, tightly regulated mechanisms.

Extensive research efforts are being employed to elucidate the complex pathways and interactions that lead to OA disease development, with the aim of developing more targeted and effective treatments that can halt or reverse the disease's progression.[14–16] To address the gap in effective arthritis treatments, the rise of nanomedicine, a medical application of nanotechnology, offers a promising frontier, harnessing the power of tiny particles to deliver treatments directly to affected areas, potentially enhancing efficacy and reducing side effects.[17,18] In theory, nanoscale modalities can be applied to refine current drug formulations, introduce precise drug delivery methods, and explore cutting-edge therapies. However, the clinical translation of these innovations is hindered by incomplete knowledge of particle behavior within the body, leading to challenges in successfully applying these advances to real-world clinical care.

The work in this thesis scrutinizes the use of polymeric nanocarriers in diagnosing and treating OA. My work adds a layer of understanding to the nuanced nature of OA by providing a comprehensive view of the challenges and opportunities in OA drug delivery. Each study was designed to maintain some level of physiological relevancy to the joint and/or OA. For instance, arthritic patient synovial fluid (SF) was used to assess the formation of protein coronas (PC), an adsorbed layer of proteins and biomolecules to the NP surface. For the first time, its hindering effects were determined on small NP uptake into cartilage tissue and cells (**Paper I**). After noticing potential interactions between the NPs and extracellular matrix (ECM) components, an enzymatic ex vivo cartilage degradation model was established to confirm the ability of the NPs to not only act as nanocarriers but also as effector molecules in interactions with certain

ECM enzymes and their degradation products (**Paper II**). The following study warranted a more detailed investigation into cartilage degradation mechanisms employing two different NP species that displayed differential affinity to cartilage components including collagen and glycosaminoglycans (GAGs) demonstrating the potential for combination therapies in OA (**Paper III**). For a more accurate cartilage uptake model, a specialized chamber was designed to quantitatively utilize NPs as tools for quantifying the transport into the tissue under catabolic conditions (**Paper IV**). Finally, to underline the intricate interplay of physiological, biochemical, and biomechanical factors in OA management, the role of estrogen was investigated in an in vivo setting to deduce sex-based differences in murine OA progression (**Paper V**).

2. Joint, Cartilage and Osteoarthritis

The physiology of OA is characterized by the progressive degradation of articular cartilage, changes in subchondral bone, and inflammation of the synovial membrane, leading to joint pain, stiffness, and functional impairment.[19] The following sections detail the joint and cartilage anatomy, OA risk factors, diagnostic criteria, symptoms, conventional treatments, and the mechanisms of OA pathogenesis and its disease progression. An overview of OA-induced pathological changes is illustrated in Figure 1.

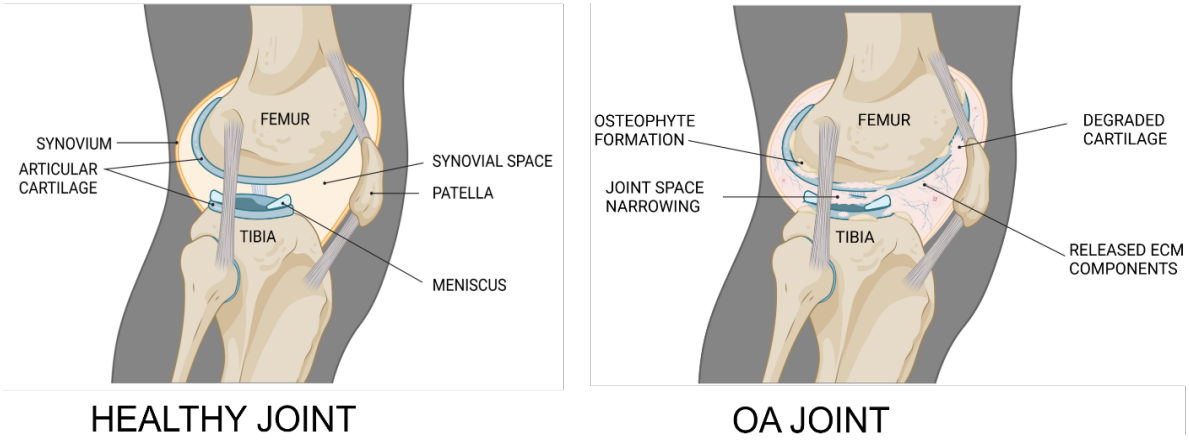

Figure 1. Anatomical differences between the health and OA-affected joint. Adapted from von Mentzer et al. (2022).[20]

2.1. Anatomy of Joints and Cartilage

Joints are the meeting points of two or more bones in the body. They play a critical role in providing the skeletal system with the flexibility and mobility needed to perform a wide range of movements, from simple daily activities to complex athletic maneuvers. Anatomically, joints are complex structures composed of various tissues and components, each contributing to the joint's overall function. Several types of joints exist and are classified based on their structure and the type of movement they allow. For example, synovial joints are characterized by a joint cavity filled with SF, an ultrafiltrate from plasma. These types of joints allow a wide range of movements and can be found in the knees, hips, wrists, and shoulders. Alternatively, fibrous joints possess no joint cavity and are composed of fibrous connective tissue, thus resulting in limited movement. Such joints can be found as sutures in the skull and the distal tibiofibular region.

Basic joint components entail a rigid bone that forms the framework of the joint. The bone is typically covered with smooth, resilient cartilage tissue that reduces friction and enables resistance to compressive forces. It is surrounded by a membrane, also known as synovium, that lines the joint capsule and produces SF, which lubricates and nourishes the joint. The entire joint system is further supported by ligaments, fibrous bands that connect the bones and provide joint stability, and tendons, connective tissue that facilitates movement by attaching the muscle to the bone.

While all joint components are essential to normal joint function, cartilage stands out for its important role in movement mechanics. Depending on its function, different types of cartilage may exhibit

variations in its structure. For instance, fibrocartilage is typically found in areas requiring great tensive strength like intervertebral discs, while elastic cartilage exhibits more flexibility due to a higher concentration of elastic fibers as is found in ears and epiglottis.[21] However, the most common and resilient type existing on the articular surfaces of bones in synovial joints is hyaline cartilage. It plays a key role by providing a smooth, lubricated surface for joint movement and serving as a shock absorber to reduce the impact on joints during activities.

Hyaline cartilage is composed of a dense network of collagen fibers and negatively charged biomolecules comprising ECM which sequesters and homes the chondrocytes, the only type of cell present in the tissue (Figure 2). The architecture of the tissue is intricately designed to fulfill its role in load distribution and shock absorption by anisotropic, depth-dependent arrangements of collagen fibers and cells. Based on collagen orientation in the tissue, cartilage sometimes is divided into distinct zones.[22] For example, at the superficial zone, which is tangential to the SF interface, collagen fibers tend to run parallel to the surface, entrapping the cells that appear to exhibit an ellipsoidal phenotype. The middle or transitional zone is characterized by a random arrangement of collagen fibers embedded in the sparsely arranged cells allowing them to appear round, while deeper in the tissue at the radial zone, collagens appear to be perpendicular to the surface, thus arranging round cells into columns. Besides, a strong and layered structural scaffold, ECM is also rich in proteoglycans and GAGs, molecules possessing negatively charged surface groups. This feature is key to the tissue's shock-absorbing properties as anionic groups attract and trap water, thus enabling tissue to easily spring back after compressional stresses associated with physical activity. Cartilage homeostasis is mediated by a fine balance of catabolic ECM enzymes such as matrix metalloproteinases (MMPs) and anabolic de novo synthesis of ECM structural components.[23]

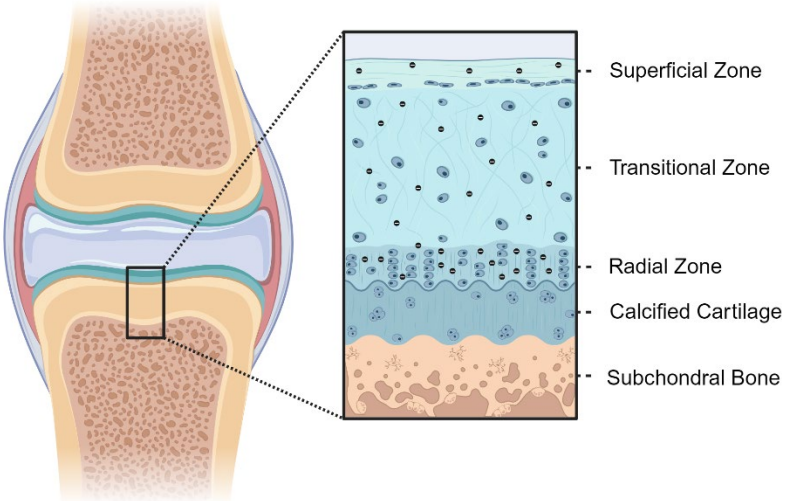

Figure 2. Articular knee joint and the structure of hyaline articular cartilage. Created with BioRender.com.

The lack of blood vessels in hyaline cartilage necessitates its dependence on diffusion from adjacent tissues, a factor that significantly slows its healing process upon injury. Since it is hard to detect early cartilage damage due to its avascularity, deterioration of hyaline cartilage has significant implications for the health of surrounding tissues and overall joint function. As cartilage deteriorates, its ability to evenly distribute load and absorb shock is diminished, leading to increased stress on the underlying bone and a higher risk of joint damage and pain. The increased stress upon subchondral bone can lead to sclerosis, bone spur formation, and alterations in bone shape.[19] Additionally, cartilage degradation often leads to inflammation of the synovium, resulting in joint swelling and pain, thus perpetuating the degradation of the joint environment. The change in joint mechanics and inflammation can alter the composition and properties of SF, reducing its lubricating and nutrient-distributing capabilities, and further exacerbating joint problems.[24]

SF plays a critical role in maintaining joint health by providing lubrication to reduce friction between cartilage surfaces during movement and by supplying nutrients to avascular cartilage. It contains hyaluronic acid (HA) and lubricin, which enhance its viscous and lubricating properties. In joint diseases including OA and even rheumatoid arthritis (RA), the quality and quantity of SF can be adversely affected.[25] Inflammatory processes can lead to an increase in fluid volume, which dilutes its lubricating properties and increases joint swelling.[26] Furthermore, the presence of inflammatory cells and enzymes can degrade SF constituents, diminishing their protective functions and contributing to the cycle of cartilage degradation, inflammation, and pain.

2.2. Systemic Risk Factors

Systemic factors play a significant role in OA pathogenesis and progression since they impact the overall health and environment of the body, thereby affecting the joints.[11,19,27] As individuals age, cartilage experiences biochemical transformations, with a notable increase in water content and a decline in the ECM integrity, rendering it more susceptible to wear and tear.[28] The cumulative effect of mechanical stress on joints through repetitive use over the years can accelerate cartilage deterioration. For instance, occupational hazards, with jobs involving repetitive motions, heavy lifting, or extended periods of standing, such as in construction, agriculture, and certain sports, predispose individuals to OA by placing continuous stress on joints like the knees, hips, and spine.

In addition, the observed sex disparities in OA prevalence, with notably higher incidences in postmenopausal women, suggest a hormonal underpinning on vulnerability to the disease.[29,30] Estrogen is believed to play a role in safeguarding the joint and cartilage health. Consequently, its diminishing levels in response to a menopause leads to a higher risk of OA development.[31]

Furthermore, metabolic disorders like diabetes and metabolic syndrome contribute to OA's development. In diabetes, high blood sugar levels promote advanced glycation end product formation, which attach to cartilage, diminishing its flexibility and increasing vulnerability to mechanical stress.[32] Obesity exacerbates OA risk, particularly in load-bearing joints such as knees and hips, due to the added

mechanical pressure and the pro-inflammatory cytokines secreted by visceral fat, which foster a systemic inflammatory state that promotes cartilage breakdown.[33]

2.3. Symptoms

OA symptoms, including joint pain, stiffness, and swelling have a direct impact on the life quality and functional ability of an individual. Pain in OA is a hallmark symptom that involves both mechanical and inflammatory processes. Mechanical stress on subchondral bone and joint structures can lead to nociception, while inflammatory mediators (such as IL-1β, TNF-α, and IL-6) released from the synovium and cartilage can sensitize peripheral nerve endings, exacerbating pain.[11,16,19] Patients with OA often prioritize pain management over other symptoms, as pain significantly impacts their quality of life and daily functioning.[34]

In addition to pain, stiffness, and swelling significantly impact patients' mobility.[16] Stiffness, particularly after periods of inactivity, restricts joint movement, complicating daily tasks and personal care, and may arise due to the degeneration of cartilage and thickening of the joint capsule. Swelling in the joints can cause additional pain and stiffness, exacerbating the cycle of reduced mobility. Understanding and addressing these symptoms are essential for managing OA effectively, as they serve as indicators of disease progression and guide treatment strategies aimed at preserving joint function, alleviating discomfort, and maintaining overall well-being. As OA progresses, the range of motion in the affected joint may decrease due to pain, muscle weakness, and mechanical interference caused by osteophyte growth, thus further increasing the risk of falls and injuries.

2.4. Diagnosis

OA is typically diagnosed through a combination of clinical evaluation, patient history, and imaging studies. The diagnosis process aims to identify characteristic symptoms of OA, assess the impact on function, and rule out other potential causes of joint pain and stiffness. The initial step in diagnosing OA involves a detailed clinical evaluation and review of the patient's medical history, followed by a medical examination. Imaging techniques such as X-rays and magnetic resonance imaging are often used to confirm the presence of OA and assess its severity by examining potential joint space narrowing, osteophyte formation, and changes in bone density around the joint.

The Osteoarthritis Research Society International (OARSI) scoring system standardizes knee OA severity assessment on radiographs, focusing on joint space narrowing and osteophyte formation to ensure consistent evaluation and correlation with clinical symptoms for research.[35] An alternative, the Kellgren-Lawrence grading scale, also assesses OA severity radiographically from grades 0 to 4, detailing the progression from no OA features to severe changes, including osteophytes and joint space narrowing.[36]

2.5. Current Treatments

The majority of OA treatment strategies currently focus on alleviating the symptoms and improving joint function. The cornerstone of OA management, especially in the early to moderate stages of OA, involves nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, naproxen, and diclofenac, or other analgesics such as acetaminophen.[37]

NSAIDs work by inhibiting enzymes (COX-1 and COX-2) involved in the inflammatory pathway, thereby decreasing the production of prostaglandins, which are compounds associated with pain and inflammation. The choice between the NSAIDs can be based on individual patient response as well as gastrointestinal, cardiovascular, and renal risks, as these drugs can have significant side effects, especially with long-term use.[38] For patients at risk of gastrointestinal complications, COX-2 selective inhibitors, which have a lower risk of gastrointestinal bleeding, may be preferred, although they may carry a higher cardiovascular risk.

Acetaminophen is often recommended as a first-line analgesic for OA pain management due to its safety profile, especially for elderly patients or those with contraindications to NSAIDs. While acetaminophen lacks the anti-inflammatory properties of NSAIDs, it is effective in reducing pain by inhibiting central pain pathways. However, care must be taken to adhere to recommended dosages to avoid liver toxicity, particularly in individuals with existing liver conditions or those consuming alcohol regularly.[39]

In cases where oral medications do not provide adequate relief, or in patients with more localized or severe symptoms, intra-articular (IA) injections may be proposed.[40] Corticosteroids are commonly injected directly into the affected joint to provide rapid anti-inflammatory and pain-relieving effects. HA or platelet-rich plasma (PRP) injections, also known as viscosupplementation, aim to improve the viscoelastic properties of SF, providing lubrication and shock absorption within the joint. Yet, the long-term efficacy of HA as well as PRP injections remains controversial. While some studies suggest that HA injections can provide relief from OA symptoms, particularly in the knee, the overall evidence regarding their long-term efficacy is mixed. Systematic reviews and meta-analyses have shown variable results, with some concluding modest benefits at best.[41–43] The American Academy of Orthopedic Surgeons has expressed reservations about the routine use of HA injections for knee OA, citing insufficient evidence to support their efficacy.[44] At the same time, the evidence base for PRP's efficacy and long-term outcomes remains controversial, with studies showing a wide range of outcomes.[45,46] The variability in PRP preparation methods, differences in study designs, and lack of standardization in treatment protocols contribute to the controversy surrounding its efficacy.[47]

It is important to note that while these pharmacological treatments can provide significant symptom relief, they do not halt the progression of OA. Therefore, these strategies are often complemented by non-pharmacological approaches such as physical therapy, weight management, and lifestyle modifications to achieve optimal management of OA symptoms and improve patients' quality of life. In advanced cases, surgical interventions such as joint replacement may be necessary to restore function and alleviate pain.

2.6. Pathogenesis and Progression of OA

Understanding the pathogenesis and progression of OA involves exploring a complex interplay of biomechanical, genetic, biochemical, and inflammatory factors. While pinpointing the exact mechanisms of OA pathogenesis is hard, it is possible to postulate potential causes that may lead to active cartilage breakdown.

Biomechanical stresses play a crucial role in cartilage degradation by inducing wear and tear, disrupting the even distribution of forces across the cartilage, creating areas of intense stress, and triggering cellular reactions that promote cartilage breakdown rather than repair.[35,48] Joints that bear weight, such as the knees and hips, are particularly prone to OA due to the substantial loads they carry, a situation exacerbated by obesity or vigorous physical activity. When the load surpasses the cartilage's ability to distribute and absorb forces, it results in mechanical deterioration. Structural anomalies may skew the normal force distribution across the joint, leading to uneven loading and heightened stress on specific cartilage regions, hastening its wear.[49] Furthermore, acute injuries to the joint, such as ligament tears or surface-impacting fractures, can disrupt normal joint function and heighten cartilage stress in localized areas.[50] Similarly, repetitive minor traumas from activities involving high impact can progressively harm the cartilage. Twisting or shearing forces are particularly detrimental to cartilage health, as they can disrupt the cartilage matrix's collagen network, causing fissures and ultimately leading to cartilage erosion.[48]

Genetic factors significantly influence the onset and progression of OA, affecting key aspects of joint health such as the strength and composition of cartilage, the body's inflammatory response, and the overall architecture of joints. Mutations in genes encoding vital cartilage proteins, like type II collagen (COL2), result in more fragile cartilage, increasing its vulnerability to damage.[51–53] Additionally, genetic variations that impact the balance of ECM synthesis and degradation, involving components like aggrecan (ACAN) and enzymes such as MMPs, are crucial for cartilage integrity and its ability to endure mechanical stress.[54,55] Genetic predispositions that alter the production and regulation of inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor-alpha TNF-α, can intensify joint inflammation and accelerate cartilage wear.[55,56] Inherited traits also influence joint structure and alignment, predisposing some individuals to joint configurations that lead to uneven stress distribution and faster cartilage deterioration. Moreover, genetic susceptibility to obesity and metabolic syndrome amplifies OA risk, especially in weight-bearing joints, due to increased mechanical stress and systemic inflammation.

Inflammation plays a complex role in OA, encompassing the synovium, cartilage, and immune response. It is characterized by the release of pro-inflammatory cytokines and mediators that drive cartilage breakdown, pain, and structural changes within the joint, accelerating OA's progression. In OA, the synovium, or joint lining, can become inflamed, leading to the production of cytokines and chemokines that worsen cartilage damage. Key cytokines, such as IL-1, TNF-α, and IL-6, produced by cells within the joint, stimulate chondrocytes to release MMPs and aggrecanases.[56,57] These enzymes degrade cartilage, and the resulting fragments can trigger a feedback loop, intensifying inflammation through

damage-associated molecular patterns (DAMPs).[20] Furthermore, inflammatory mediators like prostaglandins, nitric oxide, and reactive oxygen species (ROS) contribute to the pain and ongoing inflammation, exacerbating cartilage damage.[58] Inflammation can also lead to angiogenesis and aberrant nerve growth in typically avascular and aneural regions such as cartilage and menisci, heightening pain sensitivity and contributing to OA's pathology.[59] Despite not being a classic inflammatory arthritis, OA features immune cell infiltration, with macrophages and T cells releasing cytokines that further degrade joint tissues.[60] This complex inflammatory cascade underscores the need for targeted therapeutic interventions to mitigate inflammation in OA.

2.7. OA Model Systems

A vast variety of models have been developed to study the disease mechanisms and investigate the treatments of OA. Most often, the selection of the model system is determined based on the available resources as well as the aim of the investigation and therapeutic interventions in question. A common model to study OA is the in vitro chondrocyte culture. Ideally, the environment in vitro should resemble that in vivo, thus it is imperative to culture chondrocytes under appropriate conditions. Considering that the chondrocytes in the joint are exposed to low levels of oxygen, hypoxia is an important aspect when studying chondrocyte metabolism. To simulate the inflammatory environment of an OA joint, cells are often treated with pro-inflammatory cytokines like IL-1β and TNF-α, which are known to play significant roles in OA pathogenesis. Additionally, three-dimensional (3D) culture systems enable a more physiologically relevant setting, providing cell-matrix interactions that are important for chondrogenic gene expression.[61] 3D cultures are often employed for tissue engineering purposes, enabling custom hydrogel-based cartilage models. Despite its convenience and prominence, it is well established that the cell culture models alone are not sufficient to replicate the full picture of the joint and its underlying pathologies.

More advanced in vitro setups such as joint-on-a-chip and bioreactors are currently of interest.[62] These systems aim to replicate the dynamic mechanical and biochemical joint environment. Joint-on-a-chip allows for modeling the fluid flow and shear forces through the use of microfluidics. Bioreactors, on the other hand, are designed to resemble cell-laden tissue structures that can be subjected to various external forces including movement patterns and mechanical loading.

Another widely adapted model is the cartilage ex vivo model which employs the use of native cartilage explants.[63–65] These explants preserve the native tissue architecture and the microenvironment of chondrocytes, providing a more accurate reflection of in vivo conditions. However, tissue properties depend on the donor species, age, and health status, thus introducing potential variability in the results. Most commonly, cartilage is retrieved from porcine, bovine, and equine sources as they offer similar thickness to that of human cartilage compared to smaller animal models.

At the moment, animal models are indispensable for studying the systemic aspects of OA, including pharmacokinetics, toxicity, and off-target effects of therapeutic agents.[66] However, the majority of the in vivo studies are conducted in rodents and rabbits. The anatomical and physiological differences between

humans and small animals are apparent, thus the results of these studies are mostly appropriate for early-phase testing. While each of these models offers valuable insights into specific facets of OA pathophysiology, the inherent limitations underscore the necessity for more advanced joint-replicating systems to comprehensively study the holistic aspects of OA pathogenesis and treatment efficacy.

3. Materials in Nanomedicine

The exploration of nanoscale (1-100 nm)[67] materials opens vast opportunities for advancing medical science, particularly in diagnostics, targeted drug delivery, and pharmaceutical effectiveness. This emerging domain of nanomedicine leverages the unique properties of NPs and nanostructures to overcome limitations faced by conventional therapeutic approaches. Interactions on the nanoscale have a direct impact on biological molecules and structures, thus resulting in predicted outcomes with cellular and molecular components of the body. NPs can be engineered to bind to specific biomarkers, enabling their diagnostic application in early disease detection.[68] For instance, magnetic NPs enhance imaging techniques, providing clearer, more detailed visualizations of tissues, which is crucial for the early diagnosis of OA.[68]

Targeted drug delivery aims to transport therapeutic agents directly to the pathologically affected site, thereby maximizing pharmaceutical effects while minimizing systemic toxicity. This can be achieved through the functionalization of nanoparticles (NPs) with various ligands that recognize and bind to designated receptors on cellular surfaces.[69] Such precision not only increases the efficacy of the drug but also reduces the risk of harm to healthy cells, a common drawback of conventional therapies.

The role of nanotechnology in enhancing therapeutics extends to the design of smart drug delivery systems (DDS) capable of responding to specific physiological conditions to release their payload. Additionally, nanocarriers can protect unstable drugs from degradation in the bloodstream, improving their bioavailability and pharmacokinetics. Beyond these applications, nanomedicine is also paving the way for innovative therapeutic modalities, including gene therapy and immunotherapy. Small nanocarriers can be engineered to deliver genetic material safely and efficiently into cells, offering new avenues for treating genetic disorders.[70] In the realm of immunotherapy, NPs are being developed to modulate the immune system in a targeted manner to fight cancer and autoimmune diseases.[4,71] As a consequence, the field has experienced a significant surge in drug delivery vehicle development, with an array of types and characteristics. The following sections cover the fundamentals of the most prevalent NPs in the biomedical field, highlighting their advantages and applications.

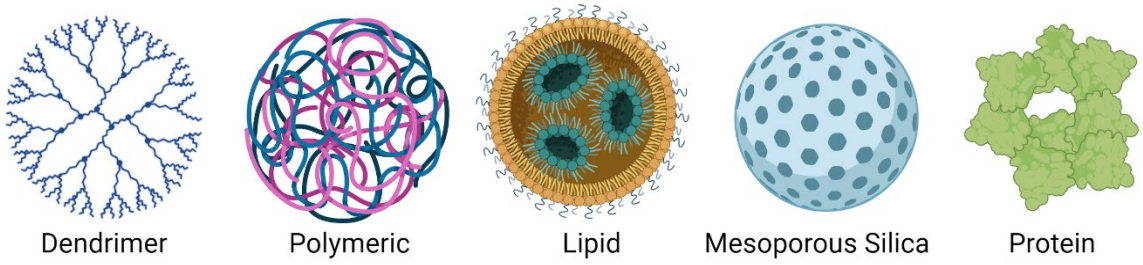

Figure 3. Common material designs for NP utilization in OA drug delivery. Created with BioRender.com.

3.1. Dendrimers

Dendrimers are branched, star-shaped polymers characterized by their uniform and monodisperse structure, which grants precision in tailoring their size and molecular compositions. Their unique architecture, consisting of numerous terminal functional groups, is receptive to easy customization and also allows the delivery of high therapeutic load making them suitable DDS. [72] Their small size and spherical dimensions are pivotal for effective biological interactions, facilitating their utility in small molecule and nucleic acid delivery.[65,73–75] Biocompatibility of dendrimers is highly dependent on their surface characteristics and molecular composition as highly positive charge arising from terminal amine groups has been linked to cytotoxic effects.[76]

3.2. Polymeric NPs

Comprising of biodegradable polymers like polylactic-co-glycolic acid (PLGA), polylactic acid (PLA), and others, polymeric NPs have been widely employed for controlled drug release applications.[77] Their biocompatibility and ability to be engineered with controlled size and surface characteristics make them effective for delivering a wide range of therapeutic agents. These NPs can be synthesized through a variety of polymerization techniques allowing the incorporation of hydrophobic, stimuli-responsive, and bioactive components. These materials are often used in smart DDS due to their highly variable physical characteristics and stimuli-responsive behavior.

3.3. Lipid NPs

Lipid NPs (LNPs) are spherical vesicles composed of one or more phospholipid bilayers, making them highly biocompatible due to their structural similarity to cell membranes. They are commonly produced through methods such as high-pressure homogenization or ultrasonication, which facilitate the formation of a solid lipid core that can encapsulate hydrophobic drugs.[78,79] In addition to lipophilic agents, hydrophilic substances and nucleic acids may be incorporated with the use of surfactants or coatings.[80] This adaptability of LNPs makes them a suitable choice for drug delivery applications, including the targeted treatment of tumors and the delivery of genetic material, such as mRNA vaccines. Clinically, LNPs have been utilized for a variety of vaccines including COVID-19.[81,82]

3.4. Silica NPs

Silica NPs, distinct from their polymeric counterparts, offer exceptional stability, large surface area, and tunable pore size, which can be precisely engineered to optimize drug loading and release profiles. Their inert nature combined with surface adaptability allows for the conjugation of various functional groups, enabling targeted delivery and enhanced biocompatibility. In the biomedical field they are used for targeted drug delivery, where their surfaces can be functionalized with ligands to bind specifically to diseased cells, such as cancer cells, ensuring that therapeutic agents are delivered directly to the target site, minimizing side effects.[83,84]

3.5. Protein NPs

Utilization of proteins as DDS represents another innovative approach for biomedical applications. Their inherent biocompatibility and biodegradability make them versatile drug delivery candidates. Silk fibroin[85], human serum albumin[86], avidin[87,88], ferritin[89], and others have been regarded for their high stability and biocompatibility as effective nanocarriers. In addition to natural proteins, synthetic peptides have been successfully utilized for targeted cartilage delivery. [64,90]

3.6. NPs for OA

In the last couple of years, a variety of NPs have been utilized to investigate their applications for OA treatments. Li et al. and a team of researchers from Shanghai modified polyamidoamine (PAMAM) dendrimers to investigate localized gene therapy.[91] This study introduced a novel approach by encapsulating modified dendrimers in hydrogel microspheres. Harnessing MMP-responsive degradation, the microspheres facilitated a sustained and targeted release of genetic cargo. Significant NP retention and reduction in cartilage degradation as well as osteophyte formation was observed in the murine OA model, thus highlighting the potential of this "nano-micron" slow-release system. The delivery of insulin-like growth factor 1 utilizing PEGylated PAMAMs by Geiger et al. improved therapeutic joint residence in rat knees by 10-fold solidifying the role of PAMAMs as efficient drug carrier systems.[65] A study by Liu et al. addressed the use of PLA and polyethylene glycol (PLA-b-PEG) based NPs for small molecule delivery. The authors report that these biodegradable, polymer-based NPs could significantly extend the longevity and therapeutic efficacy of adenosine receptor agonists, thus solidifying their role as drug-delivery vehicles.[92] Chang et al. developed an innovative OA treatment using liposomal NPs encapsulating diclofenac and dexamethasone combined with HA for enhanced pain management. This multifaceted approach showcased the versatility of liposomal NPs in delivering combination OA therapies.[93] Mehta et al. from Northeastern University have introduced a charge-optimized delivery system employing avidin and cationic peptide carriers to transport an IL-1 receptor antagonist to bovine cartilage explants, effectively reducing cytokine-induced GAG loss.[94] Alternatively, He and colleagues designed a pH-responsive DDS using mesoporous silica NPs loaded with andrographolide, an anti-inflammatory compound. This system demonstrated chondroprotective effects in IL-1β-stimulated chondrocytes and mitigated proteoglycan degradation in a rat model of OA.[83] Xue and collaborators developed a dual DDS using mesoporous polydopamine NPs decorated with a metal-organic framework and modified with collagen peptides. This innovative approach yielded significant anti-inflammatory and anti-apoptotic effects in vitro and effectively preserved cartilage integrity in vivo. [95] These diverse studies collectively highlight the vast potential of nanomedical approaches for advancing OA treatments.

3.7. Clinical Translation

Currently, the absence of NP-based active clinical trials suggests that NP-based research for OA interventions remains largely in the preclinical phase, with investigations confined to in vitro studies and animal models.[96] The development of NP-based therapies often involves proprietary formulations, which are either patented or held confidential by their developers to protect intellectual property rights. This

aspect can sometimes obscure the details of promising therapeutic candidates from the broader scientific community and may delay their clinical exploration. The transition from promising preclinical findings to human clinical trials is inherently complex and time-consuming, necessitating rigorous validation of safety and therapeutic effectiveness in biologically relevant settings to meet the stringent requirements set by regulatory agencies like the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Mandates by these agencies require comprehensive assessments to ascertain the safety, efficacy, and quality of nanomedicine products, ensuring that they meet necessary criteria for clinical application.[97] This regulatory framework is necessary for guiding the responsible advancement of NP-based therapeutics from laboratory research to potential clinical use, underscoring the importance of a thorough and transparent development process.

4. Drug Delivery Considerations

NP-based drug delivery platforms present a sophisticated modality for improvements in OA treatment by exploiting intrinsic characteristics such as nanoscale dimensions, high surface-to-volume ratio, and customizable surface properties. However, leveraging these advantages requires deliberate and strategic NP design for optimal efficacy. This section delves into key considerations for achieving advanced drug delivery therapies in OA (Figure 4).

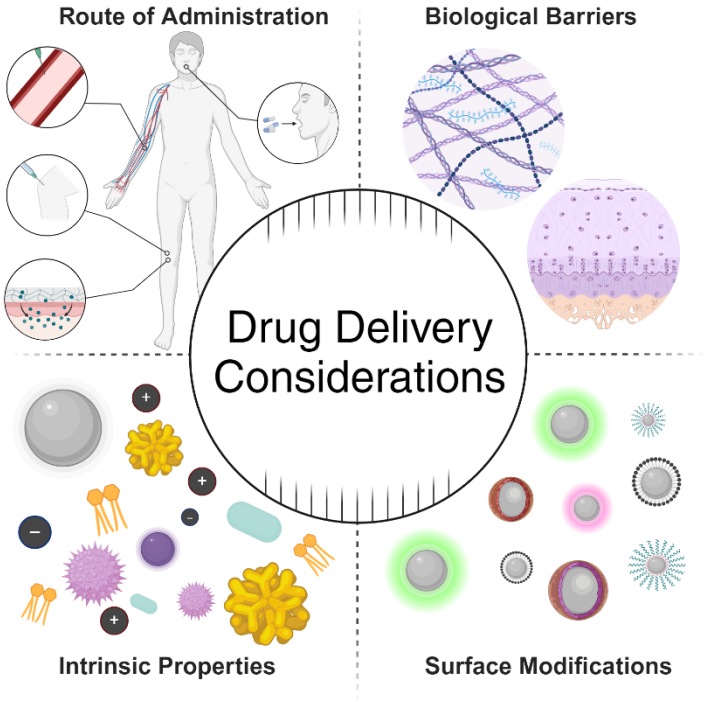

Figure 4. An illustration of the key aspects determining the design of the DDS for OA. Created with BioRender.com.

4.1. Route of Administration

The route of administration is a critical factor in the design and application of NP therapies. Traditionally, oral pharmacotherapy is one of the main types of OA management, with a broad variety of agents such as routinely prescribed NSAIDs and corticosteroids. While oral administration offers notable convenience, its long-term use is associated with adverse effects, including gastrointestinal irritation, renal dysfunction, and hepatotoxicity.[98] In the context of chronic disorders like OA, where prolonged symptom management is essential, the trade-off between effective symptom control and dosing convenience becomes particularly evident. Here, extended-release formulations are of particular interest.

For the management of mild arthritis or localized symptoms, topical pharmacotherapies such as NSAID-infused creams, gels, or transdermal patches can be administered to the surface of the affected joint. The efficacy of such treatments is dependent upon the physicochemical properties of the active pharmaceutical ingredient (API), including molecular size, polarity, and the incorporation of permeation enhancers.[99] To achieve therapeutic concentrations at the target site within the joint, the API's concentration within the topical formulation must be optimized to ensure sufficient bioavailability. This

route is advantageous for its minimization of systemic drug absorption and consequent adverse effects, thereby offering localized analgesic and anti-inflammatory benefits.

In the management of severe OA, the intravenous (IV) administration of corticosteroids and NSAIDs is employed to provide rapid alleviation of pain by ensuring the systemic presence of these analgesic agents.[100] The stability of therapeutic agents during IV administration is a critical consideration, particularly for complex formulations like NP-based DDS. The interaction of NPs with biological molecules in the bloodstream, leading to the formation of a PC, can significantly alter the pharmacokinetics, biodistribution, and efficacy of the encapsulated drugs. For this purpose, NP stealth strategies, such as surface PEGylation, have been employed to reduce potential protein adsorption.[101,102] The systemic administration of drugs may also result in off-target side effects[103], thus, IV administration is mostly reserved for cases where the benefits outweigh the potential risks.

IA administration, involving an injection directly into the joint, is a widely adopted strategy for localized symptom management in OA. Local delivery significantly limits the adverse effects of systemic adsorption, thus allowing the administration of higher doses. This approach is particularly advantageous for the administration of HA, which serves to lubricate the joint, thereby facilitating smoother joint movement and reducing pain.[43] Nonetheless, a direct exposure to the synovial environment poses several drug delivery challenges, such as rapid joint clearance, the viscosity of a SF, and dense and charged cartilage tissue.

4.2. Biological Barriers

The physiology of the joint space introduces a variety of biological barriers that determine the success of a DDS. Synovial membrane lines the joint capsule and is a critical barrier to substances entering the joint space from the bloodstream. Its selective permeability regulates the exchange of nutrients and waste products between the SF and surrounding blood vessels.[104] Lymphatic vessels within the joint space facilitate the removal of excess SF, cellular debris, foreign particles, and waste products, thereby contributing to the maintenance of joint homeostasis.[105] NP clearance of the joint is mediated by both of these components. In addition, the joint space is filled with SF which enables nutrient distribution to the cartilage and other joint tissues. However, it may also serve as a barrier due to its high viscosity, thus impeding diffusion and distribution of the DDS. The fluid dynamics of SF arise due to high protein and other biomolecule concentrations which facilitate a PC formation upon NP introduction into the joint space.[63,106] If NPs manage to bypass the latter obstacles, they still must pass through the charged cartilage ECM. While the average ECM pore size reported in the literature tends to vary from 2 nm to 100 nm[107–109], it is largely accepted that NPs with a diameter of <15 nm can penetrate the full-thickness cartilage (~4-5 mm).[65,110] In addition, the molecular permeability decreases with increasing GAG concentration and, consequently, tissue depth. Thus, while intact cartilage is impermeable to large molecules and particles, even the small particles may have trouble reaching the cells embedded in the deep tissue matrix.

4.3. Intrinsic Properties

As mentioned previously, the selection of materials is of great importance for nanomedicine-based strategies. First and foremost, the chosen material must be biocompatible to minimize adverse immune effects and cytotoxicity. It is often beneficial to ensure that the DDS are also biodegradable, allowing easy elimination from the body, and thus reducing long-term accumulation and toxicity. To optimize therapeutic outcomes and enhance targeting capabilities, these nanosystems should adapt physicochemical considerations and be able to accommodate specific functionalization.

4.3.1. Size

The size of NPs is a major factor determining NP biocompatibility and biodistribution within biological systems. By definition, "nano" applications intrinsically place particle size restrictions of <100 nm.[67] Within this realm, size variations significantly influence cellular internalization mechanisms.[111] Smaller NPs, with their higher surface area-to-volume ratio, are more readily internalized by cells. Specifically, NPs ranging from approximately 20 to 200 nm typically enter cells through endocytic processes.[112] NPs within the 20-50 nm range are predominantly internalized via clathrin-mediated endocytosis, whereas larger NPs are more likely to be taken up through caveolin-mediated pathways or macropinocytosis.[112–114] Notably, NPs <10 nm can directly diffuse across cellular membranes, entering cells through passive diffusion or specialized transmembrane channels.[112] Although nanoscale particles can achieve efficient cellular uptake, this efficiency comes with potential toxicity and off-target concerns.[115] The small size of NPs can lead to interactions that disrupt cellular functions, potentially resulting in cytotoxic effects.[116] The clearance of NPs from the body or specific sites, such as joints, is similarly influenced by their size. Studies involving systemic NP administration have identified an optimal size range of 20-100 nm for prolonged circulation.[117] NPs smaller than 100 nm are generally cleared more rapidly compared to larger particles.[118] On the other hand, larger NPs are unable to enter highly ordered tissues like cartilage but can effectively navigate through more loosely organized tissues and the synovial fluid, allowing for targeted delivery to less dense areas within the joint space.

4.3.2. Shape

The morphology of NPs plays a functional role in DDS as the shape of NPs influences the interaction with biological systems. Spherical NPs are often preferred for biomedical applications due to their resemblance to globular proteins. Studies have shown that mesenchymal stem cells exhibit a higher affinity for spherical NPs over those with rod-shaped or star-shaped geometries.[119] Furthermore, spherical NPs are less prone to phagocytic capture compared to NPs with angular shapes such as triangles or rods.[120] Nanorods and other NPs with high aspect ratios, have shown increased interactions with immune cells, leading to heightened inflammatory cytokine release.[121] This interaction not only triggers an immune response but can also activate complement pathways, as observed with rod and disc-shaped NPs, underscoring the complex interplay between NP shape and immune system activation. [122] The unique physical forces experienced by non-spherical NPs, like discs, result in distinct torques that can influence their behavior in the fluids, affecting their distribution and residence time within the

vasculature. [123] In the context of OA treatment, the clearance of NPs from joint space presents additional challenges, particularly for NPs with complex or elongated shapes. These shapes may experience slower clearance due to physical entrapment or interactions with the intricate biological structures of the joint. However, rod-shaped and ellipsoidal NPs, with their higher aspect ratios, can offer enhanced tissue penetration capabilities, facilitating better integration into the cartilage ECM and potentially improving drug delivery efficacy to affected areas.[124] While some studies report preferential uptake of spherical NPs by immune cells, others suggest that rod-shaped NPs may be internalized more efficiently, highlighting the importance of considering the context for specific applications and the interplay of other NP characteristics.

4.3.3. Hydrophilicity and Hydrophobicity

NP affinity to water is key for injection-based treatments. Hydrophilic NPs tend to be more readily dispersed in biological fluids, facilitating their circulation and reducing the likelihood of aggregation. Such NPs are typically less prone to non-specific protein adsorption, a phenomenon that can significantly influence cellular uptake mechanisms.[63] Hydrophilic particles can navigate the ECM more effectively due to their compatibility with the aqueous environment, facilitating the delivery of therapeutic agents into cartilage. A recent study by Vedadghavami et al. has reported the importance of hydrophilic NP profiles for cartilage targeting and the role of hydrophobic effects behind the cationic carrier interactions with SF.[125] Hydrophilic NPs are also less likely to be recognized and cleared by the immune system prematurely, which can further improve the efficacy of the treatment.[126]

Hydrophobic NPs offer advantageous cell uptake compared to hydrophilic counterparts due to their subsequent lipophilicity and affinity to cell membranes, however, they may also increase the risk of cytotoxicity due to membrane disruption or intracellular aggregation.[127] While these NPs can deliver lipophilic drugs and offer prolonged circulation time compared to the hydrophilic modalities, their efficacy can be compromised by prominent aggregation in aqueous environments. In addition, NPs with hydrophobic functionalities have been reported to induce inflammatory outcomes, emphasizing the need for considerate designs when incorporating hydrophobic moieties into materials for OA treatment.[128]

Alternatively, amphiphilic systems have been proposed to harness the advantages of both regimes. For instance, small amphiphilic (4-6 nm) NPs were reported to be internalized by ligand flipping. In this case, NPs get adsorbed to the lipid bilayer via electrostatic interactions and eventually diffuse into the bilayer without significant disruption.[129]

4.3.4. Surface charge

Electrostatic properties of NPs influence the interactions with the surrounding milieu. Electrostatic repulsion between NPs carrying similar charges can prevent aggregation, thereby enhancing colloidal stability within biological fluids.[130] This dispersion is essential for preserving the biological activity of NPs and preventing premature elimination from the body or the joint. However, excessive surface charge can lead to non-specific binding with serum proteins, potentially affecting NP stability and functionality as well as leading to the toxicity in biological systems.[131] For instance, cationic NPs are considered more

toxic than neutral or anionic counterparts due to their high affinity towards the negatively charged lipid membranes.[115] While this affinity may lead to increased cellular uptake via endocytosis, it may also provoke cytotoxic effects due to creation of pores within the lipid membrane. Cationic NPs can also display nuclear localization due to the proton sponge effect and subsequently escape the endosomal degradation.[132] Additionally, the positive charge can be advantageous, especially in targeting negatively charged tissues such as cartilage. The interaction with anionic molecules like GAGS within cartilage may enhance NP uptake and retention, offering a controlled release mechanism for therapeutic agents. Conversely, NPs that are neutral or negatively charged are generally less interactive with cell membranes and proteins. Neutral NPs primarily rely on hydrophobic interactions and hydrogen bonding for cellular entry at physiological pH, whereas anionic NPs may be internalized through endocytosis facilitated by positively charged sites on membrane proteins.[133] In the case of systemic administration, anionic NPs are prone to repulsive interaction with negatively charged membranes, which may lead to their rapid clearance by reticuloendothelial system uptake.[116]

4.4. Surface Modifications

Surface modifications are integral to optimizing the functionality and efficacy of DDS. Through chemical alterations or the application of coatings, it is possible to engineer systems with tailored properties that enhance their performance and aid in biological interactions. NP tracking, improved targeting, biocompatibility enhancement, and controlled release can be achieved through careful design and optimization of these modifications, thus making it possible to develop highly efficient, specific, and safe modalities for a wide range of applications.

4.4.1. Tracking

The integration of fluorescent markers that facilitate real-time monitoring of NP biodistribution and their localization within specific tissues and cellular environments is key for unraveling their behavior within biological systems. When selecting a suitable fluorescent label, several factors must be considered to ensure optimal outcomes. Fluorescent labels should exert minimal toxicity and not disrupt the normal functions of the biological system under investigation. Certain dyes, such as Nile Red, may exhibit cytotoxic properties that could compromise cell viability. In addition, the photostability of the fluorescent probe is paramount. It must resist rapid photobleaching to maintain signal integrity throughout prolonged analysis sessions. The proximity of the fluorescent probe to the NP surface, NP material composition, or any additional surface modifications can potentially induce fluorescence quenching, diminishing the emission intensity of the probe.[134] In addition, autofluorescence from biological samples also must be considered as it can obscure the detection of the fluorescent signal from the labeled NP, thus tissues rich in autofluorescent molecules, such as tryptophan, tyrosine, and phenylalanine necessitate careful fluorophore considerations or analysis methods.[135]

Popular choices for fluorophores include fluorescein isothiocyanate (FITC), known for its bright green fluorescence and application in labeling proteins, antibodies, and NPs; rhodamine derivatives, which provide robust red or pink fluorescence and excellent photostability; cyanine dyes, offering a spectrum

of emissions ideal for deep tissue imaging; and the Alexa Fluor® series, renowned for their wide-ranging color palette and superior brightness and stability.[136] When employing multiple fluorescent labels, spectral planning is essential to prevent fluorescence overlap.

4.4.2. Targeting

Targeting strategies in drug delivery require a balance between specificity, stability, biocompatibility, and therapeutic efficacy. Through the strategic integration of targeting ligands, such as peptides, small molecules, antibodies, or other targeting moieties to the NP surface, it is possible to achieve selective interaction with target receptors or biomolecules. For targeted drug delivery to cartilage, ECM-binding peptides, including those that mimic or bind to collagen[137–140], HA[141–143], and cartilage oligomeric matrix protein[144], have been successfully employed. These peptides facilitate a specific targeting of cartilage tissue, thereby extending the retention time of NPs within the tissue and potentially improving therapeutic outcomes. In addition, small molecules including receptor agonists[94], nucleic acids[145,146], growth factors[65,147–149] and other small molecules[150,151] have been utilized to target chondrocytes and macrophages, offering avenues for cell-based treatments. Alternatively, antibodies can be utilized for the same purpose but tend to be more costly than the latter modalities. Active targeting strategies may benefit from the incorporation of multiple functionalities within a single NP. In this case, dendrimer NPs stand out due to their numerous surface functional groups. Conjugation strategies of these agents should preserve the ligand's biological activity and ensure correct orientation for effective receptor binding. Common strategies include covalent bonding, bioconjugation, and click chemistry, each providing distinct benefits in terms of the resulting stability, ease of preparation, and adaptability of the conjugates.[152,153]

4.4.3. Stability

Considerations behind the colloidal and biological stability of DDS are necessary for the treatment's success. Incorporation of various surface coatings or surfactants can significantly reduce NP aggregation through steric or electrostatic stabilization mechanisms. Steric stabilization involves creating a physical barrier around each NP that prevents close contact and aggregation. For this purpose, PEG is particularly favored for its ability to form a hydrophilic and neutral "brush" around NPs.[154] This "stealth" layer minimizes protein adsorption and opsonization, effectively reducing recognition and clearance by the immune system.[101,102,155]The result of prolonged circulation time in the bloodstream may enhance the therapeutic efficacy of NPs. Other biocompatible polymers like PLGA, PLA, and polyvinyl alcohol (PVA) can be employed for this purpose.[156,157] Electrostatic stabilization, on the other hand, imparts a charge to the NP surface, leading to repulsive forces that counteract aggregation. Coating NPs with proteins or biomolecules such as albumin or HA can significantly improve their systemic circulation by evading immune detection.[143,158] Such negatively charged biomolecules create an electrostatic barrier that prevents the NPs from coming into close contact with one another. In particular, GAG coatings have been prominent for hydrogel-based DDS for tissue regeneration applications.[159] Additionally,

zwitterionic materials and biomimetic coatings can further stabilize DDS by providing a dense hydration layer or a biomimetic interface around the NPs.[160–162]

4.4.4. Controlled release

The concept of controlled release has revolutionized the field of pharmacotherapy by enabling precise modulation of drug release profiles. This dynamic process is influenced by a spectrum of degradation mechanisms inherent to the drug carrier, each adaptable to enhance drug delivery efficacy to joints. Diffusion is a universal concept in drug release, where the drug is released from the carrier matrix and propelled by the concentration gradient into the surrounding environment (SF in case of IA injection). Achieving a controlled and gradual diffusion is vital in joint applications, as it ensures sustained relief from pain or inflammation, which is particularly beneficial for managing chronic conditions such as OA. The diffusion-controlled release follows Fick's laws, with the initial release rate being relatively high due to the steep concentration gradient, which gradually diminishes over time.[163]

Recent innovative delivery systems are mainly designed to respond to specific internal stimuli, ensuring the drug is released in reaction to the unique conditions of the target environment. In the joint, such stimuli could include changes in pH, redox potential, enzyme activity, or osmotic pressure. A decrease in pH is a prominent feature of the inflammatory landscape arising from increased cellular metabolism and inflammatory mediators that induce local acidosis. A variety of pH-sensitive chemicals have been utilized in drug delivery including hydrazone and acetal-based linkers.[164] These pH-sensitive components are engineered to undergo structural changes or cleavage in acidic environments, thereby triggering the targeted release of therapeutic agents precisely at the sites of inflammation.[165] Alterations in redox potential are another significant characteristic of the inflammatory milieu within joint tissues, primarily driven by the elevated production of ROS and reactive nitrogen species during inflammation. These oxidative stress markers are a byproduct of increased cellular activity and the presence of immune cells at the site of inflammation, leading to a more oxidizing environment.[166] Redox-sensitive elements, such as disulfide bonds, are stable under normal physiological conditions but cleavable in the presence of elevated levels of reducing agents. Thiolation, the introduction of thiol (-SH) groups, is, therefore, a useful strategy to render NPs sensitive to alterations in the redox state.[167]

The presence of ECM enzymes is a distinctive aspect of arthritic disorders, such as OA and RA. These enzymes, including MMPs and aggrecanases, are upregulated in response to inflammation and play a critical role in the degradation of ECM components. Leveraging this enzymatic activity, innovative DDS have been designed to incorporate enzyme-responsive elements that can be selectively cleaved by these enzymes. Enzymatically controlled release systems often include substrates or linkers, like peptide sequences, that are specifically recognized and cleaved by the target enzymes present in the inflamed joint, thereby facilitating the controlled release of the encapsulated therapeutic agents directly in the joint space.[168]

Osmotic pressure-driven release mechanisms in DDS offer a unique approach to managing joint conditions, capitalizing on the principle of osmosis to facilitate the controlled release of drugs within the

joint space. This method involves the use of osmotically active agents such as pumps or hydrogels, which can absorb water from the surrounding SF, thereby creating an internal osmotic pressure that drives the release of the encapsulated therapeutic agents.[169,170]

5. Experimental Methods

The following chapter outlines the methodologies employed in this thesis for setting up comprehensive characterization, tracking, and detailing biological interactions of the NPs as well as OA-relevant biological models (Figure 5).

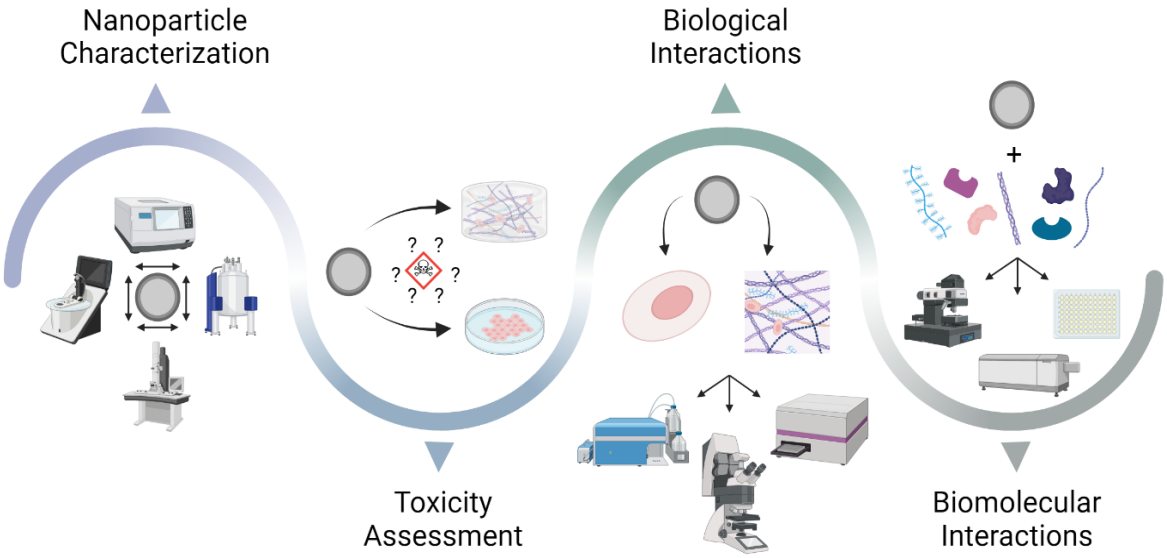

Figure 5. Experimental flow and methodology of the NP analysis. Created with BioRender.com.

5.1. Nanoparticle Characterization

Fine-tuning of physicochemical properties, morphology, and surface characteristics of the NPs is imperative due to their significant impact on NPs' interactions with biological systems and their overall therapeutic performance. Assessment of the NP stability, behavior, and safety is highly influenced by their chemical composition, size, shape, and surface charge, therefore, a detailed characterization of these aspects is essential for ensuring result validation and further research facilitation.

5.1.1. Nuclear Magnetic Resonance

Over the last decades, various nuclear magnetic resonance (NMR) techniques have been adapted for nanomaterial characterization at the atomic level.[171] Liquid and solid-state sample NMR analysis can be performed at numerous dimensions providing physical and dynamic parameters of various molecules and their conjugates. Purity, composition, and structural details can be elucidated by initially subjecting the sample to a powerful magnetic field, designed to align atomic nuclei with non-zero spin quantum numbers. This magnetic moment allows the atoms to distinctly respond to the external magnetic field. Subsequently, a specific radiofrequency is introduced to detect the transitional energy changes of the atom nuclei. Due to the shielding effect of surrounding electrons, the exact frequency at which a nucleus resonates depends on its chemical environment. This leads to small shifts in the resonance frequency, known as chemical shifts (δ), which provide information about the structure and conformation of the molecule. Work in this thesis mainly utilized one-dimensional proton NMR (^{1}H-NMR) as it provided sufficient information about the successful surface modifications at relatively low sample concentrations.

5.1.2. Electron Microscopy

Electron microscopy (EM) is one of the most powerful techniques for visualizing small particles, especially at the nanometer and sub-nanometer scales. Wave-like properties of electrons enable sample imaging down to atomic resolution due to shorter electron wavelengths compared to those of visible light. A beam of electrons is accelerated under a high-voltage environment and then used to illuminate the sample. Electron impact upon sample exposure results in electron transmission, X-rays, and secondary electron release which all offer information about the structure, composition, and topography of the sample. Advances in EM have led to the development of various specialized microscopy modes, enhancing the technique's ability to either target or circumvent particular attributes of samples, broadening its applicability, and offering a comprehensive analysis for a wide range of materials from rigid inorganic particles to delicate biological tissues.

Transmission Electron Microscopy (TEM) entails exposing a thin layer of sample to an electron beam, in turn revealing the arrangement of atoms within a bulk material or the biological ultrastructures of cells and tissues. TEM instruments may offer additional analytical tools such as diffraction patterns and elemental composition through techniques like energy-dispersive X-ray spectroscopy (EDX). It is important to ensure sample conductivity and compatibility under vacuum, thus polymeric colloidal materials often require additional heavy metal staining for enhanced contrast and drying before instrument exposure. As an alternative, cryogenic TEM (cryo-TEM) was introduced to mitigate beam damage and allow high resolution of aqueous and biological sample imaging. Vitrification of water in the sample allows the formation of amorphous ice which helps to preserve the native hydrated structure of the sample.

Scanning Electron Microscopy (SEM) involves the interactions of a focused electron beam with the atoms on the sample surface to produce various signals that are detected to form high-resolution 3D images. These signals include secondary electrons (for topographical contrast) and backscattered electrons (for compositional contrast), enabling the detailed visualization of the surface morphology and microstructure of materials. Like TEM, SEM can also be equipped with analytical tools like EDX to provide insights into the sample's chemical composition. Polymers and other non-conductive materials often require a conductive coating, such as gold or carbon, especially under high vacuum conditions to prevent the accumulation of electrical charge on the sample surface causing image distortions and reduced resolution. While SEM resolution is inferior to that of TEM, SEM provides versatility with bulkier samples and does not require a demanding sample preparation. For imaging samples in their natural, hydrated state without the need for coating or dehydration, environmental SEM or variable pressure SEM techniques allow the observation of samples under controlled pressure and humidity, thereby preserving their original morphology and minimizing electron beam damage.

5.1.3. Ultraviolet-Visible Spectroscopy

NPs possess unique optical properties which make Ultraviolet-Visible (UV-Vis) spectroscopy a valuable tool for identifying, characterizing, and studying nanomaterials. This spectroscopy technique employs

the principle of illuminating an NP suspension with UV or visible light and recording the absorption and scattering of light by the particles. Obtained spectral data can reveal information about the sample concentration and stability, which are influenced by factors such as particle size, aggregation state, surface chemistry, ionic strength and pH of the surrounding medium, providing critical insights into the interactions and behavior of NPs in various environments. UV-Vis spectroscopy may also be employed to confirm conjugation profiles of NPs by providing characteristic absorption spectra for specific functional groups or probes.

The concentration of NPs in a solution can be quantified using the Beer-Lambert Law[172] (Equation 1), which relates the absorbance of a solution to the concentration of the absorbing species.

Equation 1. $$A = \varepsilon * c * l$$

The equation above describes the linear relationship between the absorbance (A) of a solution and the concentration (c) of the absorbing species within it, as well as the path length (l) of the light through the solution. Estimation of NP concentration involves a calibration curve that can be established by measuring the absorbance at a specific wavelength for NP solutions of known concentrations. This curve is then used to determine the concentration of NPs in unknown samples by measuring their absorbance under the same conditions. It is important to note that deviations from this direct relationship may occur at elevated concentrations and high-intensity incident light due to electrostatic effects among the molecules. The stability of NP suspensions can be monitored by measuring the UV-Vis spectra at different time intervals. Changes in the absorbance spectrum such as peak broadening and shifting may indicate aggregation, dissolution, or chemical transformation.

5.1.4. Dynamic Light Scattering

Dynamic Light Scattering (DLS) is an essential tool in nanotechnology for analyzing the complex behavior of NPs in solution. By measuring the rapid fluctuations in the intensity of light scattered by particles in Brownian motion, DLS can deduce the diffusion coefficients of these particles, which are then translated into size, commonly expressed as the hydrodynamic diameter (D_h). The determination of hydrodynamic size relies on the Stokes-Einstein equation[173], which connects the particle's diffusion rate (D) to its dimensions such as radius (r), Boltzmann's constant (k_B) as well as the fluid's temperature (T) and viscosity (η) (Equation 2).

Equation 2. $$D = \frac{k_B T}{6\pi\eta r}$$

This measurement encompasses the particle's actual size plus any surrounding layers, like the solvation layer, and molecules or ions that might be attached, providing a comprehensive view of the particle's effective diameter in the medium. The distribution of NP sizes is summarized by the polydispersity index (PDI), where a low number indicates a narrow size distribution and homogenous particle suspension, whereas a high number may indicate sample impurities or aggregation.

In addition to size, DLS instruments can also assess the zeta potential (ζ), and electrical charge on the particle surface, providing a direct measurement of the electrostatic interactions among particles in fluid.

The electric charge arises due to the adsorption of ions from the solvent or due to the ionization of NP surface groups. This leads to the formation of an electric double layer, where an inner, stern layer is tightly bound to each particle and an outer, diffuse layer of counter-ions extends into the liquid. Thus, zeta potential is not the actual surface charge of the particle, but rather the potential difference between the bulk liquid and the slipping plane, a boundary that separates the layer of fluid and ions that move with the particle from the rest of the liquid.[174] Several theoretical models and equations relate the zeta potential of colloidal particles to measurable quantities, particularly in the context of electrophoresis, where particles move in a fluid under the influence of an electric field. These models help to interpret experimental data and understand the electrokinetic behavior of colloidal systems. For instance, a Smoluchowski[175] equation is particularly useful for analyzing electrophoretic mobility of larger colloidal particles where the electrical double layer is relatively thin compared to the particle size. Due to its robustness across a variety of conditions, especially in solutions with a higher ionic strength, the Smoluchowski model (Equation 3) is a widely employed in zeta potential assessments, where it relies on electrophoretic mobility (μ), dynamic viscosity (η), and dielectric constant (ε) of the material.

Equation 3. $$\zeta = \frac{\mu\eta}{\varepsilon}$$

This equation assumes no-slip boundary conditions at the particle surface and is most applicable for larger particles in low ionic strength solutions. Many modern instruments that measure zeta potential can automatically choose the most appropriate model based on the experimental conditions or use more sophisticated algorithms that cover a broader range of conditions, such as the O'Brien and White theory[176], which provides a more comprehensive treatment of electrophoretic mobility and zeta potential relationships.

5.2. Biological models

NP applications require appropriate biological models that would accurately resemble the native processes found in the joint as well as replicate the challenges NPs might encounter in an in vivo environment. Studies in this thesis have been mainly performed in fibroblast, chondrocyte, and macrophage cell lines, primary monocyte-derived macrophages and fibroblast-like synoviocyte (FLS) cells, and porcine tissue explants.

5.2.1. Cell Culture

Cell culture is a foundational technique in biological research that enables the growth and maintenance of cells outside of their original organism. This method involves using nutrient-rich media to cultivate cells on a suitable substrate within a container, typically in a controlled sterile environment of 37°C, 95% humidity, and 5% CO_2. While the cells may originate from various tissues or organisms, immortalized cell lines are typically less sensitive than freshly isolated primary cells. In these studies, mouse fibroblast (L-929) and human monocyte (U-937) cell lines have been obtained from Sigma, while the human chondrocyte (TC28a2) cell line was a gift from Dr. Cronstein's lab at NYU Langone, USA.

Anonymous healthy human donor buffy coats obtained from the blood bank at Sahlgrenska University Hospital in Gothenburg, Sweden were used to isolate peripheral blood mononuclear cells using density gradient centrifugation. Cells were then stimulated with a macrophage colony-stimulating factor to yield MDMs. RA- and OA-patient-derived FLS cells were obtained from Sahlgrenska University Hospital, Gothenburg, Sweden. All cells were cultured according to ATCC guidelines.

5.2.2. Cytotoxicity

Cytotoxicity assessment is essential for determining potential adverse effects of NPs on living cells and is required across various applications including pharmaceutical and medical industries. A variety of assays employing dyes or colorimetric indicators have been developed to assess the degree of cell viability. Among the most common assays employed is the Trypan Blue Exclusion assay which utilizes Trypan Blue, a dye that stains cells with compromised membrane integrity. The dye may be substituted with a less toxic alternative such as Erythrosine B to limit intrinsic assay interference. The premise of this assessment is based on counting the colored cells which may introduce bias if performed manually yet offer efficiency when using automated cell counters. Similarly, lactate dehydrogenase release and Annexin V/Propidium Iodide staining may also be used to assess the loss of membrane integrity and subsequent decline in cell viability.

In some cases, assays with heightened sensitivity are required. By focusing on metabolic reactions, assays like 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Alamar Blue allow the identification of subtle effects on cell health due to their reliance on mitochondrial activity. For instance, the MTT assay evaluates the reduction of the yellow MTT dye to purple formazan crystals by mitochondrial dehydrogenase enzymes in metabolically active cells. Here, the amount of formazan produced is proportional to the level of metabolic activity, serving as an indirect measure of cell viability. Likewise, Alamar Blue or Resazurin Reduction assay utilizes resazurin, a blue dye, reduction to fluorescent pink dye, resorufin, by viable cells' metabolic activities, enabling non-destructive, continuous monitoring suitable for high-throughput applications, albeit with potential fluorescence interference. This was the method used to assess the NP toxicity for the studies reported in this thesis.

5.2.3. Explant Extraction and Culture

Most NP studies in this thesis have been performed in an ex vivo porcine articular cartilage model. The porcine model was specifically chosen due to its anatomical and biomechanical similarities to human joints.[177] 3–6-month-old pig legs were obtained from the Experimental Biomedicine animal facility in Gothenburg, Sweden under the 3R principle. The 3R principle refers to a set of ethical guidelines in the use of animals in scientific research, aimed at minimizing harm and improving animal welfare i.e. replacement, reduction, and refinement. Healthy articular cartilage plugs were extracted mainly from femoral condyles using biopsy punchers and trimmed to exclude the underlying subchondral bone. Some explant batches were snap-frozen in PBS supplemented with 1% Penicillin/Streptomycin (P/S; 10,000 U/mL, Gibco) and protease inhibitors (Roche). Other times the explants were placed in culture in

Dulbecco's Modified Eagle Medium without phenol red, supplemented with P/S and GlutaMAX™, and allowed to equilibrate overnight before starting the experiments.

5.2.4. Tissue Histology

Histological examination of tissues and cells enables the visualization and comparison of structural and organizational changes in response to an intervention. The successful histological examination is highly reliant on diligent sample preparation including steps like fixation, embedding, sectioning, staining, and mounting. Cartilage preparation for histology entailed a 3-week decalcification step at 4°C. The samples were then embedded in paraffin blocks cut perpendicular to the sagittal plane and stained with hematoxylin/eosin (H&E) and Safranin-O/fast green. OARSI score was judged blindly based on a previously published murine OA scoring system[178], where a scale of 0-6 was employed to indicate pathological changes in cartilage from none to severe, respectively.

5.3. Biological Interactions with Nanoparticles

NP capacity to enter tissues and cells is dependent on the surrounding environment governed by a variety of processes and pathways. To enable NP visualization in a complex biological setting, NPs are often tagged with fluorescent probes that absorb light at a specific wavelength. Light particles, also known as photons, provide the energy that leads to electron excitation in the probe and a subsequent jump to a higher energy state. As electrons return to their ground state, the emission of lower energy photons results in the generation of a fluorescence signal. Fluorescence-based methods including spectroscopy, microscopy, and flow cytometry (FC) enable sensitive and specific NP tracking and quantification in both tissues and cells.

5.3.1. Fluorescence Spectroscopy

Fluorescence spectroscopy serves as a powerful analytical tool for probing fluorescently labeled NPs. Here, the emitted light is collected and analyzed by a spectrophotometer, which separates the light into its component wavelengths and measures the intensity at each wavelength, often resulting in a spectrum detailing the present fluorescent species. Fluorescence quantification of NPs provides an additional layer of characterization as fluorescence intensity becomes proportional to NP concentration, thus allowing the investigations on diffusion dynamics and tissue interactions. For example, NP uptake into cartilage can be determined by measuring the fluorescence intensity in the supernatant over time, which reflects the proportion of NPs remaining in the surrounding medium as they are absorbed by the tissue. The decrease in fluorescence intensity outside the cartilage directly correlates with the degree of NP penetration and accumulation within the tissue matrix. Such quantitative analyses can reveal the efficiency of NP delivery systems, the kinetics of tissue penetration, and the potential for sustained release within targeted sites. Moreover, by applying custom conditions like pH, ionic strength, and the presence of biological molecules, NP behavior can be examined to provide a comprehensive understanding of NP interactions in physiological conditions.

5.3.2. Confocal Fluorescence Microscopy

Confocal fluorescence microscopy offers optimal visualization of tissue specimens as it leverages a pinhole to eliminate out-of-focus light, thereby providing a clear image of a specimen slice at a specific depth without interference from surrounding tissue. The light that passes through the pinhole is detected by a photodetector, such as a photomultiplier tube which converts a light signal into an electrical signal, which is then digitized. This feature is particularly beneficial for the dense and heterogeneous structure of cartilage tissue, where the precise localization of NPs within the intricate matrix and among chondrocytes is important. The laser-scanning confocal microscope allows for the sequential acquisition of images at different depths, enabling the construction of a 3D representation of NP distribution within the cartilage. This z-stacking capability is fundamental in understanding how NPs penetrate, disperse, and interact within the cartilage ECM.

5.3.3. Flow Cytometry

FC is a high-throughput particle characterization technique where the particles (cells, microorganisms, biomolecules, or NPs) are suspended in sheath fluid as they individually pass through a laser beam. The same principles of fluorescence apply to FC as the previously mentioned techniques, however, in this case, the output consists of physical and chemical particle characteristics such as approximate size, granularity, and fluorescence intensity of specific fluorescent markers at single particle resolution. Furthermore, the ability of FC to sort particles based on their fluorescent properties enables the separation of NPs with different characteristics or the isolation of cells that have internalized NPs, providing valuable samples for further analysis. This sorting capability is particularly useful when studying the heterogeneity of NP uptake by different cell types complementing the spatial information obtained from confocal microscopy. Some limitations of FC include fluorescent overlap of fluorescent tags as well as the need to enzymatically or mechanically disaggregate cells which can lead to cell surface marker alteration.

5.4. Biomolecular Interactions with Nanoparticles

Interactions between NPs and their surrounding environment are inherent and often intricate. These encounters can vary significantly based on the specific modifications applied to the NPs, resulting in varying affinities for different biomolecules. While a comprehensive understanding of how NPs engage with diverse biological systems may appear daunting, several techniques allow us to determine and quantify the potential interactions.

5.4.1. Biochemical Assays

Biochemical assays serve as indispensable tools for providing quantitative and qualitative insights into various markers associated with cartilage integrity and inflammation status. By enabling precise measurements of various biomolecules such as GAGs, collagen, and others, they facilitate the evaluation of cartilage degradation and the overall state of the joint tissue.

Quantification of sulfated GAGs (sGAGs) is often assessed by dimethyl methylene blue (DMMB) assay. DMMB is a cationic dye that exhibits a high affinity for the negatively charged sulfate and carboxyl groups present on GAG molecules in cartilage tissue. This interaction causes a colorimetric shift that is proportional to the concentration of sGAGs in the sample.

Alternatively, the content of collagen can be quantified by measuring hydroxyproline, an amino acid distinctive to collagen. The assay involves multiple steps of sample processing as collagen molecules need to be broken down and rendered reactive to result in a chromophore. This enables an absorbance-based quantification of hydroxyproline that is directly related to the concentration of collagen in the sample.

For more targeted analysis, Enzyme-Linked Immunosorbent Assay (ELISA) can be utilized to assess the levels of various biomolecules based on the specific interactions between antibodies and antigens. An enzyme linked to an antibody provides a detectable signal, resulting in a color change and revealing the concentration of the analyte. This method enables a sensitive and specific quantification of ECM proteins and cytokines informative of the physical and biological effects of NPs.

5.4.2. Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a versatile technique that allows studying a variety of materials, from biological specimens to polymers and other nanomaterials. It employs a sharp, sensitive tip attached to a flexible cantilever that interacts with the sample surface at the atomic scale. By scanning the tip across the surface of a specimen, the instrument measures the chemical, electrostatic, and van der Waals forces between the tip and the atoms or molecules of the sample. These force measurements are then used to create detailed topographical maps, offering a visualization of the surface features in the sample. AFM can be utilized to assess NP-biomolecule aggregates, providing insight into their structural organization and interactions at the nanoscale. Moreover, AFM can reveal information about the mechanical properties, adhesion forces, and even electrical properties of the sample. The instrument can often be operated in multiple modes and can examine materials under various environments, thus sparing extensive sample preparation. This flexibility makes AFM an excellent choice for studying a multitude of materials, from biological specimens and polymers to nanomaterials and surfaces.

5.4.3. Circular Dichroism spectroscopy

Circular Dichroism (CD) is an analytical spectroscopy method that assesses the structural characteristics of chiral molecules by measuring the differential absorption of left and right-handed circularly polarized light in the sample. The resulting CD spectra provide insights into the secondary structure of biomolecules, their conformation states, folding patterns, and interactions with ligands or other interacting molecules. The technique requires minimal sample preparation and can be applied to solutions, making it non-destructive and suitable for studying biological molecules in conditions close to their native environment.

5.4.4. Proteomics

When NPs interact with biological environments, they invariably become enveloped by a layer of biomolecules and proteins, resulting in the formation of a PC. This corona fundamentally alters the NP properties, influencing their cellular uptake, biodistribution, and potential toxicity. The comprehensive characterization of this PC is crucial, as underscored by numerous studies, due to its significant impact on NP biological interactions and behaviors.

Proteomics emerges as a pivotal high-throughput technique in this context, offering the capability to systematically identify and quantify the proteins associated with NPs. This technique is central to the in-depth characterization of the coronas and to understanding the modifications in protein expression induced by NP exposure. Utilizing advanced mass spectrometry, alongside bioinformatic and molecular biology tools, proteomics can uncover subtle changes in protein expression levels, post-translational modifications, and interaction networks triggered by NPs. The strength of proteomics lies in its ability to capture these extensive protein dynamics within their authentic biological context.

5.5. Statistics

Various statistical tests were used to evaluate the significance of the results reported in this thesis. The choice of analysis was informed by the nature of the data and the specific hypotheses being tested. For example, Student's t-test was utilized to compare mean values between two independent groups when the data followed Gaussian distribution and there was no variation in the sample size. Alternatively, for comparisons entailing three or more independent groups, Analysis of Variance (ANOVA) was used. To pinpoint the significant group differences, several post-hoc tests were utilized. For instance, Dunnett's test was used when comparing all treatment groups to a single control group, while Tukey's Honest Significant Difference test was applied for pairwise comparisons among multiple groups. Alternatively, Bonferroni correction was utilized to adjust the significance level for each individual test when conducting multiple pairwise comparisons, thereby reducing the overall risk of Type I errors across all comparisons.

For the analysis of proteomics data, the Shapiro-Wilk test was first applied to assess the normality of the data distribution. Differential protein analysis was based on the principles of Bayesian statistics. In cases of data skewness, the non-parametric Mann–Whitney U test was utilized to compare differences between two independent samples. The false discovery rate was controlled by employing the Benjamini-Hochberg procedure for multiple hypothesis testing.

6. Original Work

This doctoral thesis aimed to build upon the recent discoveries in nanomedicine to provide new insights into important aspects of drug delivery for OA. Specifically, this work focuses on the NP design and their interactions with SF and cartilage tissue for IA cartilage targeting. Studies entailing SF focused on its effect on a) NP uptake via PC interactions (**Paper I**), b) cartilage degradation (**Paper III)**, and c) NP uptake via PC interactions in catabolic cartilage (**Paper IV**). Additionally, direct interactions between the NPs and cartilage tissue components such as HA, ACAN, disintegrin, and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (**Paper II**), and collagen (**Paper IV**) were discovered. Another part of the thesis focused on the sex-difference-induced OA progression by mimicking the post-menopausal state and attenuating its symptoms with 17β-estradiol (E2) treatment (**Paper V**). The rest of this chapter provides a more detailed summary of the research results obtained throughout these doctoral studies.

6.1. The Impact of Patient Synovial Fluid on NP Uptake

Small cationic NPs are promising modalities for cartilage delivery and have been previously reported to achieve effective cartilage tissue penetration.[64,65,179] While most conventional medicines are unable to penetrate dense and highly anionic cartilage ECM, cationic carriers can leverage electrostatic interactions to enable rapid uptake and prolonged tissue retention. Nonetheless, no studies have previously studied the effects of patient SF on NP efficacy. To address this, in **Paper I** we synthesized a panel of PAMAMs possessing a variation of sizes and charges to investigate SF implications on cartilage drug delivery (Figure 6). A PEGylation strategy was used to modify the surface charge as it has been previously reported to resist protein adsorption.[155,180] Two different PEG lengths were used while the surface conjugation ratio was ~2% for both NPs.

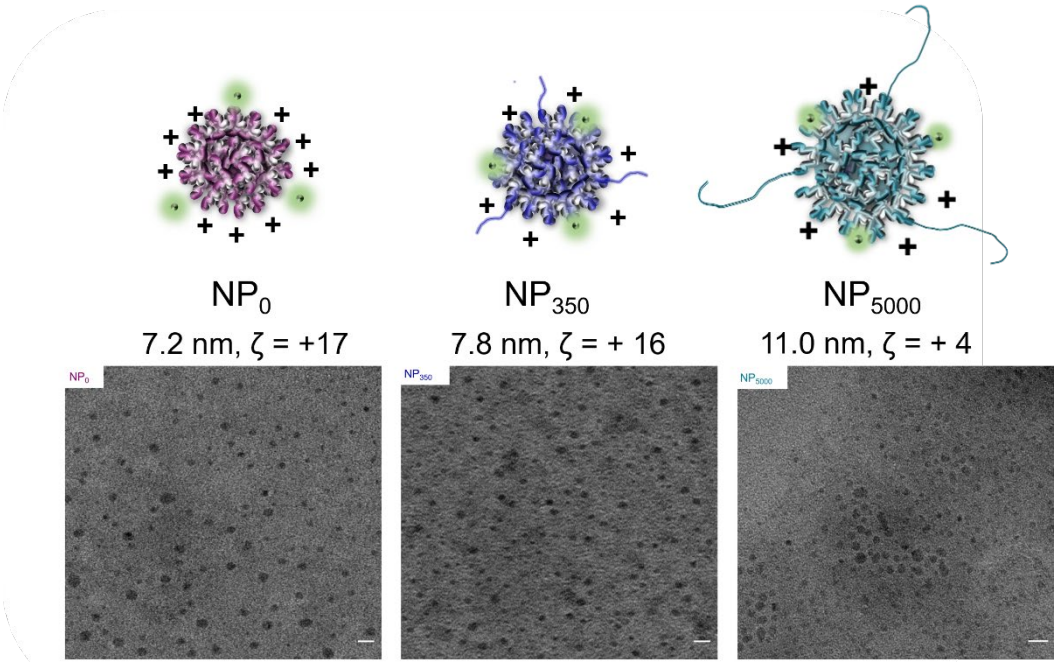

Figure 6. Characterization of PAMAM-based NP panel. NP physicochemical properties were established by DLS, while images were obtained using TEM. The scale indicates 10 nm.

To determine the effects of OA and RA patient SF on NP efficacy, NPs were incubated with the patient material for 1 hour at 37°C to enable PC formation around the NPs. The samples were then washed to remove excess proteins. NP-protein complexes were then subjected to tissue and cell studies.

To investigate the tissue uptake, porcine cartilage explants were used since porcine articular cartilage exhibits similar thickness to human. While all NPs were taken up into the tissue under PBS conditions within 24 hours (Figure 7), SF as well as fetal calf serum (FCS), which was used as a control, resulted in decreased uptake profiles compared to protein-free conditions. Comparatively, FCS-exposed NPs resulted in higher uptake than SF-exposed NPs. This may imply that the use of common serum sources such as FCS in cell and explant culture media may result in an overestimation of NP uptake profiles. Additionally, NP5000, an NP decorated with long PEG chains, resulted in a similar uptake as FCS when exposed to OA-SF, suggesting its favorability over other NPs for OA applications.

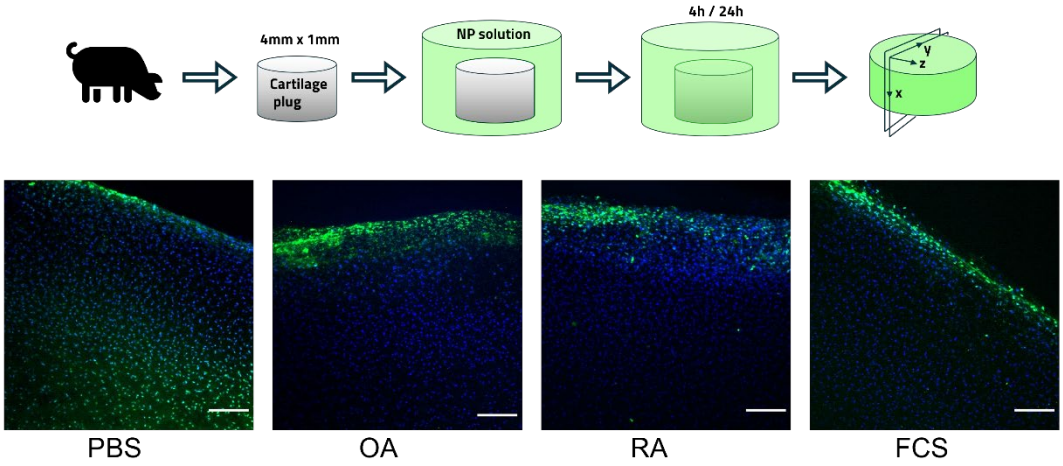

Figure 7. A representative image of NP0 uptake under different protein conditions. Here, PBS implies protein-free conditions, OA and RA indicate patient SF, and FCS indicates fetal calf serum. The scale bar indicates 50 µm.

At the same time, the uptake of NPs into joint-associated cells was also of interest. Cellular uptake was tested in both chondrocyte (Tc28a2) and monocyte (U937) cell lines (Figure 8) as well as primary OA and RA-patient derived FLS cells. Similarly, we observed a higher uptake in FCS-exposed NPs compared to SF NPs. While the highest levels of cellular uptake for SF-exposed NPs were observed for chondrocytes, overall, the overall uptake of these NPs was substantially low.

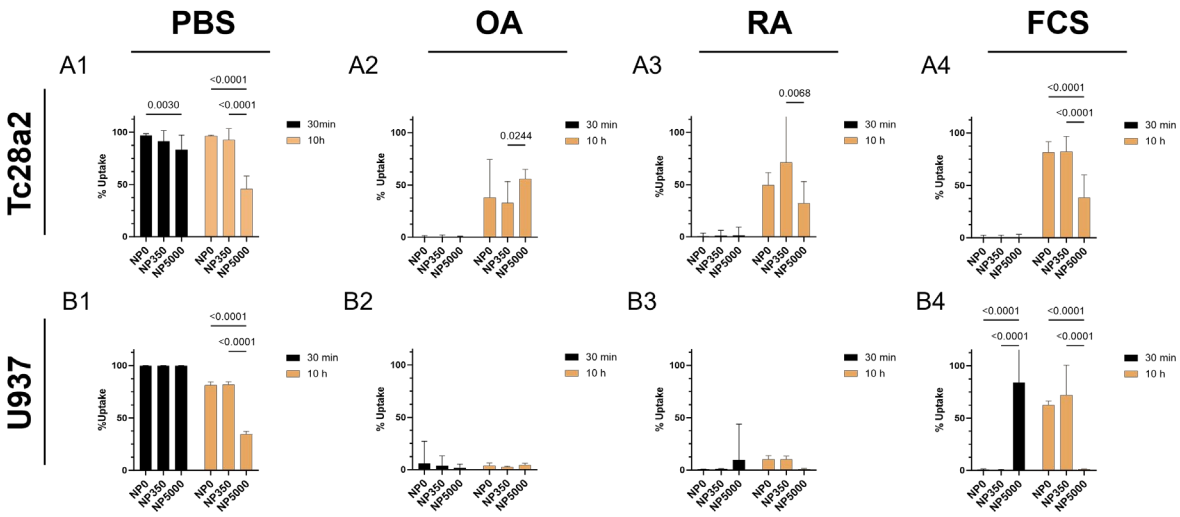

Figure 8. NP cellular uptake in chondrocyte (Tc28a2) and monocyte (U937) cell lines after 30 minutes (black) and 10 hours (yellow). Analysis was performed using one-way ANOVA with Tukey's post hoc test.

Both biological studies indicated that the varied surface chemistries of the NPs did not play a significant role in their uptake, but rather it was the PC formation that dictated the uptake profiles. Utilizing

proteomics, we detected individual corona differences on the particles as well as the differences between the protein sources (Figure 9).

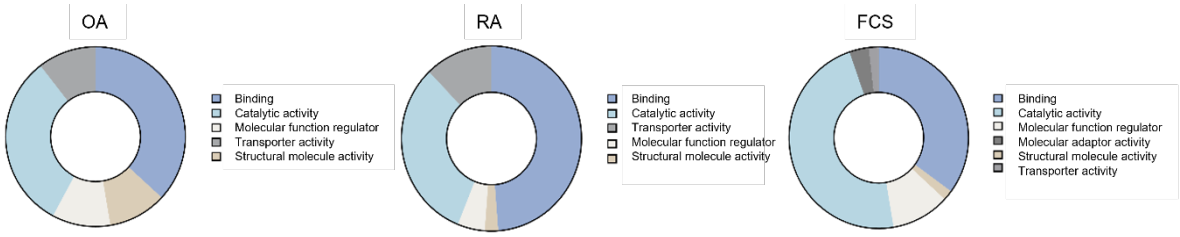

Figure 9. Proteomic pathway enrichment results for OA (A) and RA (B) patient SF and FCS (C).

This study highlights the significant role of biocoronas in influencing the uptake of NPs into cartilage tissue, particularly affecting their absorption by chondrocytes, monocytes, and patient FLS cells. We observed distinct differences related to specific NPs and proteins among the groups, and we identified key proteins that may explain these variations. This research underscores the crucial impact that PCs from SF of OA and RA patients have on NP uptake into cartilage and emphasizes the need to consider the biological microenvironment in successfully translating drug delivery vehicles into clinical applications.

6.2. NP Interactions with Catabolic Cartilage

Polymeric and lipid NPs have shown great potential for effective drug delivery, but they can be limited by interactions with the local microenvironment. In **Paper II**, the goal was to investigate two different cationic NP strategies and their interactions with ECM under OA-relevant catabolic conditions. Here, small PAMAM-based NPs such as NP0 (smaller diameter, higher charge) and NP5000 (smaller diameter, lower charge) were used for cartilage penetration and their PLGA-based counterparts, PLGA-PEI_H (larger diameter, higher charge), and PLGA-PEI_L (larger diameter, lower charge) were employed to achieve the cartilage surface binding, as they are too large to enter the tissue. Their utilization was investigated in an enzymatic OA porcine cartilage model, where prior to the NP exposure, cartilage explants were subjected to enzymatic degradation by collagenase (COL), hyaluronidase (HYA), or ADAMTS5 (ADA), enzymes that play a key role in OA progression. The study overview is depicted in Figure 10.

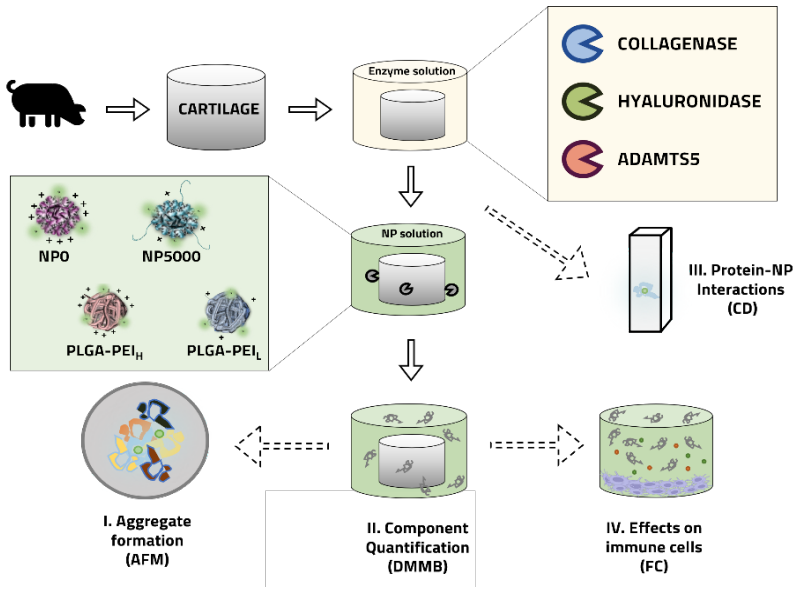

Figure 10. A schematic representation of the NP investigation in an ex vivo porcine enzymatic model of OA.

sGAG concentration in the supernatants of the cartilage explants was quantified using the DMMB assay with and without HYA, ADA, and COL catabolic stimulation (Figure 11). NP0 and NP5000 treatments resulted in a decrease in GAG concentration under no enzyme, HYA, and ADA conditions. This likely suggests that small cationic NPs are able to stabilize GAG structures before and during the enzymatic degradation, thus providing a transient yet supportive role via electrostatic interactions with sGAG. While the exact mechanism is not clear, an increased GAG release in COL condition with NP0 might indicate the synergistic degradation effects.

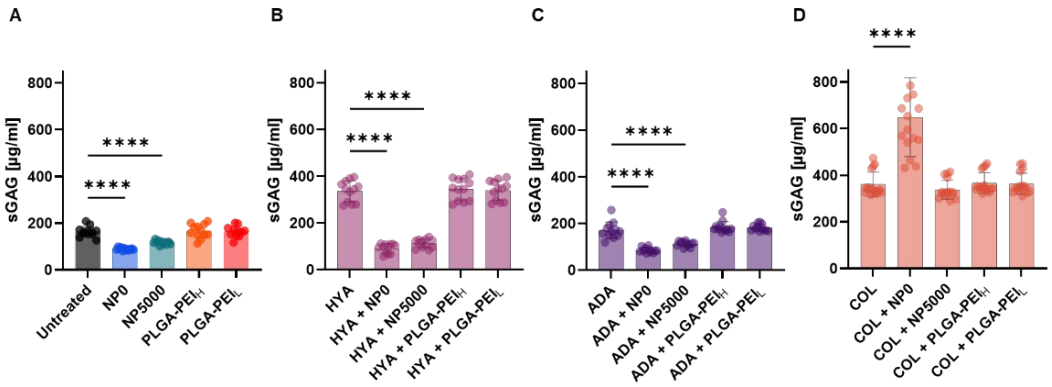

Figure 11. sGAG quantification in ex vivo cartilage culture supernatants. Cartilage was first subjected to enzymatic treatment control (A) or hyaluronidase (HYA) (B), ADAMTS5 (ADA) (C), or collagenase type II (COL) (D) for 2 hours. Subsequently, NP treatment was introduced for another 2 hours, and the harvested supernatant was analyzed using DMMB assay. Data are represented as mean values ± S.D. Analysis was performed using one-way ANOVA with Tukey's post hoc test, where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

To investigate the interactions between the ECM components and NPs, CD spectroscopy was used. Here, we investigated both the previously used enzymes and their degradation products. Shifts in CD spectra, implying secondary structure changes, were observed for HA, ACAN, and ADA (Figure 12).

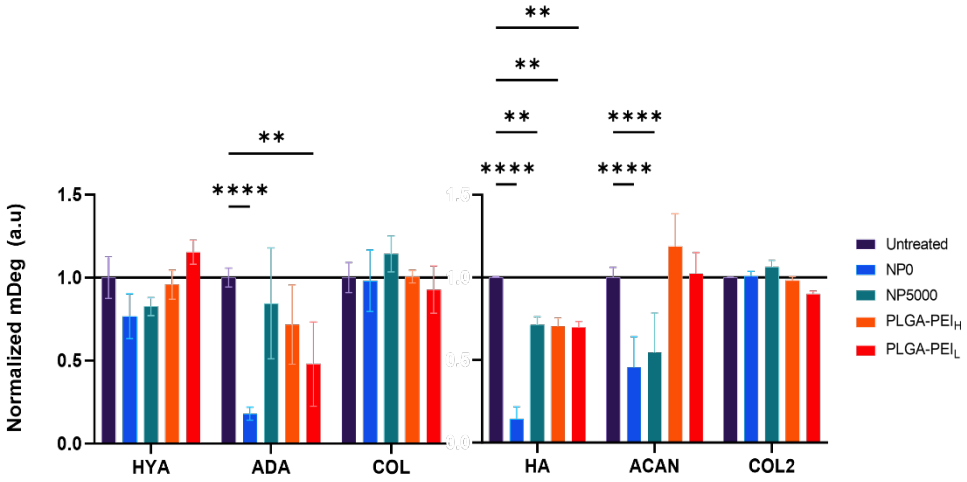

Figure 12. NP interactions with ECM enzymes (bottom) and their respective degradation products (top). The bars represent the changes in CD at the most prominent peaks that were normalized to the intrinsic CD of each biomolecule. Analysis was performed using one-way ANOVA with Tukey's post hoc test, where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

It was also hypothesized that the results of enzyme-induced degradation could contribute to NP PC formation. Considering that enzyme degradation products, especially those derived from the ECM, have been reported to possess DAMP qualities[20], we wanted to investigate the potential NP-biomolecule effects on immune cell response.

MDMs were extracted from the buffy coats, differentiated, and subjected to the ex vivo cartilage culture supernatants for 24 hours. Inflammation-related cytokines produced by the stimulated macrophages were collected and analyzed via FC (Figure 13). Interestingly, a prominent inflammatory cytokine release was observed for both NP0 and N5000, while low levels of cytokine production were observed for the PLGA NPs. Small and charged NPs can likely evoke an immune response due to biomimicry and large surface area-to-volume ratio.[181] However, surface treatments that prevent biofouling, like PEGylation, continue to play a crucial role in modulating the immune response as seen from the lower inflammatory cytokine levels of NP5000. Given the inability of larger particles, like PLGA, to infiltrate dense cartilage tissue, low levels of cytokine induction confirm that these systems are more appropriate for controlled release applications in the joint space.

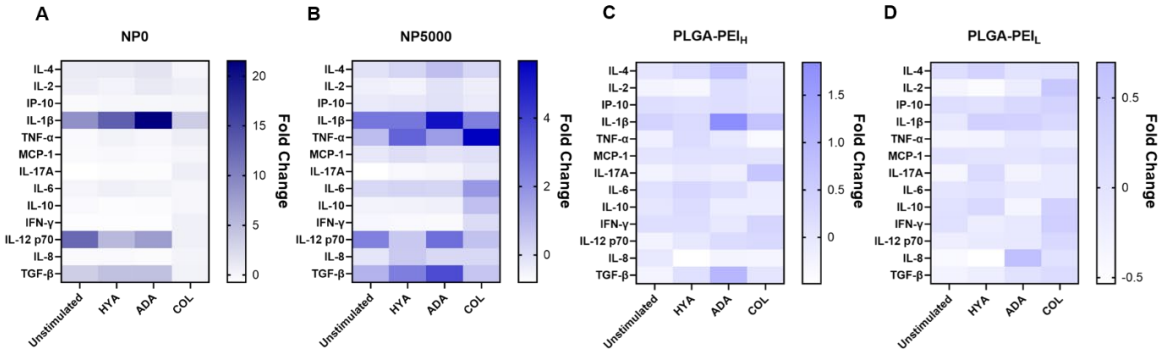

Figure 13. Cytokine expression from macrophages stimulated by ex vivo cartilage supernatants expressed as fold change. Stimulations included NP (A), NP5000 (B), PLGA-PEI$_H$ (C), and PLGA-PEI$_L$ (D) NPs.

Despite the high potential of NPs to achieve specific targeting and enhanced therapeutic efficacy, the translation into clinical practice often overlooks the pertinent clinical context with patient-specific disease profiles. This study employed an ex vivo catabolic cartilage model which may not fully represent physiological context, however, offers a fundamentally native, yet controlled environment. The results suggest that the catabolic environment present in OA cartilage results in multifaceted effects on NP interactions with the tissue ECM, its biomolecules, and immune cells. While small NPs are optimal for early-stage OA as they can penetrate the tissue, it is crucial to understand the consequences of the NP surface properties under catabolic conditions, especially in the context of inflammation-driven degeneration like in OA disease progression.

6.3. NPs as Cartilage Degradation Probes

In **Paper III**, two unique polymer-based nanocarriers, lysine-functionalized nanorods, also known as biodynamers (BD-Lys), and spherical PEG-functionalized PAMAM-dendrimers (PAMAM-PEG), were utilized to explore three distinct mechanisms of degradation within live articular cartilage tissue samples (Figure 14). Both NPs are promising nanocarriers previously employed as mRNA delivery vehicles[75,182,183], however, while PAMAM efficacy has been established by our previous studies, the implications of BD-Lys for cartilage applications are currently unknown. Thus, this study assessed the probing ability of these two NPs in a pathophysiologically relevant cartilage model.

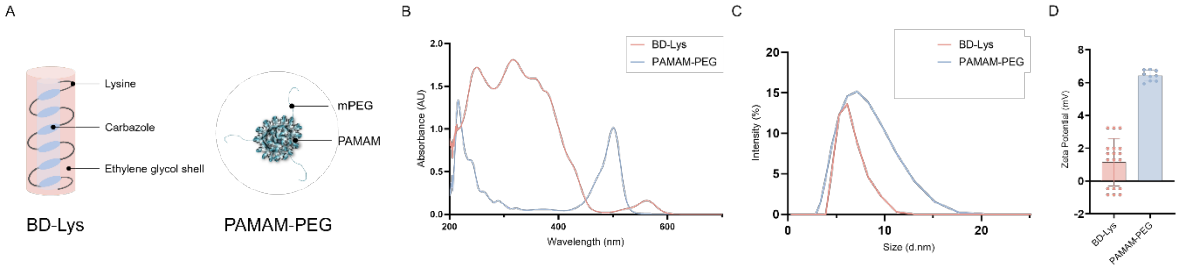

Figure 14. Illustration of the BD-Lys (pink) and PAMAM-PEG (blue) (A). NP spectral (A), size (B), and zeta potential (C) characteristics.

IL-1β, a potent inflammatory cytokine, triggers a signaling cascade in chondrocytes that leads to cartilage degradation by activating MMPs, serving as an in vitro model for OA. Alternatively, SF from arthritic patients is also known to possess prominent catabolic and inflammatory markers further driving cartilage degradation. One of the most prominent enzymes that mediate cartilage degradation is collagenase (COL). It was hypothesized that implementing proinflammatory conditions, possibly reducing tissue pH, could enhance NP electrostatic interactions with cartilage. Based on the confocal imaging, both NPs demonstrated significant penetration into the tissue (Figure 15). Interestingly, the BD-Lys signal in the confocal images revealed distinct cartilage features compared to PAMAM-PEG, indicating possible interactions with degraded tissue components.

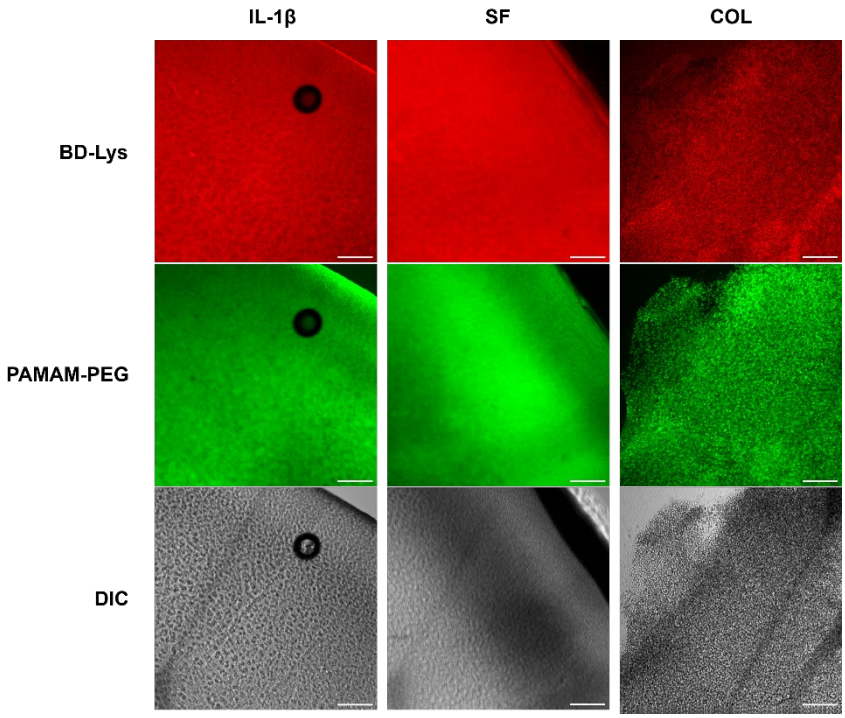

Figure 15. NP uptake into cartilage tissue treated with 1L-1β, RA patient SF, or COL and subsequently treated with BD-Lys (red) and PAMAM-PEG NPs (green). Images were obtained using confocal microscopy. Scale bars indicate 200 µm.

The signal of BD-Lys outside the tissue boundaries prompted an investigation of the possible interactions between the BDs and collagen. Utilizing AFM, the supernatants of COL-induced cartilage degradation were imaged along with BD-Lys NPs (Figure 16). When BD-Lys NPs were introduced to the COL-degraded cartilage, resulting aggregates appeared bigger in size and were surrounded by empty space. This further suggested that BD-Lys and COL2 interactions were plausible.

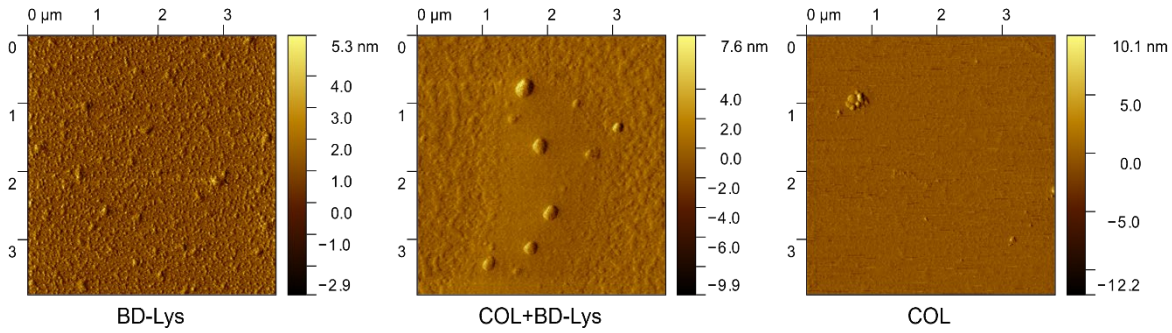

Figure 16. AFM images of ex vivo cartilage explant-derived supernatant aggregate profiles containing BD-Lys, BD-Lys, and COL, or COL only.

To determine whether the BD-Lys NPs interact with COL2 directly, CD spectroscopy was applied to probe for any conformational changes (Figure 17). Collagen is known for its unique triple-helix configuration, which is evident in its CD spectra through a characteristic negative peak near 200 nm and a positive peak in the 220-230 nm range. When COL2 was incubated with BD-Lys, there was a noticeable decrease in the random coil formations within the collagen, as indicated by a shift in the negative peak to 195 nm. This change in structural conformation towards a more ordered arrangement implies an

increase in the thermodynamic stability of the complex formed between collagen and BD-Lys NPs, suggesting that specific interactions are occurring between BD-Lys NPs and COL2.

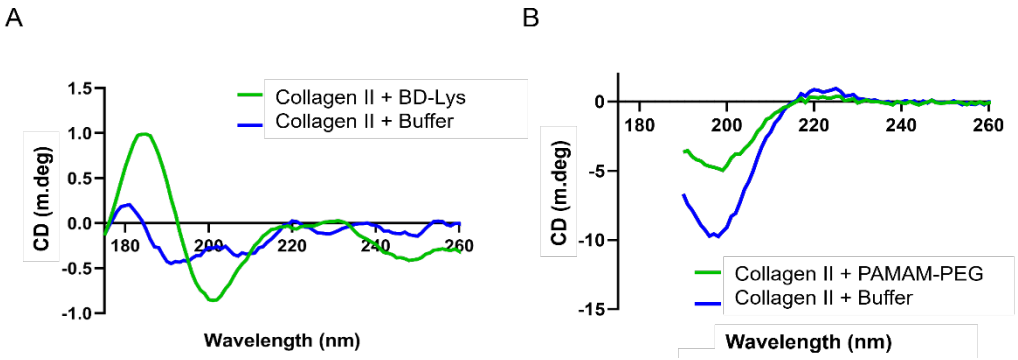

Figure 17. CD spectra of NP interactions with COL2 for BD-Lys (A) and PAMAM-PEG (B).

To investigate additional interactions between the NPs and cartilage components, we quantified GAG levels in cartilage explant supernatants (Figure 18). Based on the study performed in **Paper II**, we know that PAMAM-PEG directly interacts with ECM components such as HA and ACAN, but not COL2. Here, we further investigated whether BD-Lys would result in transient cartilage stabilization similar to PAMAM-PEG. After incubation in different conditions, the two NPs induced opposite effects on GAG release in response to RA SF-induced degradation, where BD-Lys treatment resulted in a more prominent release, while PAMAM-PEG treatment resulted in GAG levels comparable to untreated samples. These effects may be attributed to the charge differences between the NPs and their subsequent PC formations. Further studies are needed to address these mechanisms in more detail.

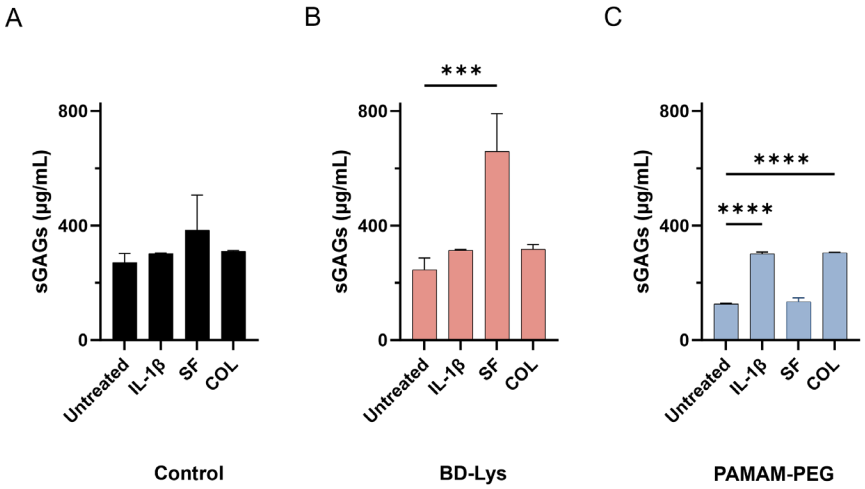

Figure 18. sGAG quantification in the ex vivo cartilage explant supernatants containing no NP (A), BD-Lys (B), and PAMAM-PEG (C). Data are represented as mean values ± S.D. Analysis was performed using one-way ANOVA with Tukey's post hoc test, where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

The complex process of cartilage tissue breakdown in OA underscores the need for personalized treatment modalities, where nanomedicine holds significant promise for creating tailored therapies. For example, certain NPs like PAMAM-PEG could target the negatively charged components of cartilage, offering temporary support to the structure via electrostatic bonding. Meanwhile, NPs with lysine modifications, such as BD-Lys, might play an essential role in detecting and tracking the degradation of collagen. Employing a combination of different NP types could overcome the shortcomings of their individual use, leading to a more flexible and successful treatment approach.

6.4. Functionalized NP Distribution in Catabolic Cartilage

To effectively study the mechanisms behind cartilage degradation, more accurate and accessible modeling systems are required, which can replicate the complex biological and mechanical environment of human joints. In **Paper IV**, we developed a 3D-printed unidirectional diffusion-driven transport chamber where the various aspects of NP transport into cartilage can be addressed and studied (Figure 19). Unidirectional transport allows the NPs to enter the tissue at the superficial zone of cartilage mimicking the joint space, relevant for IA injections. Utilizing prominent ECM-targeting enzymes such as COL and HYA as well as arthritic patient SF, we employed NPs as tools for understanding NP transport under various physiological conditions.

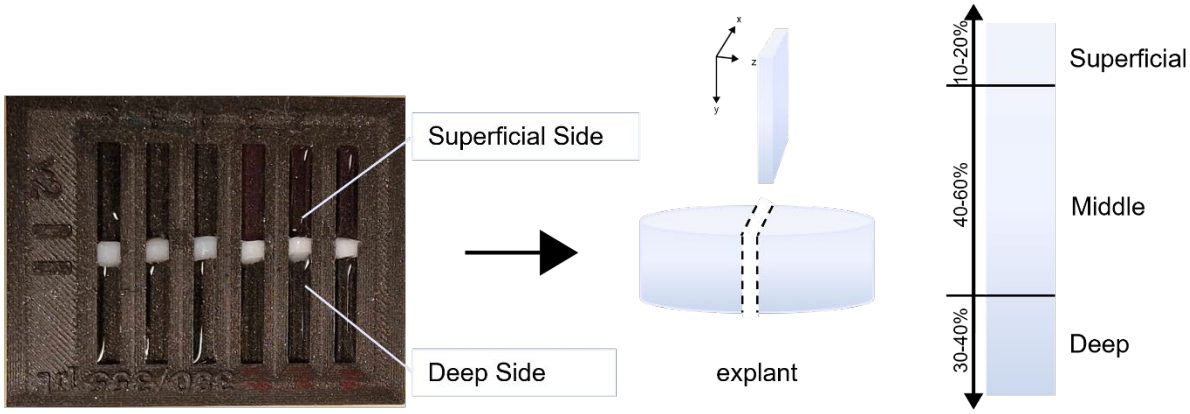

Figure 19. The design of the 3D printed unidirectional flow transport platform.

To build on our previous studies, PAMAMs were chosen as model NPs. In this study, however, three different functionalities were employed, where the NPs were either solely labeled with a fluorophore (PAMAM-FITC), or additionally functionalized with PEG (PAMAM-PEG), or a HA-binding peptide (PAMAM-PEP). By employing these surface modifications, we aimed to identify the differences in the NP cartilage uptake.

PAMAM-FITC NPs resulted in a high uptake profile under no enzyme and HYA conditions. COL had a more notable impact on the NP uptake resulting in a decreased signal intensity observed in the cartilage tissue (Figure 20). Similar results were observed for PAMAM-PEG uptake, however, it was substantially reduced by COL. PAMAM-PEP, on the other hand, exhibited a slower uptake in the presence of HYA, likely due to initial interactions with cleaved HA. Despite the HA-binding peptide modifications, PAMAM-PEP penetration was also significantly impacted by COL-induced degradation.

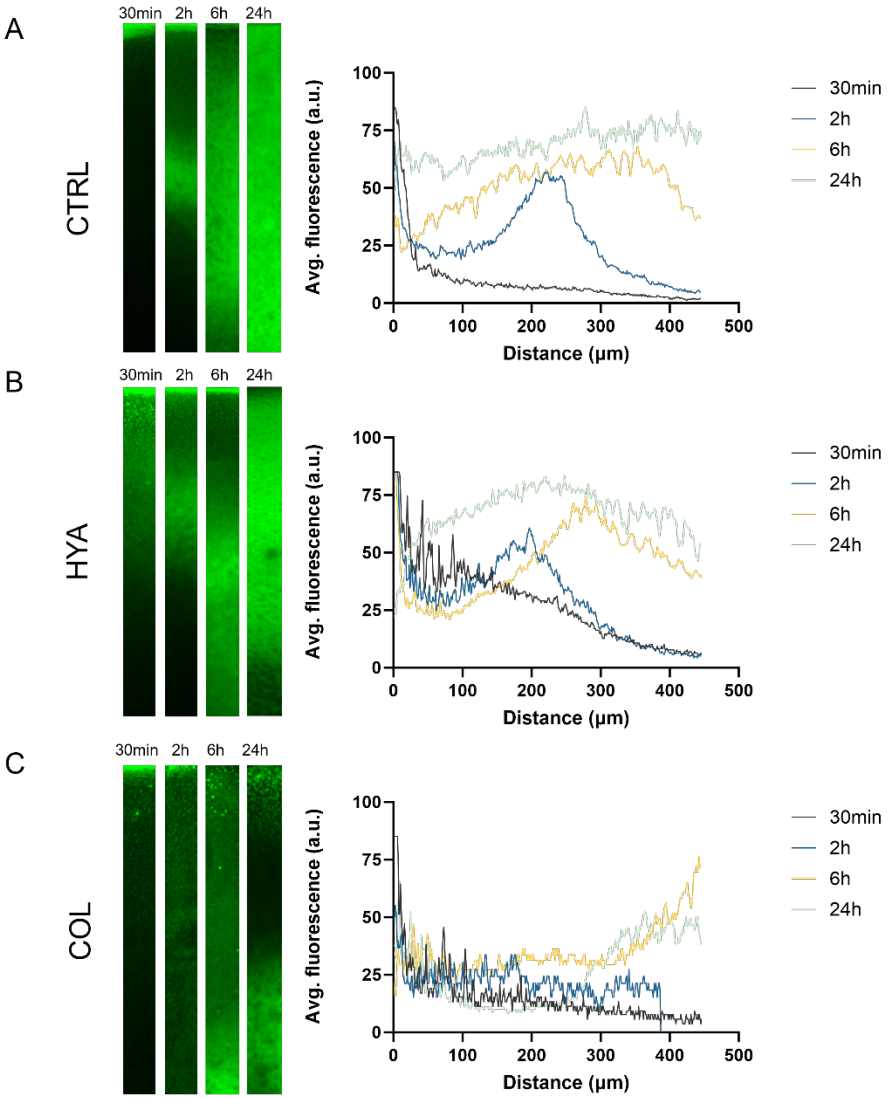

Figure 20. PAMAM-FITC transport in articular cartilage under no enzyme (A), HYA (B), or COL (C) conditions.

To assess the effects of SF on NP binding, the fluorescence profiles of NP uptake were compared to regular media control (Figure 21). SF resulted in a decreased fluorescence for PAMAM-FITC and PAMAM-PEG. On the other hand, PAMAM-PEP fluorescence detected in the cartilage tissue resulted in a slightly diminished signal when no enzymes were present and an increased signal in the presence of both HYA and COL.

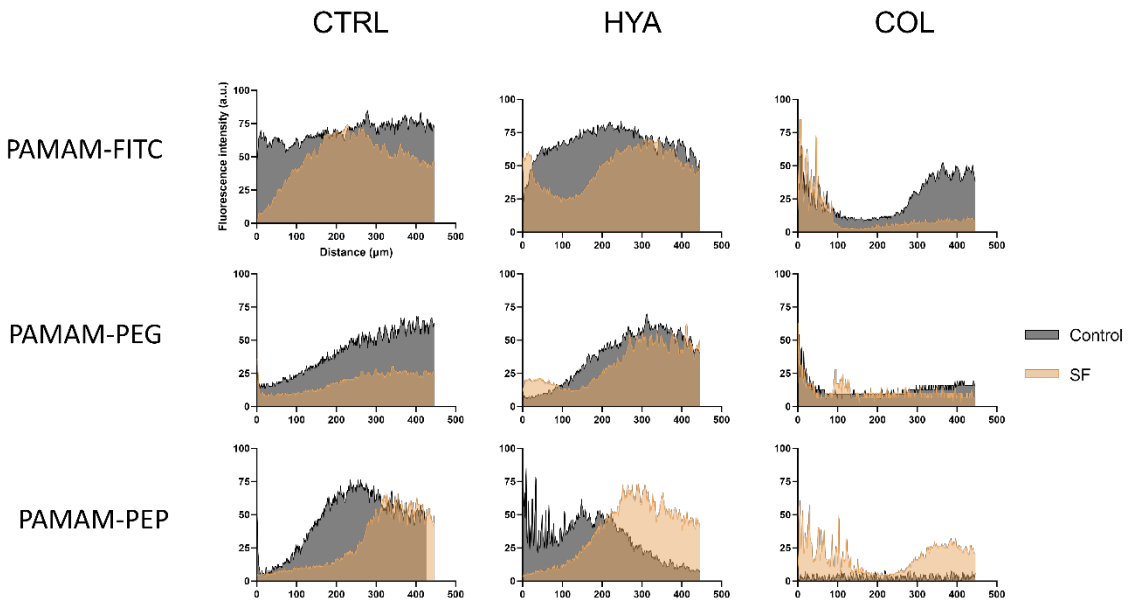

Figure 21. Comparison between 24-hour fluorescence intensities in articular cartilage explants under regular (grey) and SF (yellow) conditions.

This study highlights how NP transport varies under different catabolic and physiological states. PAMAM-FITC showed superior uptake in PBS control conditions, whereas functionalized PAMAM-PEP demonstrated enhanced efficiency in SF. These results deepen the comprehension of how charged NPs interact with SF and complex cartilage environment, offering valuable perspectives for designing customized DDS.

6.5. Estradiol Effects on Murine OA Progression

To develop effective nanocarrier-based therapeutic strategies, a thorough understanding of the underlying disease mechanisms is essential. **Paper V** delves into population-specific aspects of OA, such as the effect of sex-dependent hormone changes on OA progression. Here, the aim was to investigate how the lack of female sex hormones and the addition of estradiol (E2) to ovariectomized (OVX) mice affect the advancement of OA. In an early stage of OA, OVX mice, used as a model for the condition, received a physiological dose of E2. E2 was given in intermittent doses to mimic the natural hormone variations seen in the mouse estrous cycle (Figure 22).

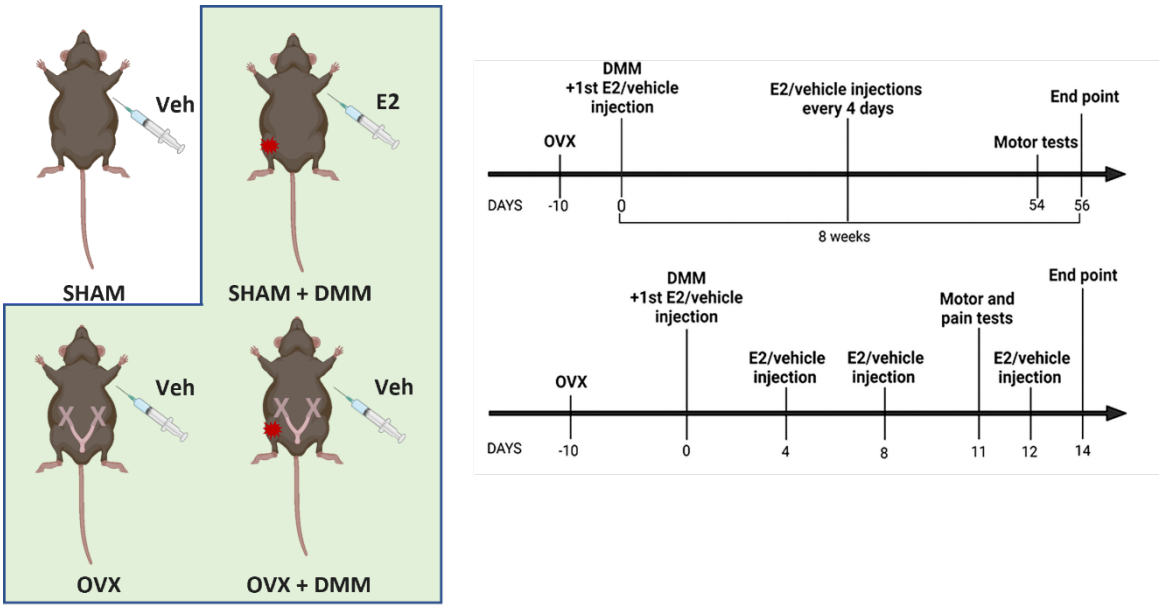

Figure 22. Experimental plan of the murine OA experiment in vivo. Mice were subjected to destabilization of the medial meniscus (DMM) to induce OA and were subjected to ovariectomy (OVX) to mimic a postmenopausal state. The schemes on the right describe the two timelines of the experiments including the 8 weeks (top) and 2 weeks E2 (bottom).

The administration of E2 in a pattern mimicking the natural hormone fluctuations during the murine estrous cycle showed that OVX in mice led to a slight, non-significant increase in cartilage degradation and synovial thickness in OA conditions (Figure 23), contrasting with some previous findings that reported worse OA outcomes in OVX models. Histological examination of murine articular cartilage demonstrated that E2 replacement alleviated proteoglycan depletion, surface fibrillation, and synovial thickening, emphasizing the hormone's protective role in joint tissues. We also observed a significant decrease in testosterone levels in OVX+Veh mice, suggesting a compensatory mechanism for estradiol loss post-OVX, which might explain the absence of significant differences in OA severity and synovial thickness between OVX and sham-operated mice. Additionally, an increase in lumbar spine bone mineral density (BMD) in OA mice reflects changes observed in human OA, where knee OA severity correlated with vertebral BMD alterations.

The study also recognized OA's inflammatory component, noting increased CD4+ and CD8+ T cells in the lymph nodes of OVX mice, which was reduced by E2 treatment, indicating an anti-inflammatory effect. Additionally, OVX mice exhibited decreased motor activity and increased pain sensitivity, both of which improved with E2 replacement, suggesting E2's potential to enhance mobility and reduce pain in OA. These findings contribute to the understanding of OA as a multifaceted disease influenced by hormonal and inflammatory factors, highlighting the complexity of OA progression and the potential therapeutic role of hormonal treatments.

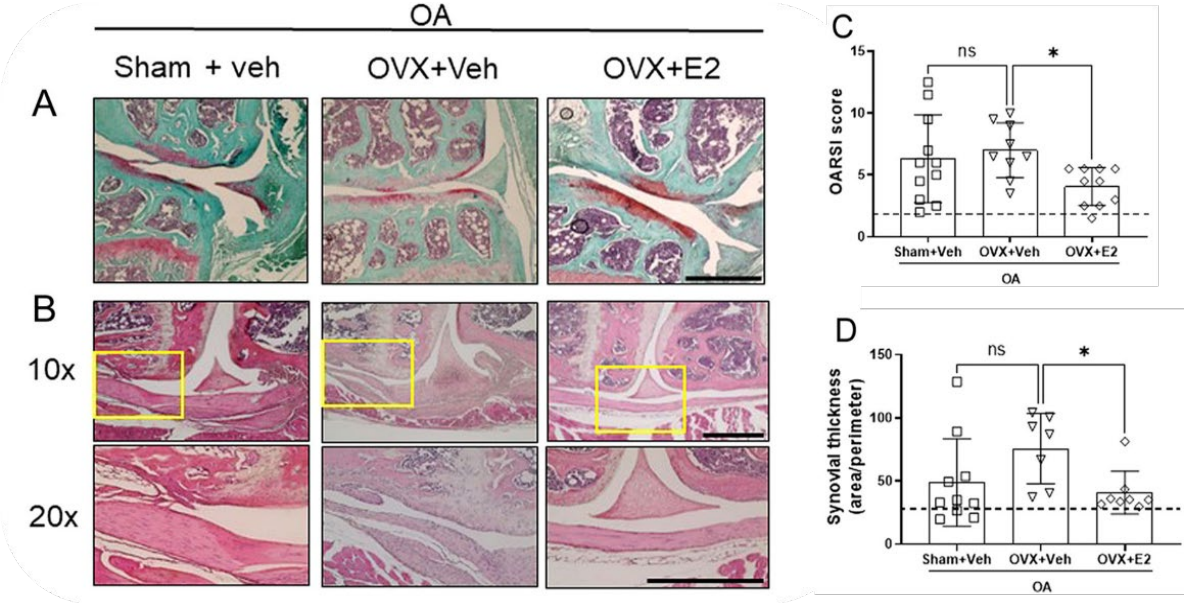

Figure 23. Representative images of the knee articular surfaces stained with the Safranin-O/fast green (A) and H&E staining of the knee joint (B). Based on the histological data, OARSI scores (C) and synovial thickness (D) were quantified. Data are expressed as mean ± S.D. and analyzed by one-way ANOVA followed by Dunnet's post hoc test.

6.6. Concluding Remarks

OA represents a significant clinical challenge due to its widespread prevalence and the complexity of its pathophysiology. NP-mediated therapeutic strategies offer the potential to improve the quality of life for OA patients by enabling an improved penetration and localized drug delivery to the joint.

The research presented in this thesis delineates several key insights. Primarily, it's apparent that the effectiveness of NPs is intricately linked to their environmental context. IA injections subject NPs to SF which alters their surface chemistry via protein adsorption. Based on the findings in **Paper I**, this resulted in hindered uptake of PAMAM NPs into both cartilage tissue and joint-associated cells. Nonetheless, PEGylation of NP surfaces remains a viable strategy for reducing PC effects in cartilage delivery. An important consideration here is the surface modification density. PEG brush conformation in an aqueous environment is caused by the steric repulsion of the neighboring chains, thus a low surface conjugation percentage may not lead to the most optimal protein adsorption resistance.

While studying NPs in physiologically relevant settings is important, the vast abundance of proteins and biomolecules in SF complicates the studies of more specific interactions between the NPs and cartilage biomolecules. In **Paper II**, cartilage stimulation with various ECM-targeting enzymes enabled to detect charge-dependent interactions between their degradation products like HA and ACAN and the cationic NPs. However, the implications of these interactions on the immune level were size-dependent. Small cationic PAMAM dendrimers triggered a more pronounced release of pro-inflammatory cytokines such as IL-1β compared to their larger PLGA counterparts, despite similar charge properties. The findings of this study imply that smaller PAMAM NPs can induce pro-inflammatory signaling. Conversely, PLGA NP treatment resulted in lower inflammatory cytokine release. Small NPs may necessitate careful consideration for their use in advanced stages of disease, whereas larger NPs could be advantageous for targeting the cartilage surface and providing controlled release in the later stages of disease progression.

An alternative treatment approach, outlined in **Paper III**, evaluated the potential of biodynamer, a small lysine-functionalized nanorod, used for probing cartilage breakdown, revealing their affinity for COL2. These NPs may be complemented with PAMAM-PEG NPs, which have also been previously investigated in Papers **I** and **II** and have been shown to possess an affinity for HA and ACAN. Together, the interdependent, ECM-specific effects of these two NPs could be leveraged for probing tissue degradation as well as synergistic cartilage targeting mechanisms.

Building on these insights, **Paper IV** delved into the effects of PAMAM surface modifications within an enzymatic cartilage environment. A unidirectional transport chamber allowed a physiologically relevant transport of NPs entering the tissue strictly from the superficial zone. This model stands out for explant-based drug delivery studies by establishing a baseline for NP entry, contrasting with traditional methodologies employed in microplate-based assays. The results revealed a compromised uptake for all NPs in the context of collagen-induced degradation, particularly affecting the uptake of functionalized PAMAMs, including PAMAM-PEG and PAMAM-PEP variants. The effects of SF were similar to the ones reported in **Paper I**, where the uptake of NPs decreased compared to protein-free conditions.

Interestingly, peptide-functionalized PAMAM uptake improved under SF enzymatic conditions. This suggests that active targeting-mediated delivery may be a strategy to avoid extensive PC formation. Peptide-based surface functionalization possibly exhibits a protective effect, limiting the extent of undesirable protein adsorption and preserving the intended surface characteristics that favor cellular uptake. This model provides the foundation for studying drug delivery aspects in cartilage with implications for the NP functionalization, dosing, and pharmacokinetic profiles.

Finally, comprehending the broader spectrum of drug efficacy is crucial for a holistic understanding of OA management. **Paper V** sheds light on sex steroids like E2 on OA progression, encompassing pain reduction, cartilage conservation, and inflammation modulation. Exploring diversity within patient populations enables the development of tailored drug delivery strategies, thereby reducing the adverse effects associated with systemic treatments.

The insights gained from these investigations pave the way for the development of more sophisticated NP-based delivery systems, particularly those that can mimic or respond to the physiological conditions of the joint. The potential of NPs to target and interact with different tissue components uncovers new avenues for site-specific therapy and further exploration of OA disease mechanisms.

7. Future Outlook

It is becoming increasingly clear that the future of drug delivery in OA will entail pathology-specific and personalized treatments, a paradigm shift accentuated by the insights arising from NP-mediated therapeutic approaches. This approach, established on the determination of distinct protein corona profiles, diverse pathophysiological environment, and an integration of individual genetic and environmental aspects, promises to revolutionize the treatment paradigm. By tailoring therapeutic regimens to the unique molecular and phenotypic characteristics of each patient, personalized medicine aims to optimize therapeutic efficacy while minimizing the potential for adverse reactions.

From the patient's perspective, the shift towards personalized and combined therapies represents a significant departure from the conventional, analgesic treatment approaches. It embodies a more patient-centric model of care, wherein treatments are meticulously tailored to the individual's specific disease attributes, genetic predispositions, and personal preferences. This paradigm shift not only fosters a more engaged and informed aging patient population but also aligns more closely with the ethical imperatives of autonomy and informed consent in medical practice.

As our understanding of the intricate nature of age-related diseases deepens, the idea of a "magic bullet" — one perfect NP formulation — seems increasingly naive. The field of nanomedicine opens the door to the advantages of combination therapies, allowing for a more nuanced approach to disease treatment. By strategically combining pharmaceuticals, biological treatments, and non-drug interventions, healthcare providers will be able to tackle the complex causes of conditions like OA more effectively. This integrated strategy goes beyond merely interfering with the various molecular pathways involved in the onset of the disease. It also aims to alleviate the wide range of symptoms and complications that impact the patient’s quality of life.

Looking forward, the integration of cutting-edge technologies such as artificial intelligence and machine learning in the analysis of complex biological data sets holds great promise for advancing personalized medicine in OA care. These technologies offer unparalleled capabilities in discerning patterns and predicting treatment outcomes from vast arrays of genomic, proteomic, and clinical data. Nevertheless, the implementation of these advanced therapeutic strategies is inherently dependent on a cohesive interdisciplinary framework - one that effectively merges the diverse expertise of scientists, clinicians, and engineers. Moreover, the incorporation of the patient's perspective into this collaborative model is imperative. Ensuring that the development and implementation of personalized therapies are guided by not only scientific and clinical considerations but also by the values, preferences, and life experiences of those they aim to serve.

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