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Polymeric and Lipid-based Nanoparticles for Anti-inflammatory, Anti-fibrotic and Regenerative Applications in Tendon Diseases

by

Giuseppina Molinaro

ACADEMIC DISSERTATION

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Abstract

Nanomedicine includes the use of nano-biomaterials and nano-therapeutics for the prevention, diagnosis, and treatment of various diseases. Nanoparticles (NPs), due to their small size and ability to be functionalized, offer a targeted approach that enhances drug accumulation at the site of action while minimizing systemic toxicity and off-target effects, leading to improved therapeutic outcomes.

Tendinopathies are chronic disorders often resulting from repetitive strain injuries and which have a significant social and economic impact globally, due to loss of working hours and medical care costs. In the US the incidence of tendinopathy was estimated to increase by 2030, with 11.9 million new cases reported in 2020. Conventional treatments, such as corticosteroids and non-steroidal anti-inflammatory drugs, often fail to address the complex pathophysiology of tendinopathy, providing only temporary symptomatic relief and leading to repeated relapses. This highlights the need for new therapies aimed at completely resolving the pathology. Nanomedicine offers promising opportunities to improve the treatment of tendinopathy through precise drug delivery strategies. NPs, with their physicochemical properties and customizable surface functionalities, are ideal for delivering therapeutics directly to the site of tendon injury. Encapsulating anti-inflammatory drugs, growth factors, and regenerative molecules within nanocarriers allows for targeted and sustained drug release, optimizing therapeutic efficacy while minimizing systemic toxicity.

Thus, the main aim of this thesis focuses on the development novel polymeric and lipid-based nanosystems, with anti-inflammatory, anti-fibrotic and regenerative properties, for tendon diseases treatment.

First, lignin-based NPs loaded with curcumin, coated with tannic acid (TA), and cloaked with platelet cell-membrane were designed for treating atherosclerosis, a potential risk factor for tendinopathy. The biomimetic NPs were evaluated for stability in cell-culture media and for cytocompatibility, anti-inflammatory and anti-fibrotic activities, in human coronary artery endothelial cells.

Additionally, acetalated-dextran NPs encapsulating curcumin were prepared and coated with TA to assess drug-release profiles, cytocompatibility and anti-inflammatory and anti-fibrotic activity in human primary tenocytes.

Finally, a lipid-based nanosystem was optimized for delivering collagen I mRNA and SMAD3 siRNA for tendon injury regeneration. The therapeutic efficacy was evaluated in human primary tenocytes, using the real time quantitative polymerase chain reaction, collagen I immunofluorescence staining and SMAD3 intracellular staining, demonstrating the pro-regenerative and anti-fibrotic properties of the nanosystem.

Overall, polymeric and lipid-based NPs were developed, showing to be promising platforms for drug delivery in tendon diseases treatment.

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In distant August 2018, when I became part of the Santos' Lab for the first time, I realized that what I truly wanted to do in life was conducting scientific research in a cool group like that one, where I could not only acquire scientific skills but also grow personally. I remember the last day of my master's thesis in Helsinki: I was sad and didn't want to leave. Returning to Italy, I secretly hoped I could come back someday.

♦ ♦ ♦

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Doing a PhD is always a big risk, it's a journey that you can love and sometimes even slightly hate. But one thing about these three years is certain: I will be eternally grateful to P4FIT for the people it allowed me to meet, without whom it wouldn't have been the same.

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♦ ♦ ♦

Some statistics say that Finland is the happiest country in the world, and even though it's not the easiest place to live, with its long winters, the cold that sneaks in as early as September, and my greatest fear: ice on the roads, I almost have to agree.
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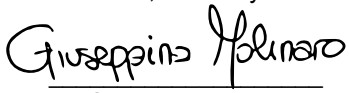
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“ Be less curious about people and more curious about ideas.”
Marie Skłodowska Curie

“ E quindi uscimmo a riveder le stelle “
Dante Alighieri (Inferno XXXIV, 139)

Table of contents

Abstract i

Acknowledgements ii

List of original publications xii

Additional list of publications xiii

1. Introduction 1

2. Literature overview 3

2.1. Nanotechnology for drug delivery systems 3
2.1.1. Conventional methods for the synthesis of NPs 5
2.1.2. Microfluidics: A precise method for the production of drug delivery systems 7
2.1.3. Polymeric-based NPs as a drug delivery platform 8
2.1.4. Cell-membrane coated NPs 10
2.1.5. Lipid-based NPs for gene delivery 11
2.2. Local and systemic delivery route for nanosystems 14
2.3. Tendinopathies 17
2.3.1. Structure of the tendon in healthy condition 17
2.3.2. Tenocytes and their key roles in tendon 18
2.3.3. Tendinitis, tendinosis and tendon rupture 20
2.4. The 3 phases of tendon repair process: inflammation, proliferation and remodelling 22
2.5. Hypercholesterolemia and atherosclerosis: Potential risk factors of tendinopathies 24
2.6. Current therapeutic approaches for tendon diseases treatment 26

3. Aims of the study 29

4. Experimental 30

4.1. Nanoparticles production 30
4.1.1. Lignin-based nanoparticles production (I) 30
4.1.2. Acetalated dextran (AcDEX) synthesis and AcDEX nanoparticles production (II) 30
4.1.3. Coating with Tannic Acid-iron (I-II) 30
4.1.4. Coating with platelet-cell membrane (I) 30
4.1.5. Lipid-based nanoparticles production (III) 31
4.2. Physicochemical characterization (I-III) 31
4.2.1. Dynamic and Electrophoretic Light Scattering (DLS and ELS) (I-III) and nanoparticles tracking analysis (NTA, III) 31
4.2.2. Stability studies (I-III) 31
4.2.3. Scanning electron microscopy (SEM, I-II),Transmission electron microscopy (TEM, I-II) and Cryo-TEM (III) 31
4.3. Drug loading and release 32
4.3.1. Loading degree of curcumin and TA quantification in the polymeric systems (I-II) 32
4.3.2. In vitro drug release (I-II) 32
4.3.3. Loading of the RNAs and assay for the quantification (III) 33

4.4. In vitro cell-studies (I-III) 33
4.4.1. Cell culture (I-III) 33
4.4.2. Cytocompatibility studies (I-III) 34
4.4.3. Cell-nanoparticles interactions: Quantitative and qualitative cell-uptake (I-III) 34
4.4.4. Transfection efficiency: Silencing and expression of eGFP protein in murine macrophages (III) 35
4.4.5. Immunological profile of the empty nanosystem (I) 35
4.4.6. Real-time quantitative polymerase chain reaction: quantification of pro-inflammatory and pro-fibrotic genes (I-III) 36
4.4.7. Immunofluorescence staining for collagen I quantification (III) 36
4.4.8. ELISA for pro-inflammatory cytokines quantification (I) 37
4.4.9. Intracellular staining of SMAD3 in human primary tenocytes (III) 37
4.4.10. Ethical permit (I-III) 37
4.4.11. Statistical analysis 37

5. Results and Discussion **38**

5.1. Physicochemical characterization 38
5.1.1. size, PDI, zeta-potential, NTA and colloidal stability (I-III) 38
5.1.2. Morphological analysis: TEM, Cryo-EM and SEM (I-III) 41
5.2. *In vitro* drug release studies (I-II) 42
5.3. Cell-cytocompatibility studies of the polymeric and the lipid-based nanoparticles (I-III) 43
5.4. Quantification of the transfection efficiency of the lipid-based nanosystems (III) 45
5.5. Immunological profile of the empty nanosystem (I) 46
5.6. Qualitative and quantitative uptake studies (I-III) 47
5.7. *In vitro* studies of the anti-inflammatory and anti-fibrotic activity of the developed nanosystems (I-III) 51
5.8. *In vitro* efficacy of the lipid-based nanosystems in the tendon repair process: anti-fibrotic and pro-regenerative effects (III) 54

6. Conclusions **57**

7. References **59**

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by

Giuseppina Molinaro
born on 10th April 1995
in Vallo della Lucania, Italy

List of original publications

This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (**I-III**).

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II **Molinaro, G.**; Fontana, F.; Pareja Tello, R.; Wang, S.; López Cerdá, S.; Torrieri, G.; Correia, A.; Waris, E.; Hirvonen, J. T.; Barreto, G.; A. Santos, H. In Vitro Study of the Anti-inflammatory and Antifibrotic Activity of Tannic Acid-Coated Curcumin-Loaded Nanoparticles in Human Tenocytes. *ACS Applied Materials & Interfaces* **2023**, 15 (19), 23012-23023.

III López-Cerdá, S.[†]; **Molinaro, G**.[†];Pareja Tello R.; Correia, A.; Waris, E.; Hirvonen, J. T.; Barreto, G.; A. Santos, H. Anti-fibrotic and pro-regenerative effects of SMAD3 siRNA and collagen I mRNA-loaded lipid nanoparticles in human tenocytes. *ACS Applied Nano Materials* **2024**, 7 (15),17736-17747.

The publications are referred to in the text by their respective roman numerals (**I-III**). The papers are reprinted with kind permission from John Wiley & Sons, Inc (**I**) and American Chemical Society (**II-III**), .

In **I** and **III**, the first authorship is shared with the authors marked with †.
Paper **III** will be defended in the doctoral thesis of the co-shared author Sandra López-Cerdá.

Additional list of publications

The list below presents additional manuscripts published during the doctoral studies, which are not included in the experimental section of this thesis

I Li, W., Chen, J., Zhao, S., Huang, T., Ying, H., Trujillo, C., **Molinaro, G.**, Zhou, Z., Jiang, T., Liu, W., Li, L., Bai, Y., Quan, P., Ding, Y., Hirvonen, J., Yin, G., Santos, H. A., Fan, J., & Liu, D. (2022). High drug-loaded microspheres enabled by controlled in-droplet precipitation promote functional recovery after spinal cord injury. *Nature Communications*, *13* (1), **2022**, 1262.

II Pareja Tello, R.; Wang, S.; Fontana, F.; Correia, A.; **Molinaro, G.**; López Cerdà, S.; Hietala, S.; Hirvonen, J.; Barreto, G.; Santos, H. A. Fabrication of hydrogel microspheres via microfluidics using inverse electron demand Diels–Alder click chemistry-based tetrazine-norbornene for drug delivery and cell encapsulation applications. *Biomaterials Science* **2023**, *11* (14), 4972-4984.

III Cerdá, S. L.; Fontana, F.; Wang, S.; Correia, A.; **Molinaro, G.**; Tello, R. P.; Hirvonen, J.; Celia, C.; Barreto, G.; Santos, H. A. Development of siRNA and Budesonide Dual-Loaded Hybrid Lipid–Polymer Nanoparticles by Microfluidics Technology as a Platform for Dual Drug Delivery to Macrophages: An In Vitro Mechanistic Study. *Advanced Therapeutics* **2023***,* 6, 2300048.

IV López-Cerdá, S.; **Molinaro, G.**; Tello, R. P.; Correia, A.; Künig, S.; Steinberger, P.; Jeltsch, M.; Hirvonen, J. T.; Barreto, G.; Stöckl, J.; Santos, H. A. Study of the Synergistic Immunomodulatory and Antifibrotic Effects of Dual-Loaded Budesonide and Serpine1 siRNA Lipid–Polymer Nanoparticles Targeting Macrophage Dysregulation in Tendinopathy. *ACS Applied Materials & Interfaces* **2024,** 16 (15), 18643-18657.

V D'Amico, C.; Ziegler, A-L; Kemel, M; **Molinaro, G**; Luxenhofer, R; Santos, H.A. Self-Nanoformulating Poly(2-oxazoline) and Poly(2-oxazine) Copolymers Based Amorphous Solid Dispersions as Microneedle Patch: A Formulation Study, *Advanced Materials Technologies* **2024**, 2400766.

Abbreviations and symbols

AcDEX	Acetalated dextran
ACN	Acetonitrile
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CO_2	Carbon dioxide
COL1A1	Collagen I
COX-2	Cyclooxygenase 2
DAPI	4',6-Diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EE	Encapsulation efficiency
ELS	Electrophoretic light scattering
EPR	Enhanced permeability and retention
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food & Drug Administration
Fe	Iron
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared
GAGs	Glycosaminoglycans
GMP	Good manufacturing practice
H_2O_2	Hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCAEC	Human coronary artery endothelial cells
Hep	Heparin
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-pressure liquid chromatography
IL	Interleukin
kV	Kilovolt
LD	Loading degree
LDL	Low-density lipoprotein
LF	Lactoferrin
LNPs	Lipid nanoparticles
LPS	Lipopolysaccharides
MES	2-(N-morpholino)ethanesulfonic acid
MFI	Mean fluorescence intensity
MMPs	Matrix metalloproteinases
mPEG-PDLLA	Poly(ethylene glycol)-b-poly(D,L-lactide)
MPs	Microparticles
mRNA	Messenger-RNA
MSCs	Mesenchymal stem cells
NF-κb	Nuclear factor beta
NFKB1	Nuclear factor kappa beta subunit 1
nm	Nanometres
NPs	Nanoparticles

NSAIDs	Non-steroidal anti-inflammatory drugs
NTA	Nanoparticle tracking analysis
O/W	Oil-in-water
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil
O/W/O	Oil-in-water-in-oil
oxLDL	Oxidized LDL
oxLDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral Blood Monocytes Cells
PBS	Phosphate buffered saline
PBS	Phosphate buffered saline
PAMAM	Polyamidoamine
PCL	Polycaprolactone
PDI	Polydispersity index
PEG	Polyethylene glycol
PEI	Polyethylemeimine
PFA	Paraformaldehyde
PLGA	Poly(lactic-co-glycolic acid)
PRP	Platelet-reach plasma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
s.d.	Standard deviation
SDS-page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
siRNA	Silencing RNA
TA	Tannic acid
TB	Trypan blue
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TGFB1	Transforming growth factor-beta 1
THF	Tetrahydrofuran
TNF- α	Tumour necrosis factor-α
United Kingdom	UK
United States	US
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
W/O	Water-in-oil
W/O/W	Water-in-oil-in water

1. Introduction

Nanomedicine is defined as the application of nanotechnology to medicine,[1,2] which includes the use of nano-biomaterials and nano-therapeutics for the prevention, the diagnosis, and the treatment of various diseases.[2-4] In this context, nanoparticles (NPs) represent an excellent alternative to conventional drug therapies due to their small size and the possibility to functionalize their surface:[5] this drug targeted approach not only enhances drug accumulation at the site of action but also minimizes systemic drug toxicity and off-target effects, leading to improved therapeutic outcomes.[6,7]

Tendinopathies are chronic disorders associated with pain due to repetitive strain injuries.[8,9] Worldwide, they account for one of the largest rates of orthopaedic diseases, representing a major social and economic impact due to loss of working hours and medical care of patients.[10,11] According to a 2021 report,[12] the incidence of tendinopathy in the United States (US) was estimated to increase by 2030, registering about 11.9M new cases in 2020. Moreover, in Europe, among the top five countries (France, United Kingdom (UK), Italy, Germany and Spain), France had the highest number of new cases of tendinopathy in 2020, with about 4.5M, followed by the UK and Italy.[12]

Conventional drug treatments, such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), often fail in addressing the complex pathophysiology of tendinopathy, leading to limited efficacy and undesirable side effects.[8] Moreover, these conventional methods only provide momentary symptomatic relief, resulting in repeated relapses; hence, this goes to emphasize the need of new therapies, with the aim to completely resolve the pathology.

Nanomedicine offers promising opportunities to overcome these limitations and improve the treatment of tendinopathy through precise drug delivery strategies,[13] aiming to eliminate the primary symptoms of the disease and to address the mechanisms behind tendinopathies, to prevent future relapse.

NPs, with their physicochemical properties and customizable surface functionalities, are ideal carriers for delivering drug therapeutics, directly to the site of tendon injury. For example, by encapsulating anti-inflammatory drugs, growth factors and regenerative molecules within nanocarriers, it is possible to achieve targeted and sustained drug release, thereby optimizing therapeutic efficacy while minimizing systemic toxicity.[14]

This thesis work started with the development of lignin-based NPs characterized by loading with curcumin, coating with tannic acid (TA) and cloaking with platelet cell-membrane, as delivery platform in the treatment of hyperlipidaemia, specifically atherosclerosis, one of the possible risk factors of tendinopathy (**I**). The biomimetic platelet-coated NPs were evaluated in terms of stability in cell-culture media and cytocompatibility in human coronary artery endothelial cells (HCAEC) (**I**). Moreover, anti-inflammatory and antifibrotics studies were also conducted, to confirm the activity of the nanosystem (**I**).

Next, acetalated-dextran NPs (AcDEX-NPs) encapsulating curcumin were prepared by nanoprecipitation in a glass capillary microfluidics system,[15] followed by coating with TA (**II**). The drug-release profile of curcumin was assessed in a synovial-mimicking fluid at different pH-values (5.5 and 7) and the cytocompatibility was conducted in primary human tenocytes, derived from a patient with tendinopathy (**II**). Then, the contribution of TA-coating to the cell internalization was assessed, also in the presence of uptake inhibitors, to evaluate the mechanism used by the cells to internalize NPs (**II**). The anti-inflammatory and anti-fibrotic activity of the system, was conducted by analysing the expression of the genes nuclear factor kappa beta subunit 1 (NFKB1) and transforming growth factor-beta 1

(TGFB1) using a real-time quantitative polymerase chain reaction (RT-qPCR), in lipopolysaccharides (LPS)-treated tenocytes, to better reproduce the inflammatory-environment of tendinopathy (**II**).

Finally, a lipid-based nanosystem was optimized using also microfluidics, for the delivery of collagen I (COL1A1) messenger RNA (mRNA) and SMAD3 silencing RNA (siRNA), aiming at regeneration in a late stage of the tendon injury (**III**). After the optimization of the formulation and the evaluation of the transfection efficiency, in a murine cell-line model, all the *in vitro* studies of cytocompatibility and cell-uptake were conducted in human primary tenocytes, isolated from a patient with tendinopathy (**III**). The therapeutic efficacy of the system was evaluated using RT-qPCR, collagen I immunofluorescence staining and SMAD3 intracellular staining, aiming at demonstrating the pro-regenerative and anti-fibrotic properties of developed the nanosystem (**III**).

2. Literature overview

2.1. Nanotechnology for drug delivery systems

Nanotechnology has been revolutionizing the drug delivery field, offering several advantages over conventional drug methods.[16] At the end of the 20th century, some liposome formulations were commercialized as the first nanoparticles-based drugs marketed as delivery platforms, including doxorubicin (e.g., Doxil®),[17] for the treatment of cancer, and amphotericin B (e.g., AmBisome®),[18] for the treatment of fungal infections. The commercialization of these formulations marked a significant milestone in the field of nanomedicine, demonstrating the potential of NPs-based drug delivery in the clinical setting. Recently, the advances in nanoplatforms design and fabrication techniques have further expanded the nanotechnology field for clinical applications, leading to the development of multifunctional nanosystems capable of imaging,[19] targeting,[20] and therapeutic delivery.[21] In 2021, Pfizer-BioNTech and Moderna developed two COVID-19 vaccines,[22] representing a revolutionary achievements in the fight against the global pandemic. The success of these vaccines is due to the innovative application of nanotechnology,[23] precisely lipid nanoparticles (LNPs).[24,25] The mRNA-based vaccines use an innovative design overcoming the challenge of mRNA instability and susceptibility to degradation.[26] LNPs play a crucial role in protecting RNAs from degradation and facilitate their delivery to target cells.[27] By encapsulating the RNA within LNPs, vaccines ensure efficient delivery of the genetic material to the host cells, where the mRNA is translated into viral proteins,[25] triggering an immune response against the SARS-CoV-2 virus. The success of COVID-19 vaccines highlights the potential of nanotechnology in vaccine development,[23] infectious disease control, and moreover, confirming its potential in the development of drug delivery platforms to treat various diseases. Different properties make NPs highly versatile and adaptable to a wide range of applications. One of the defining characteristics it is the size, typically between 1 and 100 nanometres (**Figure 1**),[28] and in the context of nanomedicine, size matters.[4,29]

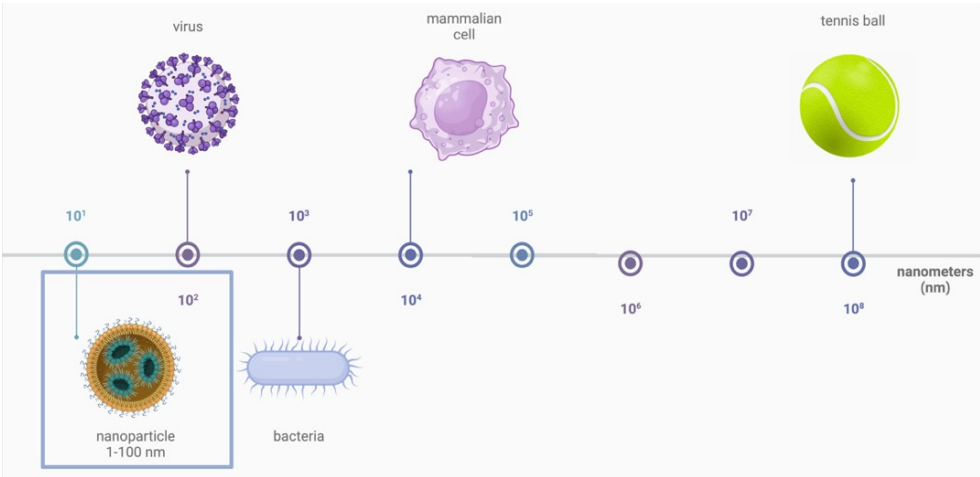


Figure 1. Nanoparticles size in comparison with a virus, bacteria, mammalian cell and tennis ball. The scale is represented in nanometres (nm). Image created with Biorender.com.

The size affects the stability, loading capacity, cellular uptake and biodistribution of a nanosystem.[30] NPs have a high surface area-to-volume ratio,[31] which enables them to effectively load and encapsulate drugs, including lipophilic ones. Their small size and different surface charge allow to more effectively penetrate biological barriers,[32] including cell membranes, facilitating cellular uptake and tissue distribution.[33] It has been demonstrated that the geometry of NPs affected the uptake: a spherical particle is more efficiently internalized compared to an asymmetric one.[34] Nanoplatforms, can also cross cell membranes through different mechanisms of endocytosis.[35-37] Targeting approaches can increase the uptake of particles, facilitating efficient delivery of drug therapeutics to specific sites.[38] Functionalization of the surface of the NPs is a strategy that can enhanced cellular uptake and intracellular delivery resulting in better therapeutic outcomes,[39] and allowing NPs to selectively accumulate in specific tissues while affecting less the healthy ones.[38] Additionally, NPs provide a protective shield for encapsulated drugs, safeguarding them, for example, from enzymatic degradation and early metabolism.[40,41] Encapsulation in particles protects drugs from harsh biological environments, prolonging their circulation time and improving their bioavailability. Furthermore, nanosystems allow controlled release of drugs, enabling sustained drug therapeutic concentrations for prolonged periods of time.[16,42] The controlled drug release kinetics minimizes fluctuations in drug levels, optimizing the drug therapeutic efficacy and reducing the drug dosing frequency.[43] This is particularly advantageous for chronic diseases that require long-term drug treatment regimens, where maintaining consistent drug levels is critical to a therapeutic success.[44] **Figure 2** gives an overview of the most common used NPs.



Figure 2. Examples of the most common polymeric, inorganic and lipid-based NPs. Image adapted with the permission from references [16]; Copyright © (2021) Nature Publishing Group.

Finally, it is important to highlight that by adjusting the physicochemical properties of the NPs, it is possible to obtain a nanoplatform with specific properties that can be applied for different therapeutic needs.

2.1.1.Conventional methods for the synthesis of NPs

Different fabrication methods are employed for producing NPs, such as single or double emulsions, nanoprecipitation, self-assembly, desolvation, and solvent exchange.[45] Emulsions, involve the mixture of immiscible liquids, yielding different emulsion types like water-in-oil (W/O), oil-in-water (O/W), oil-in-water-in-oil (O/W/O), or water-in-oil-in-water (W/O/W).[46] Despite the thermodynamic instability of emulsions, which can be solved by the use of surfactants, the techniques like double or multiple emulsions provide high payload encapsulation in drug delivery.[47]

The nanoprecipitation method involves the principle of miscibility of two solvents, usually an organic solvent miscible with water (e.g., acetone or ethanol) and an aqueous phase.[46,48] In this case, rapid diffusion of the solvent after gradual addition to the aqueous phase facilitates the almost immediate formation of particles, followed by removal of the solvent. This method is simple, energy efficient, and allows the production of particles with narrow size distributions, and it is commonly used for the entrapment of both hydrophobic and hydrophilic drugs (**Table 1**).[48]

Table 1. Advantages and disadvantages of some nanosystems synthetized using the nanoprecipitation method. Table readapted with the permission from ref.[49]; Copyright © (2022) Elsevier B.V.

Carrier	Description	Advantages	Disadvantages
Liposomes	Lipid bilayer, composed of cholesterol and phospholipids, which allows the encapsulation of hydrophobic and hydrophilic drugs.[50]	Improve the drug's pharmacokinetics, specificity, and enhance efficacy with reduced toxicity.[50]	Lack of structural integrity results in content leakage and instability during storage.[51]
Solid lipid NPs	Colloidal systems prepared from lipids, emulsifiers, and water.[50]	Biocompatibility and biodegradability.[50]	Poor drug loadings due to the compact lipid matrix network which, can be affected by polymorphic transition during storage.[50]
Nanostructured lipid carriers	Colloidal systems based on a blend of solid and liquid lipids. The liquid lipid could be either enclosed within the solid lipid matrix or located in the surfactant layer.[52]	The liquid phase provides high drug loading, and the solid lipid imparts attributes for controlled drug release.[50]	Poor colloidal stability.[53]

Polymeric nanoparticles	Polymer chains are self-assembled to form matrix particles (nanospheres). Nanospheres have a core- shell structure loaded with the drug of interest.[54]	High structural integrity, stability during storage, and controlled release behavior.[51]	Particle properties are strongly influenced by the preparation method.[55]
Lipid–polymer hybrid NPs	Core–shell structures comprising polymer cores surrounded by lipid/lipid–PEG shells that combine the characteristics of polymeric nanoparticles and liposomes or lipid particles.[51]	High structural integrity and controlled release behaviour because of the polymer core. High biocompatibility and bioavailability due to the lipid and lipid–PEG layers.[51]	Weak colloidal stability and challenging preparation to guaranteeing the particle structure.[56]

Desolvation is used for the synthesis of polymer and protein NPs,[57,58] offers simplicity and reproducibility. This thermodynamically driven self-assembly process employs two miscible solvents, often with the addition of a desolving agent such as salts, ethanol or acetone in an aqueous solution under stirring.[59] The process is usually followed by chemical cross-linking step in order to obtain NPs stable and with precise size distributions.[60] Moreover, the drug contents dependent on various factors such as ionic strength, stirring speed and pH.

In their work, Sozer *et al.*,[61] synthesized cationic Bovine Serum Albumin (BSA) NPs using the desolvation method, testing different desolvating agents (methanol, ethanol, propanol, isopropanol, acetone and acetonitrile (ACN)), and glutaraldehyde as a cross-linked agent. As a result, they achieved that the most turbid solution was obtained using in a 1:4 (v/v) proportion of ACN, resulting in a yield of NPs of 54.8%, which is a very high value when compared to the use of acetone (12.2%) and other solvents, with a yield less than 6%. In addition, the size and polydispersity index (PDI) of those NPs varies depending on the solvent used. Using methanol and ethanol, the value of hydrodynamic size, measured by Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM), was ~600 nm and PDI ~0.45; in contrast, using acetone resulted in particles with size ~119 nm and PDI lower than 0.2.

The solvent exchange, or dialysis method,[46] is based on the principle of inducing supersaturation of a solute in a solvent by rapidly mixing it with a nonsolvent. This rapid change in solubility results in the precipitation of NPs.[62,63]

In their study Lievonen *et al.*[64] demonstrated how the size and polydispersity index (PDI) of lignin-based NPs changed when the concentration of the polymer was increased from 1 mg/mL to 20 mg/mL, resulting in bigger NPs and more polydisperse. This data stand to

confirm how changing a parameter in the formulation process can affect NPs' properties. Despite the success and simplicity of conventional techniques in the formulation of NPs, some issues persist, such as inadequate control over particle preparation, significant batch-to-batch variation, and limitations in scalability. Recent studies highlight the need to explore advanced methods such as microfluidics, which promises to overcome the challenges associated with conventional NPs preparation techniques.[65]

2.1.2. Microfluidics: A precise method for the production of drug delivery systems

Microfluidics is a method used for fabricating microparticles (MPs) and NPs synthesis, offering a precise control over the process at the microscale level.[66,67] The technique involves the manipulation of fluids within microchannels, typically ranging from tens to hundreds micrometers.[68] In contrast to the conventional methods, microfluidics enables rapid mixing and precise fluid control, leading to the production of NPs with uniform size, shape, and specific properties.[69,70] In addition, the use of microfluidic reduces the volume of reagents needed, reducing the cost of the overall process and improving the yield of production per day.[71]

In their study, Liu *et al.*[72] developed a microfluidic device taking advantage of the superfast time intervals (milliseconds) between sequential nanoprecipitation process, to synthetise core/shell nanocomposites. With this approach, the yield of NPs/day obtained is ~700 g/day and the loading degree (%) of paclitaxel increases from 6.7 to 42.6% in comparison with the single microfluidics nanoprecipitation method .

One of the fundamental advantages of microfluidics is the ability to precisely control fluid flow rates: rapid mixing within microchannels promotes uniform nucleation, aiming at smaller NPs.[73,74]

Controlling the flow rates is crucial in microfluidics for achieving desired mixing, reaction kinetics, and is fundamental in drug encapsulation.[75]

Various microfluidics devices have been developed from different material such as rigid polymers, polydimethylsiloxane,[69] glass, and 3D printing material combinations. In this context, glass capillary microfluidics devices have received particular attention given the inert properties of glass, which sees it usable with organic solvents and at high temperatures, which do not alter the integrity of the chip.[15,76] Due to their versatility, it is possible to create chips with different geometries as shown in **Figure 3**. Over the years, NPs with different applications, have been synthesized in glass-capillary microfluidics by nanoprecipitation.[77-79]

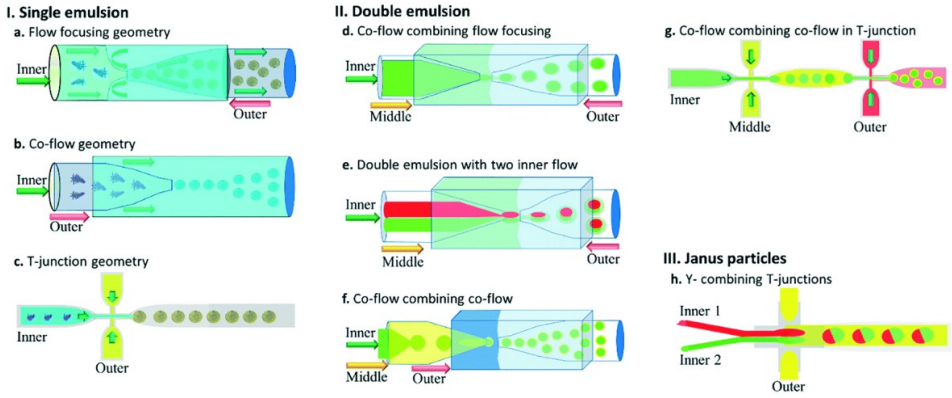


Figure 3. Different capillaries geometry for the synthesis of micro- and nanoparticles in microfluidics. **a)** Flow focusing, **b)** co-flow and **c)** T-junction geometries can be used to prepare single emulsion. **d)** Co-flow combining flow focusing, **e)** double emulsion with 2 inner flow and **f)** co-flow combining co-flow and **g)** co-flow combining co-flow in a T junction are used for double emulsion. **h)** Janus particles can be synthetize using two parallel inner fluid in a T-junction. The geometries **a**, **b**, **d**, **e**, and **f** can be used in glass capillary microfluidics. Image reprinted with permission from ref.[71]; Copyright © (2017) The Royal society of Chemistry.

In addition, a commercial microfluidic platform, the NanoAssemblr®, was developed, and it is using micromixing technology to synthesize liposomes and LNPs, operating under Good Manufacturing Practice (GMP) conditions.[80] The NanoAssemblr®, as different microfluidics devices, helps overcome the limitations of conventional methods by achieving high batch-to-batch reproducibility and controlling reaction conditions, avoiding degradation of biological agents, such as RNAs.[81,82]

2.1.3.Polymeric-based NPs as a drug delivery platform

Polymeric NPs have the flexibility to be synthetized from either natural or synthetic materials, utilizing both monomers and preformed polymers.[83,84] This NPs are developed through different techniques, including emulsification nanoprecipitation, and microfluidics, enabling the synthesis of particles with varied structures and properties.[85-87] Polymeric NPs can encapsulate drugs within their core or in the polymer matrix; moreover chemical conjugation on the surface is also possible.[88,89] This versatility means that hydrophobic and hydrophilic compounds, as well as molecules of varying molecular weight, such as small molecules and proteins, can be delivered.[16,90,91] Polymeric NPs can be classified into nanocapsules and nanospheres, further subdivided into polymerosomes, micelles and dendrimers.[92] Charged polymers such as polyethyleneimine (PEI) and polyamidoamine (PAMAM) are commonly used for dendrimer products in various clinical studies.[93,94] Polymeric NPs offer several advantages, such as biodegradability, water solubility, biocompatibility, and stability during storage.[85] In addition, their surfaces can be easily modified to improve targeting, facilitating the delivery of drugs, proteins and genetic material to specific tissues, making them valuable in cancer therapy, gene therapy and diagnostics.[73,95,96] However, issues like particle aggregation and toxicity limit the number of polymeric nanomedicines currently available and approved by the FDA but polymeric nanocarriers continue to undergo numerous preclinical studies, indicating their potential applications in drug delivery .[97-99]

AcDEX-NPs have emerged as promising carriers for drug delivery. Derived from dextran, a natural polysaccharide, AcDEX offers several advantages for therapeutic applications, including controlled drug release.[100] AcDEX-NPs are typically synthesized through dextran acetalization, a process that involves the introduction of acetal groups on the dextran backbone.[101,102] This chemical modification imparts hydrophobicity to the dextran molecule, allowing the polymer to self-assemble into NPs in aqueous solutions. The degree of acetalization can be controlled and the AcDEX can be further modified to modulate the physicochemical properties of the resulting NPs, such as size, surface charge, and drug loading capacity.[101,103] AcDEX-NPs have shown great potential for delivery of a wide range of therapeutics, including small molecules and proteins.[89,104,105] Their biocompatibility and biodegradability make them ideal candidates for systemic delivery with minimal risk of immune response or toxicity.[100,101] In addition, the properties of AcDEX-NPs allow optimization of drug release kinetics and target specificity.[106] A major advantage of AcDEX-NPs is the ability to encapsulate hydrophobic drugs within their core, protecting them from degradation and improving their solubility and bioavailability.[107,108] Furthermore, the pH-sensitive degradation of AcDEX-NPs is another important feature to highlight of these NPs. This is attributed to the chemical structure which contains acetal groups that undergo hydrolysis in acidic environments.[109] Under physiological conditions (pH 7.4), the acetal bonds remain stable, ensuring the integrity of the NP structure and prolonged circulation in the bloodstream.[110] However, when acidic environments, such as those found in inflammation or tumour microenvironments (pH < 7.4), are reached, the acetal bonds undergo hydrolysis leading to degradation of the polymer matrix and subsequent release of the encapsulated drugs.[106,111] This property can be exploited to release the compound loaded at inflamed sites, preventing release at healthy ones and decreasing possible side effacts.[112] In their study, Wang *et al.*[113] used the pH-responsive property of AcDEX to encapsulate methotrexate, a drug used to treat rheumatoid arthritis. The main aim was to decrease the toxicity of the drug while going on to increase therapeutic efficacy. The resulting NPs showed a loading degree of 88.32%, low in vitro cytotoxicity on RAW 264.7 and an extended in vivo half-life in the blood circulation (~10.8h) when compared to free methotrexate (~0.46h). In addition, the developed NPs showed a significant decrease in pro-inflammatory cytokines downregulating the JAK-STAT pathway, involved in the development of the disease.[113]

Lignin NPs have recently attracted attention as sustainable carriers for drug delivery due to the abundance of the material and biocompatibility.[114] Derived from lignocellulosic biomass, lignin is one of the most abundant biopolymer on Earth and offers a renewable and environmentally friendly source for NPs manufacturing.[115-118] Their sustainable nature and low-cost production make them attractive candidates for large-scale drug production with minimal environmental impact. Lignin NPs exhibit antimicrobial activity against some microorganisms, including fungi, bacteria and viruses,[119] and this is attributed to the presence of phenolic group and aromatic structures in lignin.[120] The hydrophobic nature of lignin allows it to interact with microbial cell membranes, disrupting their integrity and leading to lysis and cell death. Several studies have demonstrated the antimicrobial efficacy of lignin NPs against pathogenic microorganisms. In their study, Yang *et al.*[121] proposed that the damage to the bacteria is induced by lignin though ROS activity. Chen *et al.*[122] have also reported the antimicrobial activity of lignin NPs against common foodborne pathogens, including Escherichia coli, and other gram positive bacteria. In addition to the antimicrobial activity, lignin NPs exhibit potent antioxidant properties, which

result from the presence of phenolic hydroxyl groups.[123] These phenolic compounds act as free radical scavengers, neutralizing reactive oxygen species (ROS) and preventing oxidative damage to cells and tissues.[124,125] Figueiredo *et al.*[118] have also shown the potential of lignin in their developed nanosystem for drug delivery and antiproliferative effect, in cancer cells. Beside pure lignin NPs, iron (III)-complexed lignin NPs and Fe_3O_4 lignin NPs have been synthetize in this study, in order to use the superparamagnetic properties of iron for diagnosis (*e.g.*, magnetic resonance imaging). The NPs showed low cytotoxicity after 6 and 24h of incubation, in all the cell lines used, up to 100 µg/mL of NPs and haemolytic rates below 12% in red blood cells, a factor to take into consideration when NPs have to be administered intravenously.[118] Moreover, the lignin NPs were loaded with the cytotoxic agents sorafenib and benzazulene, in order to evaluate the antiproliferative effect on cancer cells. The developed nanosystems showed the maximum inhibition at 24h, using a range of concentration between 0.64 and 12.4 µM, demonstrating the potential of this drug delivery system in future applications for cancer therapy.[118]

2.1.4.Cell-membrane coated NPs

Cell membrane coated-NPs are engineered nanocarriers mimicking the surface properties and functionality of living cells by coating particles with cell membranes.[126] The process typically involves the isolation and purification of cell membranes, followed by their fusion or wrapping around the NP's surface through various techniques such as extrusion and sonication.[126]

This biomimetic approach allows NPs to have the biological properties of the cells, including surface receptors, adhesion molecules and bioactive ligands, while maintaining the core functionalities of NPs, such as drug loading capacity, stability and biocompatibility.[127] Cell membrane coated-NPs can be designed to selectively accumulate at specific pathological sites, such as tumors,[128] inflamed tissues or sites of infection,[129,130] thereby improving therapeutic efficacy and reducing off-target effects of encapsulated drugs or therapeutic agents. Furthermore, this NPs can evade immune surveillance and clearance mechanisms, prolonging circulation time in the bloodstream.[131]

In their study, Zhang *et al.*[132] developed neutrophil-membrane coated NPs by fusing the membrane in a poly(lactic-*co*-glycolic acid) (PLGA) polymeric core, to use as anti-inflammatory for the treatment of rheumatoid arthritis. In this context, the use of the neutrophil membrane on their surface was used for the NPs to mimic the starting cells, and thus, bind to immunoregulatory molecules that would otherwise target endogenous neutrophils.[132] To confirm the presence of the membrane on the surface of the particles, transmission electron microscopy (TEM) studies were conducted using uranyl acetate as positive staining, showing a unilamellar structure around the polymer core. *In vitro* studies on chondrocytes showed that treatment with these NPs decreased apoptotic events when the cells were treated with IL-1β and TNF-α. In addition, *in vivo* studies, confirmed that NPs enhanced cartilage penetration and offer chondroprotection, decreasing ligament damage and the severity of arthritis.[132]

In cancer therapy, cell membrane coated-NPs have shown potential for targeted drug delivery. By coating nanoparticles with membranes derived from tumour-targeted cells, such as cancer cells or immune cells, NPs can selectively localize to tumour sites and deliver chemotherapeutic drugs or contrast agents for imaging with high specificity, minimizing systemic toxicity and improving the overall therapeutic index.[133,134]

In regenerative medicine, cell membrane coated-NPs have shown potential for tissue engineering and repair by delivering growth factors, cytokines or stem cells into damaged or diseased tissues.[135] By coating NPs with membranes derived from stem cells or tissue-specific progenitor cells, NPs can mimic the regenerative microenvironment and enhance tissue regeneration, repair and remodelling, offering potential therapeutic solutions for a wide range of diseases.[136] Despite their significant potential, cell membrane coated-NPs face several challenges that need to be addressed to realize their full clinical translation and commercialization, including scalability and reproducibility of membrane isolation and coating processes, stability and long-term performance of coated NPs.

Platelets, the smallest cells in the bloodstream, play a crucial role in haemostasis, wound healing and immune response. By coating synthetic NPs with platelet membranes, particles can selectively target sites of vascular injury, inflammation or thrombosis, offering potential therapeutic solutions for cardiovascular diseases, cancer metastasis and inflammatory disorders.[137,138] In cardiovascular diseases, these NPs have shown promising results for targeted drug delivery to sites of vascular injury or atherosclerotic plaques.[139] By coating the particles with platelet membranes, particles can selectively adhere to activated endothelial cells or exposed collagen at sites of vascular injury, delivering active compounds directly to the targeted site.[135]

Wang *et al.,* confirmed in their study the promising efficacy of their platelet membrane-coated PLGA nanosystem, in thrombus treatment.[140] The NPs, loaded with the fibrinolytic enzyme lumbrokinase and coated with platelet cell membranes, showed *in vivo* the adhesion to the thrombus and thrombolytic efficacy, when injected in the carotid thrombosis mice model.[140] The developed nanosystem demonstrated the superior efficacy when compared to the free lumbrokinase; moreover the coated-NPs showed minor influence on the coagulation of the mice's, reducing the risk of haemorrhagic events.[140]

2.1.5.Lipid-based NPs for gene delivery

Lipid-based nanosystems offer versatile and effective platforms for drug delivery and biomedical applications.[16] Lipid nanosystems are mainly composed of lipids (natural or synthetic), amphiphilic molecules that can form various nanostructures, such as liposomes and LNPs. Liposomes are characterized by the presence of one or more lipid bilayers surrounding a central aqueous compartment.[141] LNPs typically consist of an outer phospholipid layer that encapsulates an interior that may contain non-aqueous components.[142] The composition of liposomes and LNPs typically includes lipids, such as phospholipids and cholesterol, which constitute the main structural component.[142] In lipid-based NPs cholesterol and PEGylated lipids are added to improve membrane stability;[143] moreover an helper lipid, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine(DOPE), is added to improve the delivery efficiency.[143] These NPs can encapsulate various payloads (*e.g.,* drugs or nucleic acids) within the lipid bilayer or dispersed in the lipid matrix. The difference between the lipids confers precises characteristics to NPs, influencing their stability, drug loading capacity, cellular uptake and biological interactions.

Neutrally charged lipids, such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol, have a neutral charge at physiological pH and contribute to the structural integrity and stability of NPs.[144] Moreover, cholesterol regulates the fluidity and permeability of the lipid bilayer, improving the stability and retention properties of the drug.[142] Liposomes

formulated with neutrally charged lipids can be used for hydrophobic drug delivery, such as chemotherapeutic agents.[145]

In their study, d'Avanzo *et al*.[146] highlighted the potential of loading two chemotherapeutic drugs, doxorubicin and sorafenib, in DPPC-liposomes, to treat breast cancer. The liposomes were synthetized using the thin layer evaporation method and then functionalized with a peptide targeting the protein p32 (LinTT1), overexpressed in cancer cells. The developed NPs showed good stability in human plasma, making them suitable for intravenous administration and LinTT1-liposome showed higher internalization in human breast adenocarcinoma cells (MDA-MB-231), when compared to not functionalized liposomes.[146]

Ionizable lipids contain ionizable functional groups that give NPs pH-dependent solubility and charge characteristics. In acidic pH environments, such as within endosomes or lysosomes, ionizable lipids are protonate and acquire a positive charge, facilitating escape from the endosome and intracellular drug release.[147,148]

Billingsley *et al.*[149] in their work, formulated different ionizable LNPs with a library of 24 lipids, to deliver mRNA in human T cell. The LNPs, loaded with luciferase mRNA, were screened in JURKAT cells, showing that only 7 formulations were suitable for the expression of luciferase over the positive control (lipofectamine), and that particles synthesized with C14-4 lipid gave the highest transfection.[149] This study goes to emphasize how the structure of the ionizable lipid can influence the formulation and the activity of the RNAs delivered from different nanosystems.[149]

Cationic lipids are positively charged at physiological pH due to the presence of quaternary ammonium groups or other functional groups. The lipids interact with negatively charged cell membranes and nucleic acids through electrostatic interactions, facilitating cellular uptake and intracellular cargo delivery.[150] In addition, they can activate immune responses and stimulate antigen presentation, making them effective vectors for vaccine delivery.[150]

Lopez-Cerdá *et al.*[78] demonstrated in their work the potential of the cationic lipid CKK-E12 to deliver the green fluorescence protein siRNA (eGFP-siRNA) in murine macrophages (RAW 264.7). Due to the ability of fusogenic lipids, such as DSPC and CKK-E12, the developed hybrid lipid-polymer NPs transfected the cells (eGFP-expressing RAW 264.7) at 24h and showed only 2% eGFP+ cells at the concentration of 0.5 µg/mL of loaded siRNA, proving the potential of this system for siRNA delivery in macrophages.[78]

Endosomal escape is a crucial step in the intracellular delivery of therapeutic drugs, particularly for the nucleic acids, such as siRNA and mRNA, which require access to the cytosol to have their therapeutic effects.[16] NPs formulated with ionizable or cationic lipids play a key role in facilitating endosomal escape via pH-sensitive mechanisms or membrane disruption.[78,151] When ionizable lipids are protonated in acidic endosomal environments, they undergo a change in their physicochemical properties, leading to membrane destabilization and disruption. This pH-dependent transition allows NPs to escape from the endosomal compartment into the cytosol, where they can release their cargo and apply their therapeutic effects.[148] Similarly, cationic lipids interact with negatively charged endosomal membranes through electrostatic interactions, destabilizing the membrane and promoting endosomal leakage.[152] Cationic NPs can disrupt endosomal membranes through various mechanisms, including pore formation, allowing the release of encapsulated cargoes into the cytosol.[152] By enabling efficient cytosolic delivery, NPs can overcome the barrier posed by endosomal entrapment and degradation, allowing nucleic acids to exert their therapeutic effects on target cells. Two examples that underline the potential of lipid-based nanosystems are DOXIL® and the COMIRNATY® vaccine. DOXIL®,

also known as liposomal doxorubicin, is a liposome-based formulation of the chemotherapy drug doxorubicin. It is approved for the treatment of various cancers, including ovarian and breast cancer. DOXIL® encapsulates doxorubicin within liposomes, allowing the controlled release of the drug and reducing its toxicity to healthy tissues.[153] The liposomal formulation of doxorubicin offers several advantages over conventional doxorubicin formulations. By encapsulating doxorubicin within liposomes, DOXIL® reduces the risk of cardiotoxicity, a common side effect associated with free doxorubicin.[154] Furthermore, the prolonged circulation time of liposomal doxorubicin increases its accumulation in tumour tissues due to its enhanced permeability and retention (EPR) effect, resulting in improved therapeutic efficacy and reduced systemic toxicity.[153] The COMIRNATY® vaccine, developed by Pfizer-BioNTech, is a COVID-19 vaccine. It uses LNPs to deliver mRNA encoding the SARS-CoV-2 virus spike protein into host cells. Once inside the cells, the mRNA instructs the cells to produce the spike protein, stimulating an immune response and triggering the production of antibodies against the virus.[25] The LNPs used in the COMIRNATY® vaccine have multiple functions, protecting mRNA from degradation by ribonucleases in the extracellular environment, improving cellular uptake of mRNA by endocytosis, and facilitating the endosomal leakage to transport mRNA into the cytosol.[26,27] This allows efficient translation of mRNA into proteins and induction of a robust immune response against the virus.

Overall, liposomes and LNPs represent promising platforms for drug delivery, as they offer a combination of biocompatibility, versatility, targeting capability, controlled release, stability and enhanced cellular uptake (**Table 2**). Due to their ability to encapsulate a wide range of drugs and achieve targeted delivery to specific tissues or cells, liposomes and LNPs hold great promise for the advancement of personalized medicine, precision therapies and improved patient outcomes in different therapeutic area.

Table 2. Summary of lipid nanosystems properties and their description, highlighting the potential of these drug delivery systems.

Feature	Description
Biocompatibility	Lipids are naturally occurring molecules in the body, making lipid-based nanosystems biocompatible and well-tolerated. This enhances their safety profile and reduces the risk of adverse reactions.[155]
Versatility	Lipid-based nanosystems can encapsulate a wide range of drugs, including hydrophobic, hydrophilic, and amphiphilic compounds, allowing for efficient drug loading and delivery of diverse therapeutic agents.[155]

Targeting	Surface modification with targeting ligands or antibodies enables specific delivery to diseased cells or tissues. This approach enhance therapeutic efficacy and minimize off-target effects and systemic toxicity.[156]
Controlled release	Engineered to release drugs in a controlled manner, lipid-based nanosystems offer sustained release profiles and prolonged therapeutic effects, improving drug bioavailability.[157]
Stability	Exhibiting good stability in biological fluids, lipid-based nanosystems prolong circulation time and enhance drug bioavailability, improving drug efficacy and reducing the frequency of dosing.[25]
Protection of Nucleic Acids	LNPs shield nucleic acids from degradation by nucleases, ensuring their stability and integrity during transport to target cells.[26]
Scalability and cost-effectiveness	Relatively simple and cost-effective synthesis and production make lipid-based nanosystems attractive for large-scale manufacturing and commercialization.[158]

2.2. Local and systemic delivery route for nanosystems

Systemic administration of drugs involves delivering medications to the bloodstream, allowing them to circulate throughout the body and reach target tissues or organs. Common methods of systemic drug administration include oral, intravenous, intramuscular routes.[159]

Oral administration is one of the most common and convenient methods of systemic drug delivery. Medications taken orally are absorbed through the gastrointestinal tract and enter the bloodstream, where they are distributed to various tissues and organs.[160] Oral administration offers advantages such as ease of administration, patient compliance, and non-invasiveness. However, it is subject to factors such as first-pass metabolism in the liver, variability in absorption rates, and potential degradation by stomach acid and enzymes.[161]

Intravenous administration involves delivering drugs directly into a vein, allowing for rapid onset of action and precise control over drug dosage. Medications administered intravenously bypass the gastrointestinal tract and are delivered directly into the bloodstream, resulting in immediate systemic distribution.[162] This route of administration is commonly used in emergency situations, critical care settings, and for drugs that require rapid and reliable delivery. NPs engineered for intravenous drug delivery provide a versatile platform to encapsulate therapeutics, enabling targeted delivery to specific tissues or cells while minimizing systemic side effects.

Intramuscular administration involves injecting drugs into muscle tissue, where they are absorbed into the bloodstream through the capillary network. This route of administration offers advantages such as rapid absorption, sustained release of medications from depot formulations, and avoidance of first-pass metabolism. Intramuscular injections are commonly used for vaccines, antibiotics, and certain medications that require prolonged action.[163] However, intramuscular injections may cause pain or discomfort at the injection site and carry a risk of tissue damage or infection if not administered properly.[164]

In contrast to the systemic administration, the local administration of drugs involves delivering medications directly to the site of action or target tissue, bypassing systemic circulation.[159] This route of administration offers several advantages, including targeted delivery, reduced systemic exposure, enhanced therapeutic efficacy, and lower dose requirements.[165] **Table 3** highlights the advantages of local administration, such as targeted delivery, reduced systemic exposure, enhanced therapeutic efficacy, lower dose requirements, minimized systemic side effects, rapid absorption at the site of action, sustained release for prolonged therapeutic effects, and minimization of first-pass metabolism, compared to systemic administration. Nevertheless, it is important to note that usually local administration involves local injections or implants, which require expert intervention, and patients often cannot administer these drugs themselves.[165]

Intra-articular administration provides injection directly into a joint space, where they exert their effects locally. This route of administration is commonly used for the treatment of inflammatory joint conditions, such as osteoarthritis, rheumatoid arthritis, and tendinopathies, as well as for the delivery of certain medications to the synovial fluid.[166] Recently studies have demonstrated that injectable hydrogels and NPs are excellent local drug delivery systems, performing their effects at the site of action, minimizing systemic side effects.[166,167]

Table 3. Comparison between the advantages of a local administration *versus* the systemic administration route.

Advantages of local administration	Advantages of systemic administration
Targeted delivery to specific tissues or organs	Widespread distribution throughout the body
Reduced systemic exposure minimizing first-pass metabolism	Rapid onset of action
Enhanced therapeutic efficacy	Convenient for patients
Lower dose requirements	Accessibility for drug administration
Minimized systemic side effects	Systemic effects on multiple organs or tissues

Non-invasiveness	Suitable for treating systemic diseases or conditions
Rapid absorption at the site of action	Systemic effects on multiple organs or tissues
Sustained release for prolonged therapeutic effects	Rapid absorption (IV injection)

Kim *et al.*[168] developed a poly(ethylene glycol)-b-poly(D,L-lactide) (mPEG-PDLLA) and PLGA NPs loaded with rebamipide, a drug used to treat osteoarthritis (OA), to allow a sustained release of the drug, which when is administered alone has an half-life to 2h. In this way, no repetitive intra-articular injection are needed, making the formulation more patient-friendly compared to the administration of the drug alone. The round-shape of the developed NPs was confirming using TEM imaging and the DLS analysis showed almost the same size, for empty (241.7 ± 38.3 nm) and loaded NPs (231.0 ± 40.8 nm).[168] To confirm the *in vitro* activity of rebamipide NPs, a RT-PCR was conducted in chondrocytes treated with IL-1 and LPS, showing the decreasing levels of some interleukins and matrix metalloproteinases (MMPs), when cells were treated with NPs.[168] Moreover, *in vitro* studies in intra-articularly injected monosodium iodoacetate-induced OA rats showed that a single injection of rebamipide-NPs could suppress the inflammatory process and decrease cartilage degradation, showing to be more effectively than the oral or intra-articular injection of the drug alone.[168]

Ye *et al.*,[169] in their study, exploited the properties of rapamycin (RAPA) to block fatty infiltration by muscle-derived stem cells (MDSCs), to treat rotator cuff tears in the shoulder tendon. Since systemic administration of RAPA causes side effects such as nausea and infection, in this study the authors investigated the potential of loading RAPA into polycaprolactone (PCL)-PEG NPs for local injection, to achieve sustained release of the drug, leading in a decreased cytotoxicity in tendon cells (tenocytes).[169] The developed NPs showed an average size of 78.33 ± 0.13 nm, PDI of 0.017 ± 0.01 and a spherical morphology, accordingly to DLS and TEM analysis.[169] *In vivo* studies, on rats with shoulder cuff injury, demonstrated the potential of this nanosystem showing a decreasing in the fatty infiltration and muscle atrophy, when animals were treated with PCL-PEG NPs compared with the RAPA administered alone.[169] Moreover, the *in vivo* studies showed the promising capacity of the nanosystem to promote tissue healing and improve the mechanical properties of the healed tendon.[169]

Freedman *et al.*[170] developed an adhesive hydrogel loaded with a corticosteroid (triamcinolone acetonide) to use in tendon healing. The hydrogel showed strong adhesion to patellar and Achilles tendon, in live rats; moreover the formulation improved healing while reducing scar formation in a model of Achilles-tendon rupture rat.[170] Furthermore, *in vivo* studies in patellar tendon injury rats, demonstrated a decrease of the inflammatory process when in the animals were treated with the hydrogel. This is due to the modulated release of triamcinolone which promotes the macrophage polarization to the M2-phenotype while modulates the chemokine secretion.[170]

These various studies have demonstrated the potential of the local administration in treating various musculoskeletal diseases, including tendinopathies, and that NPs and

hydrogels can be harnessed to go beyond the limitations that conventional methods have not yet been able to break down, such as complete tissue regeneration and the ability to prevent possible relapse of the disease.

2.3. Tendinopathies

Tendinopathies is a common term used to refer to a group of conditions characterized by pain, swelling and impaired function of tendons.[171] Tendons are bands of connective tissue that connect muscles to bones, facilitating movement and transmitting force. When tendons become injured or damaged, due to overuse, repetitive strain, or acute trauma, tendinopathies can occur.[171]

Tendinopathies commonly affect various tendons throughout the body; some of the most frequently affected areas are the Achilles tendon and the tennis/golfer's elbow.[9] Symptoms typically manifest with localized pain and stiffness at the affected tendon, which often worsens with activity and improves with rest.[172] Swelling and tenderness may also be present, accompanied by a reduction in strength and range of motion.[9]

Although injuries limited to tendons and ligaments account for about 50% of all musculoskeletal disorders;[10] the diseases are responsible for an high decrease of work-time productivity.[173] Musculoskeletal injuries involving tendons or ligaments can have a high impact on an individual's ability to maintain pre-injury capabilities, regardless of the treatment approach chosen. Recovery from such injuries, whether managed surgically or nonsurgical, often involves prolonged recovery periods and may not fully restore pre-injury levels of function.[9] On a personal level, the chronic pain and functional impairment associated with tendinopathies can have a big impact on physical and mental well-being.[174]

In addition, medical costs associated with tendinopathies include diagnostic procedures, conservative treatments, and potential surgery, contributing to significant health care costs for both individuals and health care systems.[10]

2.3.1. Structure of the tendon in healthy condition

Tendons are composed primarily of tightly packed collagen fibres incorporated in a matrix of proteoglycans, water, and other proteins. This collagen-rich composition imparts tensile strength to tendons, enabling them to withstand the mechanical forces generated during muscle contraction and movement.[175] Type I collagen predominates as the primary collagen (**Figure 4**), supplemented by small amounts of other types such as collagen III, V, VI, XI, XII, and XIV, each of which contributes distinct roles to tendon function and structure.[175]

While fibrillar collagens (I, III, V, and XI) form the majority of the tendon's composition with long triple-helix regions, nonfibrillar collagens, such as type VI, have specific pericellular distributions, and fibril-associated collagens (XII and XIV) play key roles in stabilizing collagen fibres and regulating fibril diameter during development.[176] The extracellular matrix (ECM) surrounding collagen fibres plays a crucial role in maintaining tendon structure and function. Proteoglycans, such as decorin and aggrecan, are important components of the ECM and provide hydration and lubrication to the tendon while regulating fibrillogenesis and collagen organization.[175] Water, which accounts for about 55-70% of the wet weight of tendon tissue, also contributes to the viscoelastic properties of the tendon, allowing it to deform and recover its shape in response to mechanical stress.[177] The cellular components of tendons, mainly tenocytes, are responsible for the synthesis and maintenance of the ECM. Tenocytes have a characteristic elongated

morphology, with cytoplasmic extensions extending between collagen fibres within the tendon matrix.[178] This elongated shape allows the tenocytes to align parallel to the direction of mechanical stresses within the tendon, optimizing their ability to sense and respond to mechanical stimuli. Tenocytes play a crucial role in the turnover, remodelling, and repair of the ECM in response to mechanical loading and tissue injury.[178]

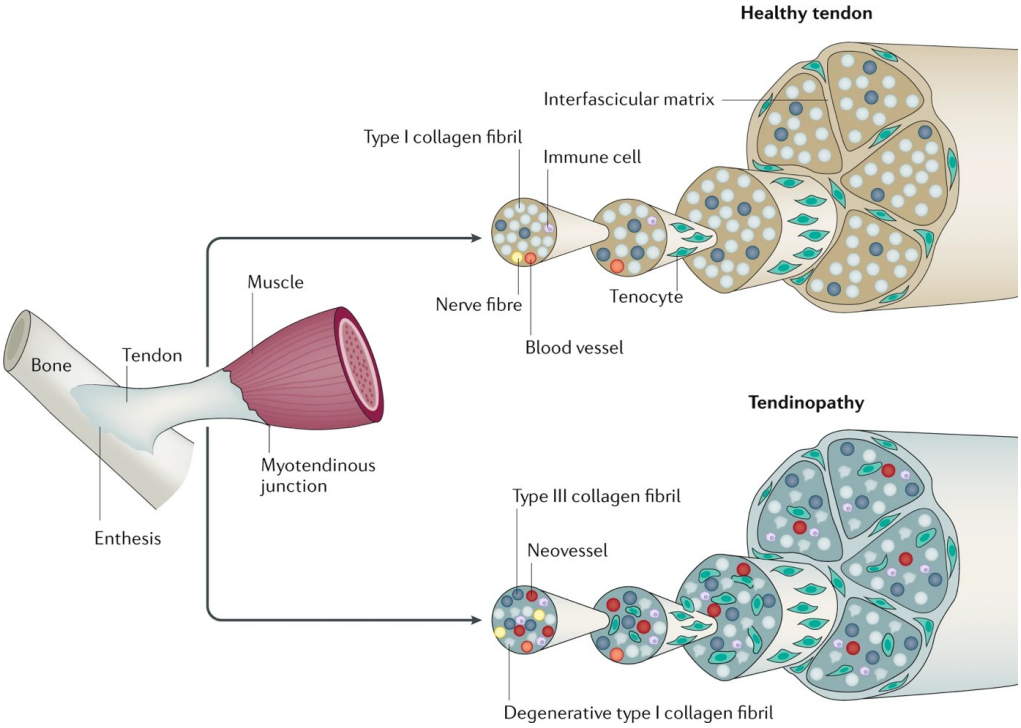


Figure 4. Comparison between the structure of a healthy and a pathologic tendon.
In a normal tendon the collagen fibrils are well organized and collagen I is predominant; in contrast, when tendinopathy occurs, the fibril are composed mainly of collagen III and are degenerative. Image reprinted with permission from ref. [9]; Copyright © (2021) Springer Group.

2.3.2.Tenocytes and their key roles in tendon

One of the main roles of tenocytes is collagen synthesis, particularly the production of type I collagen, which is the predominant type of collagen in tendons.[175,179] Type I collagen forms the structural framework of tendons, providing the tensile strength and elasticity needed to withstand mechanical forces during movement.[175,179] Tenocytes continuously synthesize and deposit new collagen molecules into the ECM, contributing to the growth, repair, and adaptation of tendons to changing mechanical demands. In addition to collagen synthesis, tenocytes also regulate ECM turnover and remodelling through the secretion of MMPs, enzymes responsible for the degradation and remodelling of ECM components. This dynamic process of ECM turnover ensures the maintenance of tendon integrity and function over time. **Table 4** provides a comprehensive overview of the major MMPs involved in tendon biology, their alternative names, the primary substrates they target within the ECM.[180,181]

Table 4. Overview of the principal MMPs involved in tendon biology. The Table provides also information about their specific functions in physiological ad pathological conditions.[180,181]

MMP	Alternative Names	Main Substrates	Function	Conditions
MMP-1	Collagenase-1	Collagen I, II and III	Degradation of fibrillar collagens.	Tendinopathy, Tendon Tears
MMP-2	Gelatinase A	Denatured collagen, collagen IV Gelatin, Elastin	Degradation of collagen, gelatin, elastin, and other ECM components.	Tendon Remodelling, Tissue Repair
MMP-3	Stromelysin-1	Proteoglycans, Collagen (III, IV, V), Fibronectin	Degradation of a wide range of ECM components including proteoglycans, collagen, and fibronectin.	Tendon Remodelling, Inflammation, Tendinopathy
MMP-9	Gelatinase B	Collagen, Gelatin, Elastin	Degradation of collagen, gelatin, elastin, and other ECM components.	Tendon Repair, Inflammation, Tendinopathy
MMP-13	Collagenase-3	Collagen I, II and III	Degradation of collagen.	Tendinopathy, Cartilage Degradation, Osteoarthritis

In addition, tenocytes play a crucial role in the response to mechanical loading and injury by modulating their metabolic activity and ECM production. Mechanical loading stimulates tenocyte proliferation and collagen synthesis, promoting tendon adaptation and strengthening in response to increased demand.[182] Moreover, excessive or repetitive mechanical stress can lead to tenocyte apoptosis (cell death) and ECM degeneration, contributing to the development of tendon pathologies and tendon tears.[182]
Tenocytes are also involved in the inflammatory response to tendon injury, secreting cytokines and growth factors that recruit immune cells and promote tissue repair.[179] Following injury, tenocytes undergo to a phenotypic changes, switching to a more active and synthetic state, characterized by increased ECM synthesis and remodelling. This

reparative process involves the proliferation and migration of tenocytes to the site of injury, where they cooperate with immune cells and other cell types to facilitate tissue healing.[179] In conclusion, tenocytes play an important role in maintaining tendon structure and function, from collagen synthesis to ECM remodelling and tissue repair.

2.3.3.Tendinitis, tendinosis and tendon rupture

Tendinitis, also known as tendonitis, is a common condition characterized by inflammation of a tendon.[183] It usually occurs due to repetitive overuse or sudden trauma, which causes microtears in the tendon tissue and triggers an inflammatory response.[179] The characteristic symptoms of tendonitis are pain, tenderness, swelling, and stiffness of the affected tendon and surrounding area.[179] The pain often worsens with movement or activity related to the affected tendon and may be accompanied by a creaking sensation within the tendon. Treatment of tendinitis focuses on reducing pain and inflammation, promoting tendon healing, and restoring function. Initial management often includes rest, activity modification, ice therapy, and over-the-counter pain medications, such as NSAIDs.[183]
Figure 5 provides an overview of the main biological mechanisms involved in the development of tendinitis.[184-186]

Choi *et al.*,[7] in their study, evaluated the anti-inflammatory and the healing effects of their nanosystem in rat tenocytes, *in vitro*, and in a *in vivo* collagenase-induced Achilles tendinitis rat model. The developed NPs, lactoferrin-immobilized, heparin-anchored, poly(lactic-co-glycolic acid) NPs (LF/Hep-PLGA NPs) showed a decreasing of pro-inflammatory factors, such as IL-1β , TNF$-\alpha$ and MMP-3 and an increasing in the level of anti-inflammatory cytokines (IL-4 and IL-10), in both *in vitro* and *in vivo* studies.[7] Moreover, in the *in vivo* evaluation of tenogenic markers, as collagen I, decorin and tenascin-C, the RT-PCR results showed an increasing in the expression of this markers, when animals were treated with LF/Hep-PLGA NPs, compared to the controls, demonstrating the effective contribution of the nanosystem in the healing process of the tissue in tendinitis.[7]

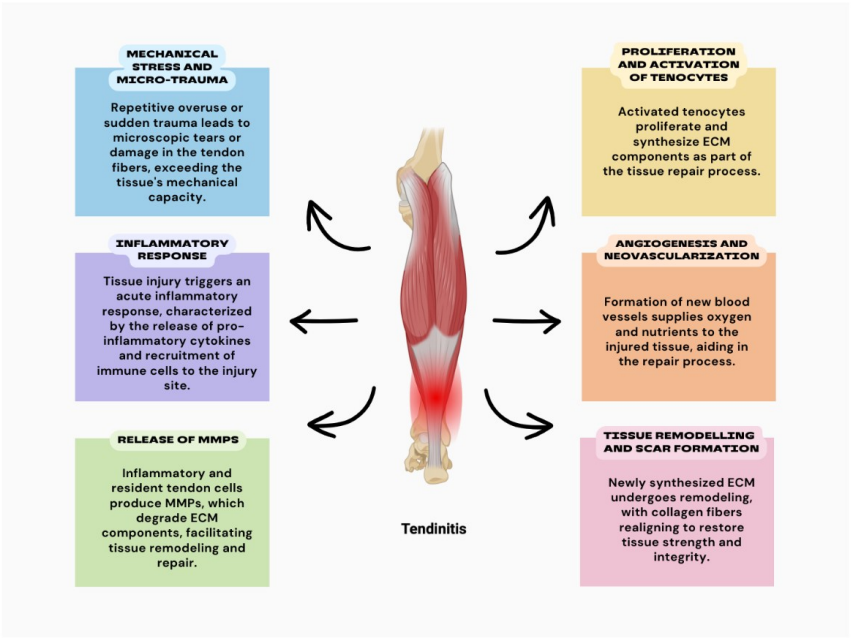


Figure 5. Biological mechanism involved in tendinitis. Effects of tendonitis: after mechanical stress and microtrauma, there is an inflammatory phase with release of cytokines and recruitment of immune cells, which activate MMPs. In addition there is proliferation and activation of tenocytes, with tissue remodelling, angiogenesis, and initial formation of scar tissue.[184-186]

Tendinosis represents a distinct pathologic condition characterized by degenerative changes within the tendons, in contrast to the inflammatory nature of tendonitis.[183] It arises due to repetitive microtrauma, overuse, or failure of healing responses after tendon injury. Unlike tendinitis, tendinosis lacks acute inflammatory markers and is characterized by an imbalance between degeneration and synthesis of the tendon matrix.[179]

Macroscopically, tendinosis is manifested by mucoid degeneration, which causes softening of the affected tendon region, which loses its characteristic white appearance, taking on a greyish-brown, opaque hue.[187] Histologically, the disease is defined by an aberrant collagen composition, particularly a high ratio of type III to type I collagen.[188] In addition, collagen fibres become disorganized, and collagen fibrils exhibit reduced uniformity in length and diameter. Tendon tissue becomes hypercellular, with metabolically active tenocytes showing a more rounded morphology.[189] Moreover, there is an increased incidence of vascular growth and neovascularization, along with accumulation of glycosaminoglycans (GAGs) between tendon fibrils and potential calcification of the tendon.[190] These histologic changes suggest a potential shift toward a chondrogenic phenotype in the tendon, probably due to disturbances in cell differentiation or altered loading conditions.[189]

Furthermore, tendinosis predisposes individuals to muscle atrophy, weakness, and a high risk of tendon rupture. **Figure 6** provides an overview of the key biological mechanisms involved in the development and progression of tendinosis.[179,185,188,189]

Cetik *et al.*[191] evaluated the effects of PLGA-b-PEG TGF-β3 loaded NPs, in the process of tendon healing, in a rat Achilles tendon injury model. The NPs were loaded in chitosan films, with the aim to have a controlled release, as demonstrated in the *in vitro* drug release study. The release of TGF-β3 from the NPs showed the release of more than 60% of the drug up to 2h, followed by a sustained profile up to 72h.[191] In contrast, the TGF-β3 released from the NPs loaded in the chitosan films was less than 25% at 2h, and only the 40% of the total drug was released after 72h, demonstrating the potential of the nanoplatform for the controlled release.[191] In *in vivo* studies , immunohistochemistry analysis demonstrated that 6 weeks after the treatment with PLGA-b-PEG TGF-β3 loaded NPs, collagen I was predominant over collagen III; moreover, the collagen III production, at gene level, results to be high after 6 week, and this can be explained by the fact that increasing the concentration of TGF-β3 results in an accelerated remodelling process, with an increase in the turnover of collagen III.[191]

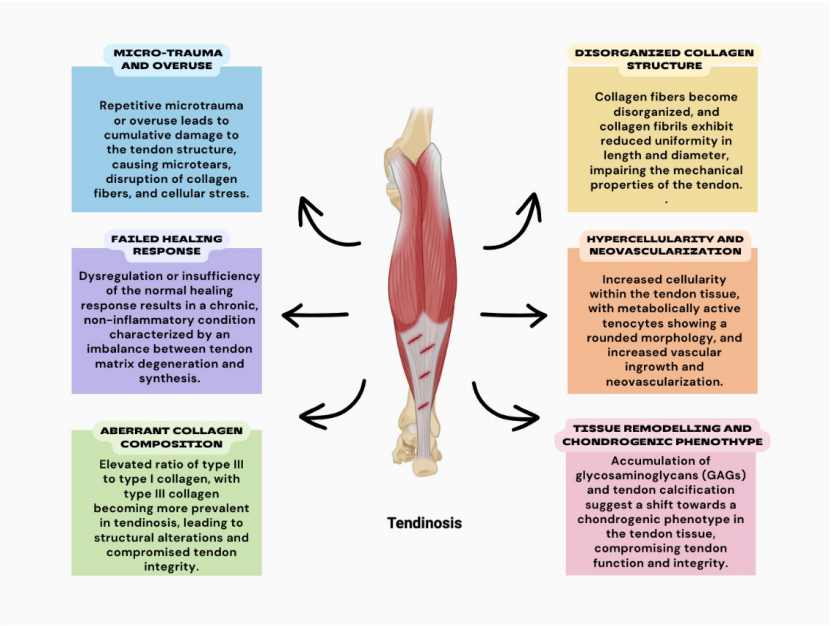


Figure 6. Biological mechanism involved in tendinosis. Effects of tendinosis: after mechanical stress and microtrauma, there is a failed healing phase with an imbalance between ECM synthesis and degeneration. Collagen III prevails over collagen I, causing disorganization of fibers and reduction in their length and diameter, compromising tendon strength. Tenocytes change morphology from an elongated to a round shape-all these mechanisms negatively impact tendon function and integrity.[179,185,188,189]

Tendon rupture refers to the partial or complete tearing of tendon fibres, which can occur suddenly as a results from an acute event or develop gradually as a chronic condition. Acute ruptures typically result from a single high-load impact, while chronic ruptures occur when the tendon is weakened due to factors such as tendinosis or aging, causing it to rupture at a lower load threshold.[192] Following rupture, tendons typically undergo healing with the formation of scar tissue, but this process often results in tissue that lacks the same composition, and mechanical properties as the healthy tissue. Consequently, there is an increasing risk of subsequent re-rupture.[188,193]

2.4. The 3 phases of tendon repair process: inflammation, proliferation and remodelling

Tendon repair is a complex process that involves three distinct phases represented in **Figure 7.**[193] Each phase is characterized by specific biological mechanisms and the presence of various cells, contributing to the restoration of tendon integrity and function.[194]

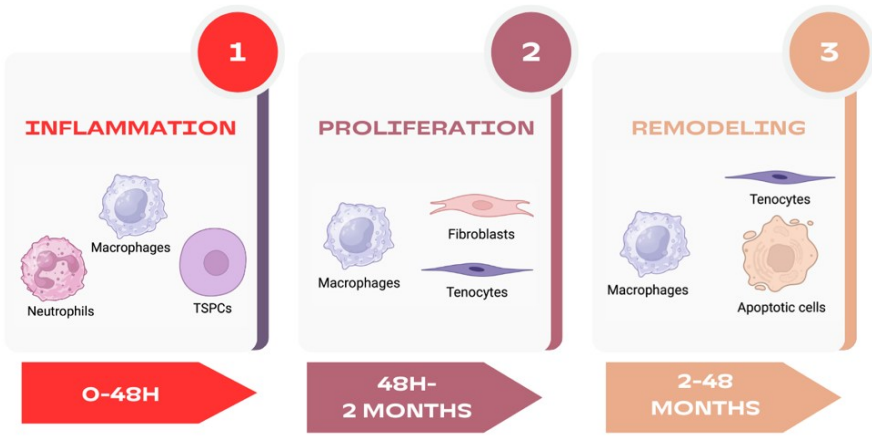


Figure 7. The three phases of tendon healing. In the early inflammatory phase, immune system cells go to accumulate at the site of injury. Later, during the proliferation phase tenocytes and fibroblasts are predominant, going on to produce the components of the ECM. Finally, during the remodelling phase macrophages and fibroblasts continue to redefine ECM turnover, while tenocytes contribute to the alignment of fibres to ensure that a strong tissue with healthy-like properties is obtained. The duration of the phases varies according to the severity of the injury and from patient to patient. Adapted with permission from ref. [194], Copyright© by the authors.

The **inflammation phase** marks the initial response to tendon injury, aiming to remove damaged tissue and prepare the site for healing. Injured tendon tissue releases inflammatory mediators such as cytokines (*e.g.*, interleukin-1β, tumour necrosis factor-alpha (TNF- α)) and chemokines, which attract immune cells to the site of injury.[195] Neutrophils are the first cells to arrive within hours at the site of injury, to phagocytize debris, and release proteases to degrade the damaged tissue. This is followed by macrophages, which clear cellular debris and secrete growth factors that stimulate the next stages of healing.[193] The release of inflammatory mediators triggers vasodilation and increased vascular permeability, leading to the influx of immune cells to the injury site. While tenocytes are not as prominent in the inflammation phase, they may contribute to the release of signalling molecules that modulate the inflammatory response.[179] Additionally, tenocytes at the periphery of the injured tendon may become activated and undergo phenotypic changes in response to inflammatory signals,[196] preparing them for the subsequent phases of repair.[195,197]

The **proliferation phase** involves the synthesis of new ECM and the formation of granulation tissue, laying the foundation for tissue repair and regeneration. Fibroblasts, recruited from adjacent healthy tissue and resident tendon cells, play a crucial role in ECM synthesis. These cells proliferate and migrate to the injury site, where they produce collagen III, proteoglycans, and other ECM components to rebuild the damaged tendon structure.[194] Angiogenesis, the formation of new blood vessels, also occurs during this phase, and it is important for providing oxygen and nutrients to support tissue repair.[198] Macrophages continue to play a crucial role during the proliferation phase by releasing growth factors that promote fibroblast proliferation and ECM synthesis. They also help

regulate the inflammatory response, transitioning from a pro-inflammatory to a pro-repair phenotype.[199]

The **remodelling phase** focuses on the maturation and organization of newly synthesized ECM, optimizing tissue strength and function. Collagen fibres realign along the lines of mechanical stress, enhancing tendon strength and elasticity. Excess collagen is removed, and the ECM undergoes cross-linking and remodelling to achieve structural integrity. The balance between collagen synthesis and degradation is crucial during this phase to prevent scar formation and to ensure tissue strength, given that the mechanical properties of an healed tendon are often inferior when compared to the healthy tissue.[194,200] During the remodelling phase, fibroblasts and macrophages continue to play a critical role in ECM turnover and remodelling. They secrete MMPs that degrade excess collagen and other ECM components, allowing for the refinement of tissue structure and the removal of scar tissue.[184,201,202]

Tenocytes remain active during the remodelling phase, contributing to the alignment and organization of collagen fibres along the lines of mechanical stress, by continuing to synthesize and modify ECM components, helping to optimize tissue strength and function.[203]

In summary, during the 3 phases of tendon repair, communication between the cells of the immune system and the cells residing in the tissue is crucial to recover the normal condition of the tendon.

2.5. Hypercholesterolemia and atherosclerosis: Potential risk factors of tendinopathies

Tendinopathies risk factors include overuse and repetitive stress from activities like sports, poor biomechanics, age-related degenerative changes, muscle imbalances, sudden increases in activity intensity, obesity, genetic predisposition, and certain systemic diseases or medications.[9] Moreover, some studies have demonstrated that hypercholesterolemia, characterized by elevated levels of cholesterol in the bloodstream, is a key contributor to the development of atherosclerosis and they are linked with tendinopathies.[204-206]

Atherosclerosis is a multifaceted disease characterized by plaque buildup in arteries, resulting in arterial narrowing and hardening. It is a major contributor to cardiovascular diseases such as stroke and coronary artery disease.[207] Atherosclerosis typically begins with damage to the endothelial cells lining arterial walls (**Figure 8**), caused by factors like high blood pressure, smoking, hypercholesterolemia, and inflammation. This damage leads to endothelial dysfunction, characterized by increased permeability, adhesion molecule expression, and inflammation.[207]

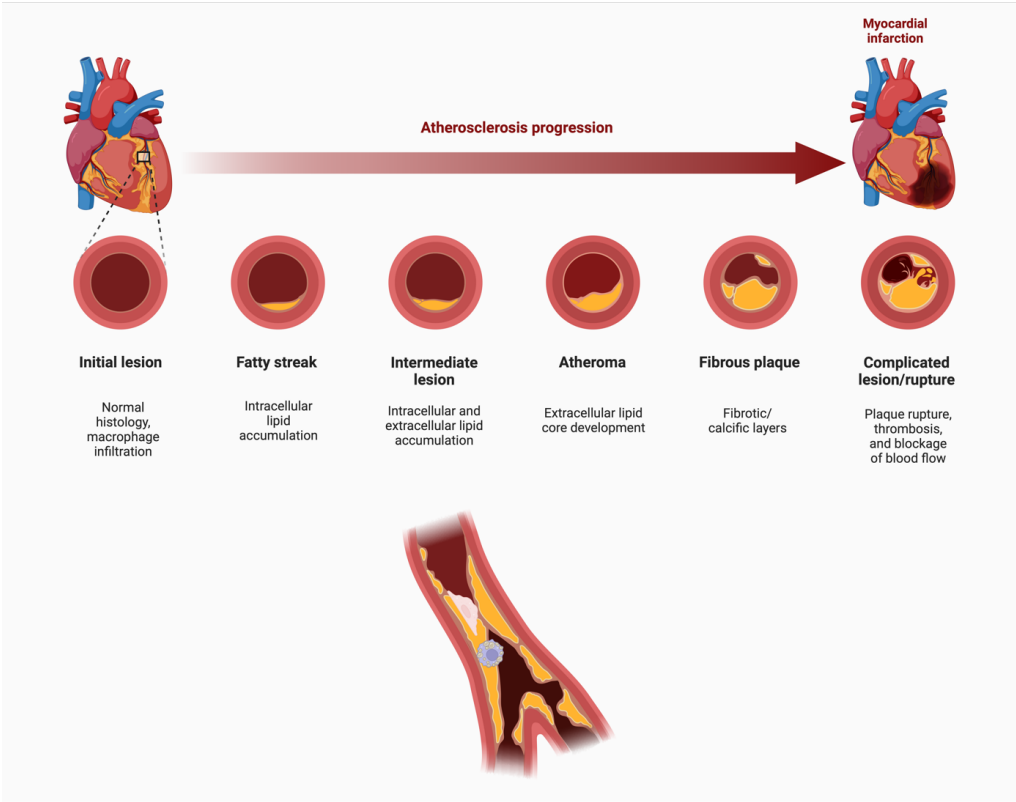


Figure 8. Progressive stages of atherosclerosis: From initial lesion to atheroma and plaque formation.[208] After the initial injury, oxLDLs accumulate in the arteries triggering an inflammatory reaction with the recall of macrophages. Over time, the cumulation of the cells initially forms an atheroma, which will then form fibrous plaque, unstable, that in case of rupture can cause blockage of blood flow and thrombosis. Image created with Biorender.com

Consequently, low-density lipoprotein (LDL) cholesterol penetrates arterial walls, undergoes oxidation, and transforms into oxidized LDL (oxLDL), initiating an inflammatory response.[207,209] The inflammatory response triggers the recruitment of monocytes and macrophages to the site of injury, where they engulf oxLDL particles and become foam cells. These foam cells accumulate within the arterial intima, forming fatty streaks—the earliest visible lesions of atherosclerosis. Meanwhile, inflammatory mediators, such as cytokines and chemokines, further promote immune cell recruitment, perpetuating the inflammatory process.[210]

In response to inflammatory signals and growth factors released by activated immune cells, smooth muscle cells contribute to the formation of the fibrous cap—a protective layer covering the atherosclerotic plaque—and produce ECM components like collagen and elastin. Over time, the accumulation of foam cells, inflammatory cells, smooth muscle cells, and ECM proteins leads to the formation of atherosclerotic plaques.[207] These plaques consist of a lipid-rich core containing cholesterol crystals, foam cells, and cellular debris, surrounded by a fibrous cap composed of smooth muscle cells, collagen, and elastin.[211] Atherosclerotic plaques can undergo structural changes, leading to plaque instability and

rupture. Plaque rupture exposes the thrombogenic core to the bloodstream, triggering platelet activation and aggregation, and the formation of a thrombus.[212]
The immune system plays an important role in both the initiation and regulation of atherosclerosis, exerting significant influence on the progression and outcome of the disease. Dysregulated inflammation contributes to plaque formation and instability, increasing the risk of cardiovascular events.[207]

The CANTOS study, known as the Canakinumab Anti-inflammatory Thrombosis Outcome Study, examined the impact of targeting interleukin-1β (IL-1β) on inflammatory responses and thrombosis outcomes.[213] Canakinumab, a monoclonal antibody targeting IL-1β, significantly reduced the risk of recurrent cardiovascular events compared to placebo, highlighting the potential therapeutic benefit of targeting inflammation in atherosclerosis.[214]

Zhao *et al.*[215] developed kaempferol biomimetic NPs, for the repolarization of macrophages to M2, in the treatment of atherosclerosis. The NP's core was coated with cell membrane derived from RAW 264.7 macrophages, to enhance the targeting for the adhesion of the particles at the injured site. The presence of the successful coating was evaluated with the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), to check the protein composition in the cell extracted membrane and cell-coated NPs.[215] Moreover, the presence of the CD47, using Western Blot (WB) analysis, was assessed, since the protein is a unique marker present on the surface of macrophages, confirming the integrity of the membranes on the coated-NPs.[215] The developed nanosystem showed in the *in vivo* studies the ability to target the atherosclerotic plaques and suppress the inflammation, switching the macrophages from M1 to M2 phenotype, in $ApoE^{-/-}$ mice, confirming the potential of this platform for atherosclerosis treatment.[215]

In cases of hypercholesterolemia, increased concentrations of LDL and ox-LDL contribute to systemic inflammation and tissue damage, affecting various organs and tissues, including tendons.[216] LDL cholesterol is a key player in atherosclerosis, but emerging evidence suggests its involvement in pro-inflammatory responses within the tendon.[217] LDL and ox-LDL can infiltrate the tendon matrix, where they interact with resident cells such as tenocytes and immune cells. Upon entry into the tendon, ox-LDL triggers inflammatory signalling pathways, leading to the production of pro-inflammatory cytokines, chemokines, and adhesion molecules.[216] These inflammatory mediators recruit immune cells to the site of injury, perpetuating the inflammatory response and contributing to tendon degeneration and dysfunction. Furthermore, ox-LDL-induced inflammation within the tendon microenvironment can disrupt tendon homeostasis, impair collagen synthesis and turnover, and promote matrix degradation.[218] Some studies have demonstrated that this dysregulated matrix remodelling may weaken the tendon structure, rendering it more susceptible to injury and degeneration. Further studies are warranted to elucidate the connection between hypercholesterolemia, atherosclerosis, and tendinopathy, as well as to delineate the underlying mechanisms and identify potential therapeutic targets for intervention.

2.6. Current therapeutic approaches for tendon diseases treatment

Current therapeutic approaches for tendon disorders include conservative management strategies and surgical interventions.
Conservative management strategies are often employed as an initial treatment modality for tendon diseases. These may include rest and activity modification to enable tendon healing and recovery. Physiotherapy plays a crucial role in the management of

tendinopathies, focusing on exercises to improve the strength, flexibility, and biomechanics of the tendon.[179] In addition, modalities such as ultrasound, electrical stimulation and manual therapy can be used to relieve pain and promote tissue healing. NSAIDs are commonly used to reduce pain and inflammation in tendinopathy, although their long-term use must be monitored due to potential adverse effects on tendon healing and gastrointestinal health.[219]

Injection therapies are another treatment option for tendinopathies. Corticosteroid injections can be administered directly into the tendon sheath or surrounding tissue to reduce pain and inflammation. However, repeated doses can weaken the tendon and increase the risk of rupture.[219]

In the treatment of tendinopathies, several cell-based strategies have been developed, including platelet-rich plasma (PRP) and stem-cells therapy. PRP therapy implicates the injection of platelets derived from the patient's own blood into the injured tendon to promote healing and tissue regeneration.[179,220] Neverthelss, PRP therapy for tendinopathies remains controversial since some studies have shown no efficacy compared with the control group.[221,222] Stem cells are attractive for the treatment of tendinopathies because of their ability to differentiate into various specialized cell types, including tenocytes.[223] Stem cells can also release growth factors and molecules that help reduce inflammation and promote healing. Some studies have demonstrated the beneficial role of injecting mesenchymal stem cells (MSCs), alone or in combination with PRP.[224] Furthermore, more studies need to be conducted to better understand the real efficacy and reproducibility of this therapy. In fact, Chun *et al.*[225] in their study showed that cell- therapy had no benefit compared with control injections, and that further investigations are needed to better understand the application of MSCs in the treatment of tendon diseases.[221,222]

In the case of severe tendon damage or failure to respond to conservative treatments, surgery may be necessary. Surgical procedures, such as tendon repair or reconstruction, aim to restore the integrity and function of the tendon. Tendon transfer surgery involves the transfer of a healthy tendon to replace a damaged or ruptured tendon, while tendon grafting can be performed using autografts or allografts to reconstruct the tendon in cases of extensive tendon loss or irreparable damage.[226,227] New therapeutic approaches for tendon diseases are currently underway, including the use of novel biological agents and tissue engineering strategies. NPs, hydrogels and scaffolds play an important role in the development of new delivery systems in injured tendon. These innovative therapies aim to modulate the inflammatory response, promote tissue repair, be patient-personalized and improve functional outcomes in patients with tendinopathies.[228-230]

In the context of tendinopathies, curcumin has attracted particular interest because of its anti-inflammatory and antioxidant properties,[231] which can be used in the early phase of tendinopathy, when pro-inflammatory cytokines are released.[232] Curcumin promotes the decrease of the inflammatory process by going to inactivate the nuclear factor beta (Nf-κb), and reducing the expression of cytokines such as IL-6 and TNF-α, as demonstrated in the study conducted by Kim *et al.*, where curcumin was loaded into porous microspheres.[233] To confirm the uniformity of the drug loaded, confocal laser microscopy study was performed, showing that the curcumin was distributed throughout the microspheres, and the *in vitro* release study resulted in a fast profile at day 1, due to the diffusion from the porous of PLGA microspheres.[233] *In vitro* studies were performed on rat tenocytes treated with LPS, to mimic the inflammatory environment. RT-PCR showed that in cells treated with NPs there was a decrease in the expression of cyclooxygenase 2 (COX-2), TNF-α and MMP-3, resulting in a suppression of the inflammation.[233] Moreover, *in vivo* studies on

collagenase-induced Achilles tendinopathy rat model, were conducted, confirming the decreasing of pro-inflammatory cytokines and the increasing of the tensile strength of the tissue, when the biomechanical test was performed on tendon extracted from the NPs-treated animals, compared to the collagenase positive control.[233]

López-Cerdá *et al.*[228] developed a novel lipid-polymeric hybrid nanoplatform, for the dual delivery of budesonide and serpine1 siRNA, in order to tackle with the same system, inflammation and fibrosis in macrophages dysregulation in tendinopathy. At gene and protein levels, the NPs showed to decrease the expression of Nf-κb and TGF-β, both in murine and human macrophages.[228] Moreover, the NPs shifted the phenotype to M2, modulating the production of pro and anti-inflammatory markers. In addition, in the cells treated with NPs, the expression of PAI-1 protein decreased , due to the delivery of serpine1 siRNA, hence leading in tendon ECM remodelling process.[228] Furthermore, it has been shown that by going to inhibit TGF-β1/Smad2/3 signalling during the tendon healing process, it would increase collagen I production and enhance biomechanical properties.[234] Therefore, delivering in NPs a siRNAs targeting SMAD3 may be a good strategy to improve tendon healing.

Wang *et al.*, in their study investigated how the inhibition of Smad3 affected the rotator cuff tendon healing, using an *in vivo* rat model.[234] The study confirmed that, the treated animals with Smad3 inhibition, resulted in higher production of collagen I and better healed tissue with collagen fibres continuity, showing higher biomechanical properties, compared to the other groups.[234] Moreover, by also going to load an mRNA that goes to promote the production of collagen I, which is the predominant element in the structure of healthy tendon, the regeneration of tissue in a late stage of tendinopathy can be targeted.

In conclusion, in the management of tendinopathies, tailoring therapeutic strategies to individual patient characteristics is essential to optimize clinical outcomes and improve patients' quality of life. In this context, the use of novel drug delivery approaches emerges as a promising prospective. New therapies, which can be tailored to meet the specific needs of each individual, could represent a significant breakthrough in the treatment of tendinopathies, offering potential for an effective tissue healing and to improve living standards for patients.

3. Aims of the study

Nanosystems with different compositions have been synthetized for different stages of tendon injury. First, a lignin-based nanosystem was developed to treat one of the possible risk factors of tendinopathy: atherosclerosis; then polymeric NPs were synthesized to be able to act as anti-inflammatory in the early phase of tendon injury. Finally, LNPs were developed and loaded with two RNAs, with the aim to decrease fibrosis, in the late tendon injury phase, and at the same time stimulate the proliferation of collagen I, the main component of tendon fibrils in healthy condition.

More specifically, the objectives of the study were:

1. To develop three reproducible and scalable nanoplatforms for the delivery of therapeutics **(I-III)**.
2. To evaluate the interaction of the lignin-based NPs on human coronary artery endothelial cells (HCAEC) and the anti-inflammatory activity of the loaded curcumin **(I)**.
3. To investigate the cytocompatibility and the cell-NPs interaction of the polymeric and lipid-based nanosystem, in human primary tenocytes derived from patient with pathological tendon condition (**II-III**).
4. To evaluate the anti-inflammatory and anti-fibrotic activity of the polymeric NPs in LPS-treated human primary tenocytes (**II**).
5. To investigate the anti-fibrotic and proregenerative effects of the RNAs loaded LNPs in human primary tenocytes (**III**).

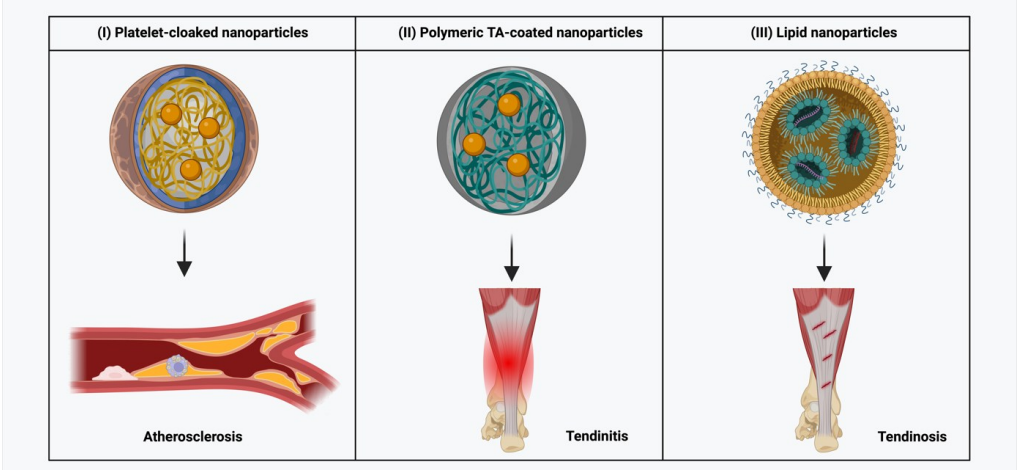


Figure 9. Overview of the 3 nanosystems developed, starting with (I) lignin-NPs loaded with curcumin and coated with TA and platelet cell-membrane; (II) AcDEX- NPs loaded with curcumin and coated with TA; and (III) LNPs loaded with SMAD3 siRNA and COL1A1 mRNA. Image created with Biorender.com

4. Experimental

This section summarizes the methods used for the studies described in the thesis. The detailed materials and methods can be found in the original publications (**I–III**).

4.1. Nanoparticles production

4.1.1. Lignin-based nanoparticles production (I)

Lignin and curcumin-loaded lignin NPs were prepared by solvent exchange dialysis following a previously developed protocol.[118] 1 mg of curcumin was dissolved with 15 mg of lignin in tetrahydrofuran (THF). After the production, the NPs were collected, then centrifuged at 16100×*g* for 5 min and washed twice to remove residues of THF. The NPs were then stored at +4°C in MQ-water.

4.1.2. Acetalated dextran (AcDEX) synthesis and AcDEX nanoparticles production (II)

AcDEX was synthetized following the protocol already described by Bachelder *et al.*[102] AcDEX loaded NPs were prepared in a glass-capillary microfluidics device by nanoprecipitation.[15] Briefly, 10 mg of AcDEX were dissolved in 900 µL of ethanol and 100 µL of 5 mg/mL curcumin in ethanol were subsequentially added, representing the inner phase. A solution of 1% Poloxamer-188 (pH 8) in MQ-water was prepared as the outer phase. The two miscible liquids were pumped into the chip at the constant flow rate of 2 mL/h and 60 mL/h, for the inner phase and the outer phase, respectively, using the Harvard PHD 2000 syringe pumps. The NPs were collected and pelleted by ultracentrifugation, washed twice with MQ-water and stored at +4°C.

4.1.3. Coating with Tannic Acid-iron (I-II)

AcDEX and lignin NPs were coated with TA-iron (Fe^{3+}) as reported elsewhere.[235] TA and iron were dissolved in MQ-water at the concentration of 40 mg/mL and 6 mg/mL, respectively.

Then 0.5 mg of AcDEX NPs were resuspended in 500 µL of MQ-water and 1 mg of lignin NPs in 1 mL of MQ-water, to have a final concentration of 1 mg/mL. After, 5 µL and 10 µL, respectively, of the solution of TA were added to the particles, and the solution was vortexed for 30s. The same process was made for the iron. AcDEX TA-coated NPs were centrifuged at 11,200×*g* × 4 min and the lignin-TA NPs were centrifuged at 13,200×*g* × 5 min. Then the supernatant was collected for the indirect quantification of TA and iron, using the Folin-Ciocalteu's method and the isothiocyanate colorimetry method, respectively.[236,237]

4.1.4. Coating with platelet-cell membrane (I)

Platelets were isolated from the blood of an healthy anonymous donor, accordingly to the licenses of the Finnish Red Cross. The cells were isolated following a protocol described elsewhere,[137] and then stored at -80°C at the concentration of 3×10^9 platelets/mL. Platelet cell-membranes were extracted using the freeze and thaw method. The platelet from -80 °C were thawed at room temperature (RT) and centrifuged for 10 min, using a speed of 20,800×*g* and a temperature of +20°C, to avoid the activation of the cells. Then, the supernatant was removed, the pellet suspended in 1 mL of MQ-water and freeze in liquid

nitrogen. The above mentioned process was repeated 4 times and the last centrifugation time was settled at 15 min. Subsequentially, the platelet-membrane were used for the NPs cloaking.
Lignin@TA and curc@lignin@TA NPs, at the concentration of 1 mg/mL, were resuspended and 0.6 x 10^9 platelets/mL were mixed together with the particles, in a ratio 1:1 (v/v). Subsequentially, the sample was bath-sonicated for 2 min and centrifuged at 5,900g for 3 min. The NPs were resuspended in MQ-water and stored at +4°C.

4.1.5. Lipid-based nanoparticles production (III)

LNPs, siRNA LNPs, mRNA LNPs and dual-loaded LNPs were synthetized using a co-flow glass-capillary microfluidics system, previously optimized in our lab.[15] The lipids, at the molar ratio of 50:10:38.5:1.5 (cKK-e12:DSPC:cholesterol:DSPE-PEG) were dissolving in ethanol, representing the inner phase, and the outer phase used was 1% polyvinyl alcohol (PVA) (w/v) in diethyl pyrocarbonate (DEPC) MQ-water. The COL1A1 mRNA and the SMAD3 siRNA were dissolved in the outer phase. At the flow rates of 1 mL/min (inner phase) and 20 mL/min (outer phase) the LNPs were synthetized and then dialyzed overnight against a solution of MQ-water.

4.2. Physicochemical characterization (I-III)

4.2.1. Dynamic and Electrophoretic Light Scattering (DLS and ELS) (I-III) and nanoparticles tracking analysis (NTA, III)

The hydrodynamic diameter (z-average), the PDI and the surface charge of the developed NPs were characterized using a Zetasizer Nano ZS instrument (Malvern Ltd, UK). All the measurement were done diluting the samples in MQ-water and the analysis were run at RT.
The particles concentration and size were also measured by ZetaView PMX-120 NTA instrument (**III**), (Particle Metrix GmbH, Ammersee, Germany). LNPs were diluted in 1 mL of MQ-water, using different dilution factors, and the analysis was performed at 22 °C.

4.2.2. Stability studies (I-III)

The colloidal stability of the developed nanosystem was studied in different relevant media for each study. 0.6 mg of NPs were suspended in 200 µL of MQ-water and added in 1.3 mL of different media (**II-III**), stirred at 150 rpm at 37 °C and, at each time point, 200 µL were taken, diluted in 800 µL of MQ-water and analysed by DLS. Lignin NPs (**I**) were redispersed in MQ-water at the concentration of 1 mg/mL and 180 µL of the solution were added in 1mL of RPMI medium, stirred at 200 rpm at 37 °C. At each time point 180 µL of samples were diluted in 800 µL of MQ-water and analysed using DLS.

4.2.3. Scanning electron microscopy (SEM, I-II),Transmission electron microscopy (TEM, I-II) and Cryo-TEM (III)

The morphology of the different nanosystems was investigated using SEM microscopy (**I-II**). The shape of lignin NPs was analysed using Hitachi S-4800 (JAP) SEM (**I**): about 10 µL of a solution of 1 mg/mL of NPs were added on a silicon wafers and left drying overnight; then particles were coated with gold-platinum alloy and imaged. The external morphology of the AcDEX NPs (**II**) was analysed using SEM Quanta FEG 250 (FEI USA) at the voltage

of 5.0 kilovolt (kV). Briefly, 5 µL of a solution of 0.5 mg/mL of particles were added on a silica substrate, coated with a 10 nm platinum layer and then analysed with the microscope. TEM studies were also performed to analyse the shape of NPs (**I-II**). About 5 µL of NPs suspension (1 mg/mL and 0.5 mg/mL, I-II respectively) were added on a carbon-coated copper grid, left drying overnight and the analysed using Jeol JEM-1400 microscope. For Cryo-EM analysis (**III**), LNPs were vitrified on glow-discharged EM grids, using a Leica EM GP plunger. The grid screening was performed using a ThermoFisher Scientific Talos Artica microscope, operating at 200 kV.

4.3. Drug loading and release

4.3.1. Loading degree of curcumin and TA quantification in the polymeric systems (I-II)

The amount of curcumin loaded in the NPs was evaluated by loading degree (LD) following Equation (1):

$$LD\ (\%)\ = \frac{Concentration\ of\ curcumin\ quantified\ (\mu g\ mL^{-1})\ x\ Volume\ of\ NPs\ (mL)}{Mass\ of\ NPs\ (\mu g)}\ x\ 100 \quad (1)$$

For the AcDEX NPs (**II**), the concentration of the drug in the samples was quantified using high performance liquid chromatography (HPLC, Agilent 1200 series, Agilent Technology USA) and a Gemini 5 µm reversed-phase column (100 × 4.6 mm, Phenomenex). For the lignin NPs (**I**) the HPLC Agilent 1100 series (Agilent Technologies, GER) and 3 µm C-18 Gemini- NX column (100 × 4.6 mm, Phenomenex, USA) were used. For the detection of curcumin the mobile phase chosen was 0.2 % phosphoric acid and ACN (44:55), with a flow rate of 1 mL/min, injected volume of 10 µL and wavelength of 280 nm (**II**) and 480 nm (**I**). The temperature of the column was settled at 25 °C. The amount of TA present on the surface of the NPs was quantified indirectly, using the Folin-Ciocalteu's method (**I-II**).[236] After centrifuging the NPs, about 100 µL of supernatant were mixed with 200 µL Folin-Ciocalteu's reagent (Merck, Germany), and vortex for 30s. Then 800 µL of a solution 0.7 M of Na_2CO_3 in MQ-water were added and incubated for 2h at RT. A calibration curve of TA was used to quantify the amount of the compound. Finally, the absorbance of all samples was read with a Varioskan multimode plate reading, setting the wavelength at 765 nm. The quantity of iron was determined adding 100 µL of a solution of ammonium thiocyanate 1M to 100 µL of the supernatant, and incubated for 15 min at RT. A calibration curve of the iron was used to quantify the amount of the compound. The absorbance was then read with a Varioskan multimode plate reading, setting the wavelength at 490 nm.

4.3.2. *In vitro* drug release (I-II)

The *in vitro* drug release for the lignin NPs was performed in PBS + 40 mM of ascorbic acid + 1% w/v Tween80 at pH 7.4 and in 2-(N-morpholino)ethanesulfonic acid (MES) + 40 mM of ascorbic acid + 1% w/v Tween80, at pH 5.5, to mimic both the homeostatic and inflamed condition (**I**). The samples were stirred at 150 rpm at 37°C and at each time point 200 µL of samples were taken, replaced by fresh buffer, and analysed by HPLC. The *in vitro* drug release of curcumin from the Drug-Loaded and TA-Coated NPs was assessed in a synovial-mimicking fluid,[238] with the addition of 1% of Tween-80 (Sigma-Aldrich, USA), agent which help the stability of curcumin for the quantification (**II**). The release was studied at pH 7.4 and 5.5, in order to analyse the effect of a physiological and pro-inflammatory

environment. The samples were stirred at 150 rpm at 37°C and at each time point 200 µL of samples were taken, replaced by fresh buffer, and analysed by HPLC. In both case, the cumulative percentage of drug release was calculated using Equation (2):

$$Cumulative\ Drug\ Released\ (\%) = \frac{released\ drug\ \ (mg)}{total\ amount\ of\ drug\ loaded\ (mg)}\ x\ 100 \quad (2)$$

4.3.3.Loading of the RNAs and assay for the quantification (III)

The amount of COL1A1 mRNA and SMAD3 siRNA loaded in the NPs was determined using the Quant-it™ RiboGreen® RNA assay kit. LNPs were dissolved in 1% Triton X-100 (w/v) to quantify the total amount of RNA, complexed and non-complexed; 1× TE buffer was used to quantify the non-complexed RNA. The calibration curves of SMAD3 siRNA, COL1A1 mRNA and mixture of both RNAs were prepared in a range from 1 to 100 ng/mL, in both Triton X-100 and TE buffer. The concentration of RNA was determined using the Varioskan multimodal plate reader (ThermoFisher Scientific, USA) setting the wavelength at 485nm and 520 nm, for excitation and emission, respectively. The EE was calculated using Equation (3):

$$EE\ (\%) = \frac{concentration\ of\ RNA\ quantified\ \left(\frac{ng}{mL}\right) x\ volume\ of\ sample\ (mL)}{total\ amount\ of\ RNA\ loaded\ (ng)}\ x\ 100 \quad (3)$$

4.4. *In vitro* cell-studies (I-III)

For the *in vitro* studies, the following cell lines were used: Perypheral Blood Monocytes Cells (PBMC) isolated from the blood of anonymous healthy donors; human macrophages (KG-1, ATTC© CCL-246™); HCAEC (Sigma- Aldrich, USA); human primary tenocytes, isolated from the extensor *indicis proprius* of a 58-years-old patient and human macrophages (KG-1, ATTC© CCL-246™) (**II**); human primary tenocytes isolated from the flexor *digitorum profundus* of a 25-years-old patient, RAW 264.7 (American Type Culture Collection, USA) and eGFP-expressing RAW 264.7 (Cellomics Technology, Rockville, Maryland,USA) (**III**). PBMC, KG-1, HCAEC and eGFP RAW 264.7 cells were maintained in an incubator (BB16 gas incubator, Heraeus Instruments GmbH, Germany) with 5% of CO_2 and 95% relative humidity, at 37 °C. To grow human tenocytes the incubator used was ESCO CelCulture® CO_2 (ESCO Scientific), with. 5% of CO_2 and 95% relative humidity, at 37 °C. Human tenocytes used for the *in vitro* studies of publications **II** and **III** were between passage 2 and 7.

4.4.1.Cell culture (I-III)

PBMC were resuspended in tissue culture flasks (Corning Inc., USA,) in RPMI 1640 medium, supplemented with 10% of heat-inactivate Fetal Bovine Serum (FBS) and 1% of penicillin-streptomicyn (100 IU/mL Penicillin and 100 IU/mL Streptomicyn, PEST). KG-1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM), with addition of 10% FBS, 1% PEST, 1% L-glutamine and 1% NEEA. HCAEC were cultured in MesoEndo Cell Growth Medium, supplemented with 10% FBS and 5 ng/mL of vascular endothelial growth factor (VEGF). eGFP- RAW were cultured in RPMI 1640, supplemented with 10% of FBS. Finally, human primary tenocytes were cultured in DMEM-F12 medium, supplemented with 10% FBS, 1% PEST and 200 µM of ascorbic acid.

4.4.2.Cytocompatibility studies (I-III)

The cytocompatibility of the developed nanosystems was assessed using the CellTitler-Glo® luminescence assay, quantifying the adenosine triphosphate (ATP) produced by cells, when treated with the NPs. Adherent cells (HCAEC and human tenocytes) were seeded at the concentration of 20,000 and 10,000 cells/well, respectively, in a 96 well-plate (Corning, USA), and let to attach overnight. Complete cell medium and 1% Triton X-100 were used as negative and positive control. Then, cells were treated with different concentration of NPs, and after the established time of incubation, were washed 2 times with Hank's balance salt solution-(N-[2-hydoxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HBSS-HEPES, pH 7.4). Finally, 100 µL of CellTiter-Glo and HBSS–HEPES (1:1) were added and the luminescence was read with the Varioskan multimodal plate reader. For the non-adherent cells (KG-1 and PBMC), cells were seeded at the concentration of 20,000 cells/well and treated with different concentration of NPs, reaching a final volume of 100 µL/well. Then, 100 µL of CellTiter-Glo were added and the luminescence was read with the Varioskan multimodal plate reader.

4.4.3.Cell-nanoparticles interactions: Quantitative and qualitative cell-uptake (I-III)

The interactions between NPs and different cell lines were studied quantitively by flow cytometry and qualitatively using confocal microscopy (**I-III**).

In the first study (**I**), cells were seeded at the concentration of 200,000 cells/well in a 6-well plate (HCAEC) and in a 12-well plate (KG-1) (Corning, USA) and left to attach overnight (HCAEC). Then cells were inflamed with 10 ng/mL of LPS or left untreated, adding PBS at pH 7.4. After 24h the NPs were added in HCAEC cells at the concentration of 50 µg/mL and incubated for 1h at 37°C, washed with HBSS, then detached with trypsin neutralised by trypsin inhibitor. Cells were centrifuged at 220×*g* for 5 min, washed twice and resuspended in 500 µL of HBBS, in FACS tubes (Falcon, Corning Brand). Finally samples were analysed with LSRII Flow Cytometer (BD Bioscience, USA) in fluorescein isothiocyanate (FITC) channel. KG-1 were also treated with 25 µg/mL of NPs and incubated for 30 min at 37°C. The cells were then centrifuged at 300×*g* for 5 min, washed 2 times with PBS and resuspended in 500 µL of PBS, in FACS tubes. The samples were analysed with LSRII Flow Cytometer (BD Bioscience, USA) in the FITC channel and the results processed with FlowJo™ v.10 (BD Biosciences, USA).

Confocal microscopy studies were conducted on HCAEC treated with lignin NPs. 6,000 cells/well were seeded in a 8-well chambers (LabTekSlides, ThermoFisher, USA) and then cells were treated with 10 ng/mL of LPS in PBS or PBS at pH 7.4. After 24h, NPs were added at the concentration of 50 µg/mL for 1h, at 37°C. Cells were washed with HBSS, following by membrane staining with CellMask DeepRed™ (Life Technologies, USA) at 37°C for 3 min. Then cells were washed with HBSS and fixed with 4% paraformaldehyde (PFA) for 15 min at 37°C. Finally, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue, Vector Laboratorie, USA) for 5 min at 37 °C, and after the cells were washed with HBSS and stored at +4°C before imaging. The images were acquired using a Leica TCS SP5II HCS A confocal inverted microscope (Leica Microsystems, Germany), with a water immersion objective. The image were then processed using LAS AF (2.6.0 build 7266) software.

For study **II**, human tenocytes were seeded at the concentration of 200,000 cells per well in a 12-well plate, and let attached at 37°C overnight. Then cells were incubated with

50 µg/mL of NPs and 1.4 µg/mL of curcumin, as positive control, for 3h. After, cells were washed with PBS, collected in 500 µL of PBS in FACS tubes and then analysed with LSRII, evaluating the fluorescence quenching with trypan blue (0.005% v/v, Gibco, USA), processing the results using FlowJo™ v.10. To study the mechanisms of cell-uptake, tenocytes were pre-treated with selected pathway inhibitors for 1h and then incubated with the NPs for 3h. Then cells were detached and analysed by LSRII as described above.

Moreover, confocal imagining in human tenocytes was performed to assess the qualitative uptake of NPs. Cells were seeded at concentration of 30,000 cells per well in a 8- well chamber, let attach overnight and then treated with 200 µL of a solution of 25 µg/mL of NPs. After, the protocol used for the study **I** was used to prepare the samples before imaging. The images were acquired with Leica TCS SP8 STED 3X CW 3D Inverted Miscoscope (Leica Miscosystems, Germany), using a 63x water objective.

In publication **III**, human tenocytes were seeded at the concentration of 100,000 cells per well in a 12-well plate, let to attach at 37°C overnight and then incubated with 100 µg/mL of FITC-labelled LNPs, for 1,3,6 and 12h. Then, cells were washed with PBS and collected in 500 µL of PBS. Samples were analysed with LSRFortessa™ Cells Analyzer (BD, Bioscience, USA). The results were analysed with FlowJo™ v.10. Confocal imaging studies were also performed in study **III**, following the protocol used in study **II**, with the only difference of the CellMask Deep Red staining, that was performed before the treatment with FITC-LNPs (100 µg/mL).

4.4.4. Transfection efficiency: Silencing and expression of eGFP protein in murine macrophages (III)

RAW 264.7 and eGFP-expressing RAW 264.7 were used to study the *in vitro* transfection of eGFP-mRNA and eGFP-siRNA LNPs, respectively. Briefly cells were seeded in 24-well plate, at the concentration of 20,000 cells per well and left to attach overnight. Then eGFP-mRNA and eGFP-siRNA LNPs were added in RAW and eGFP-RAW cells, respectively, at the concentration of 100 and 200 µg/mL. Following the protocol suggested by the manufacturer, lipofectamine RNAiMAX was used as positive control and cell media was used as a negative control. After 48h of incubation cells were washed with PBS-etylenediaminetetracetic (EDTA), detached with trypsin, washed twice with PBS-EDTA and resuspended in 200 µL PBS-EDTA. Samples were analysed using BD Accuri C6 Plus (BD,USA) flow cytometry, measuring the percentage of eGFP+ expressing cells. The results were then analysed with FlowJo™ v.10.

4.4.5. Immunological profile of the empty nanosystem (I)

The effect of the empty nanosystem in the expression of CD86 was evaluated in KG-1 and PBMC cells. Cells were seeded at the concentration of 28,000 cells per well in a 12-well plate and treated with 10 ng/mL of LPS in PBS (LPS-activated cells), or only with PBS pH 7.4, for 24h. Then NPs were added for other 48h. After, cells were centrifuged and transferred in a 96-well-V-bottom plate (Corning, USA) and the human FC block solution (TruStain FcX™, BioLegend®) was added to each sample (2 µL in 28 µL of PBS), incubating the cells for 10min. 7 µL of mouse anti-human CD86-APC antibody (BD, Bioscience, USA) were added in each well and the plate was incubated in ice for 20 min. Samples were centrifuged, washed twice with PBS, resuspended in 200 µL of PBS and analysed with BD Accuri C6 Plus flow cytometry.
The results were processed with FlowJo™ v.10.

4.4.6. Real-time quantitative polymerase chain reaction: quantification of pro-inflammatory and pro-fibrotic genes (I-III)

In study **I**, the anti-inflammatory effect of curcumin was evaluated by RT-qPCR on HCAEC. 60,000 cells per well were seeded in a 12-well plate and allowed to attach overnight. After, HCAEC were treated with 10 ng/mL of LPS in HBSS, pH 7.4, or with only HBSS, for 24h. Then, NPs were added to cells for other 24h, leaving the negative control with only HBSS and the positive with only LPS in HBSS. The RNA was isolated using TRIzol reagent (ambion,USA) and Phase Lock Gel system (5PRIME. Lock gel heavy, QuantaBio), following the manufacturer's instruction. First-strand cDNA synthesis kit (Transcriptor First strand cDNA synthesis kit, Roche, Germany) was used for the cDNA synthesis and the samples were analysed with a lightCycler 480 qPCR machine (GE Healthcare Lifecience), using Taqman chemistry. The probes used were *18S* (4333760T), *NFKB1* (Hs00765730_m1) and *TGFB1* (Hs00998133_m1) (ThermoFisher Scientific). The ΔΔCT of the sample was determined normalizing all the results to the housekeeping gene *18S*.

In study **II**, the anti-inflammatory and anti-fibrotic effects of curcumin and TA were verified using RT-qPCR in human primary tenocytes. Briefly, cells at concentration of 100,000 cells per well in a 12-well plate, and let to attach overnight. After cells were treated with 250 ng/mL of LPS for 24h, and then treated with the NPs for other 24h. Curcumin, TA, DMEM-F12 and LPS were used as control. The RNA isolation, cDNA synthesis and samples analysis were performed following the protocol used in study **I.** The probes used were *18S* (4333760T), *NFKB1* (Hs00765730_m1),*TGFB1* (Hs00998133_m1), MMP3 (Hs00968305_m1) and MMP9 (Hs00957562_m1) (ThermoFisher Scientific).

In study **III**, the RT-qPCR was assessed to evaluate the anti-fibrotic effect of SMAD3 siRNA and pro-regenerative effect of COL1A1 mRNA. 50,000 tenocytes per well were seeded in a 24-well plate and left to attach overnight. Then cells were treated with Empty LNPs (100 µg/mL), siRNA LNPs (100 µg mL, corresponding to 0.3 µg mL siRNA), mRNA LNPs (100 µg/mL, corresponding to 0.3 µg/mL of mRNA) , dual-loaded LNPs (100 µg/mL, corresponding to 0.3 µg/mL of siRNA and mRNA), mix LNPs (100 µg/mL of siRNA LNPs + 100 µg/mL of mRNA LNPs, equivalent to 0.3 µg/mL of siRNA and mRNA) and the controls SMAD*3* siRNA (0.3 µg/mL) and COL1A1 mRNA (0.3 µg/mL), for 48h. The RNA isolation, cDNA synthesis and samples analysis were performed following the protocol used in study **I.** The probes used for the study were *18S* (4333760T), *TGFB1* (Hs00998133_m1), *COL3A1* (Hs00943809_m1), *BMP2* (Hs00154192_m1) and *SMAD3* (Hs00969210_m1) (ThermoFisher Scientific).

4.4.7.Immunofluorescence staining for collagen I quantification (III)

Human primary tenocytes (7,500 cells per well) were seeded in a 96-well plate and allowed to attach overnight. After, human TGF- β1 (20 ng/mL) was added as positive control, and different LNPs were added to treat the cells for 48 h. DMEM-F12 was used as negative control. Tenocytes were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 and the blocking was performing by incubating with 1% BSA for 30 mins. After the of COL1A1 primary antibody (Abcam, USA, ab138496, 1:500 dilution) was added in each well and incubated at 4°C, overnight. Cells were washed three times with PBS and were incubated with the secondary antibody Alexa Fluor® 568 (Abcam, USA, ab138492, 1:1000 dilution), for 1 h. Cells were washed again with PBS and 50 µL of PBS were added in each well for imaging.

32 images per well were acquired with a MolecularDevices Nano Instrument and processed using the MetaXpress software for the quantitative analysis of the collagen I production. The standard global Otsu thresholding was used to segment the cells and the morphology and the Texas Red intensity values were calculated.

4.4.8.ELISA for pro-inflammatory cytokines quantification (I)

The supernatant of HCAEC samples from RT-qPCR study, was used to quantify the production of IL-6 and IL-1β using commercially available ELISA kit: IL-6 human Elisa kit (**EH2IL6,** Thermo Fisher Scientific, USA) and IL-1 beta human Elisa kit (**BMS224-2,** Thermo Fisher Scientific, USA), following the protocol provided by supplier.

4.4.9.Intracellular staining of SMAD3 in human primary tenocytes (III)

Human primary tenocytes (75,000 cells/well) were seeded in a 24-well plates to let the cells attach overnight. Then, human TGF-β1 (20 ng/mL), and LNPs were added for 48h. TGF-β1 treated cells were used as control of SMAD3 overexpression. After 48 h incubation, intracellular staining was performed using a PE-labelled anti-SMAD3 antibody (562586, BD Biosciences). Briefly, tenocytes were detached with trypsin and were fixed with IC Fixation buffer (Paisley, UK). Then, cells were centrifuged at 400×*g* for 5 min, the supernatant was discarded and 1× permeabilization buffer (Paisley, UK) was added. Cells were washed again, and the pellet was resuspended in a 1:1 mixture of the permeabilization buffer with an addition of 5 µL of the antibody. The cells were incubated with the antibody for 40 min at RT and protected from light. Finally, cells were washed twice with PBS, resuspended in 400 µL of PBS and analysed with BD LSRFortessa™ Cells Analyzer (BD Bioscience, USA). The results were processed with FlowJo™ v.10.

4.4.10.Ethical permit (I-III)

PBMC cells were isolated according to the internal blood sampling licenses of the Finnish Red Cross (**I**). Patient's recruitment, participation, and sample collection were obtained after receipt of a signed informed consent, approved by the Helsinki and Uusimaa Hospital District ethics committee (HUS/2785/2020) and by the institutional review board (HUS/234/2020) (**II-III**).

4.4.11.Statistical analysis

All the experiments assessed in these studies were performed at least in triplicates, and the values are expressed as mean ± standard deviation (s.d.). The statistical analysis was performed using GraphPad Prism v.6, v.9 and v.10. A detailed description of the statistical method used to analyse the data is reported in each figure legend.

5. Results and Discussion

NPs have proven in recent years to be excellent alternatives to conventional methods and have demonstrated their potential in various applications. In this thesis, polymeric NPs were developed and tested in terms of formulation, colloidal stability, cell compatibility and anti-inflammatory and anti-fibrotic activity, in reference cell lines. Furthermore, a system of lipid NPs was formulated and its physicochemical characteristics were tested, along with compatibility, and *in vitro* studies, to demonstrate its pro-regenerative and anti-fibrotic activity in primary human tenocytes derived from a patient with tendinopathy.

5.1. Physicochemical characterization

5.1.1.size, PDI, zeta-potential, NTA and colloidal stability (I-III)

The first parameters investigated in the development of the new nanosystems were the size, with PDI and Zeta potential, in order to confirm the reproducibility of the NPs' production process.[239] DLS was used to perform the analysis.

In publication **I**, Curc@Lignin showed a size of 291 nm, increasing in Curc@Lignin@TA to 459 nm, due to the coating with the TA-iron complex (**Figure 10a**). Compared to the TA-Coated formulation, when the particles were then further coated with the platelet membranes, the size decreased to 321 nm, due to the use of sonication during the cloaking process, which decreased the aggregation between the NPs. The same trend can be observed for polydispersity, ranging from a value of 0.25 to 0.15 for Curc@Lignin@TA@PL. Furthermore, all formulations presented a negative surface charge, due to the presence of the polymer and coatings, resulting in -72 mV for the final formulation (**Figure 10b**). After the loading of curcumin in the nanosystem and then the coatings, the LD was not affected by the surface modifications, resulting in 5.5% for Curc@Lignin@TA and 6.3% for Curc@Lignin@TA@PL. Following a protocol already optimised, the quantification of the amount of TA and iron deposited on the surface of the particles showed an average of 321 µg of TA and 53 µg of iron, for 1 mg of NPs.

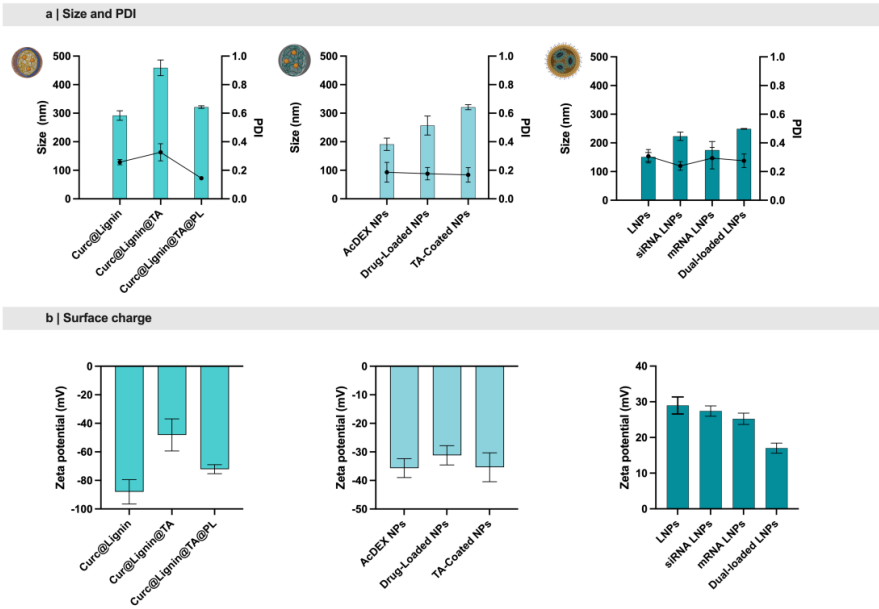


Figure 10. Physicochemical characterisation of the NPs developed in this study, in term of (a) size and PDI and (b) zeta-potential. Results are presented as mean ± s.d. (n ≥ 3 independent batches of particles prepared in different days).

In publication **II**, the empty AcDEX NPs showed an initial size of 191 nm, with an increasing in the Drug-Loaded and TA-Coated NPs to 257 nm and 321 nm, respectively (**Figure 10 a**). This increasing in the size suggests that the curcumin was successfully loaded and that the layer of TA-coating had deposited on the surface of the particles. The PDI remained below 0.2 for all the formulation, without showing any statistically differences, and the zeta-potential remained around -30 mV for all the samples (**Figure 10b**), due to the presence of AcDEX and TA-coating, which was not affecting the overall surface charge. The LD calculated was 4.3% of curcumin for the Drug-Loaded NPs and 2.8% for the TA-Coated NPs, displaying that a small amount of curcumin was lost during the coating process. Following a protocol already optimised,[236] the quantification of the amount of TA and iron deposited on the surface of the particles calculated were 248.6 µg of TA and 22.5 µg of iron, for 1 mg of NPs.

In publication **III**, the size of the loaded LNPs was higher than the empty particles, (~250 nm for the dual-loaded) (**Figure 10a**); however, the PDI was always around 0.2, confirming the size homogeneity of all the nanosystems. In **Figure 10b**, the surface charge showed an decreasing in the dual-loaded LNPs to +18 mV, confirming that part of the RNAs is complexed on the lipidic surface.[240] The EE, calculated using the Ribogreen assay, showed a percentage ~63% for both siRNA and mRNA LNPs (**Figure 11a**); moreover, the EE of the dual-loaded LNPs was 67%, confirming that the process of encapsulation was similar for all the formulations. In addition, NTA analysis were performed to confirm that an acceptable amount of NPs were obtained during the microfluidics process, and the results were 4.5×10^{11} and 1.0×10^{10} particles per mL for Empty LNPs and Dual-loaded LNPs, respectively, taking into account the dilution factors (**Figure 11b**).

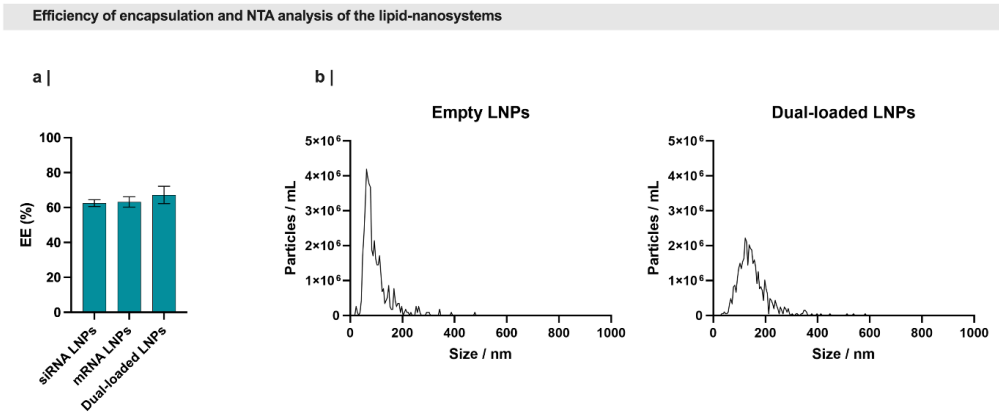


Figure 11. Physicochemical characterisation of the optimised lipid nanosystems in terms of (a) EE (%) and (b) size distribution and concentration as measured by NTA (samples diluted 10,000 times and 5,000 times, respectively).

The colloidal stability of the developed nanosystems was investigated in different relevant media, due to the importance of this parameter for the *in vitro* studies.

In publication **I**, the stability of the polymeric NPs was performed in RPMI 1640 medium with 10% of FBS, showing no major increases in the size and PDI, demonstrating that the particles were stable over the 2h of incubation at +37°C (**Figure 12a**).

In publication **II**, the colloidal stability of the Drug-Loaded and TA-Coated NPs was assessed in DMEM-F12 with 10% FBS, cell culture media of human tenocytes. **Figure 12b** highlights an increasing in size and PDI for the Drug-Loaded NPs over the 2h of incubation, when compared with the TA-Coated NPs, where no major increases were detected. These results demonstrated that the TA-coating gives stability to the particles by reducing the interaction with the serum proteins.[91] The stability of the lipid nanosystem (**III**) was also performed in DMEM-F12 with 10% of FBS, media used to conduct all the *in vitro* studies. **Figure 12c** shows an increasing in size over the time (7 days) and this phenomenon of the protein corona effect has been previously reported by others with the, where the proteins of the medium interact with the NPs establishing surface interactions.[241,242]

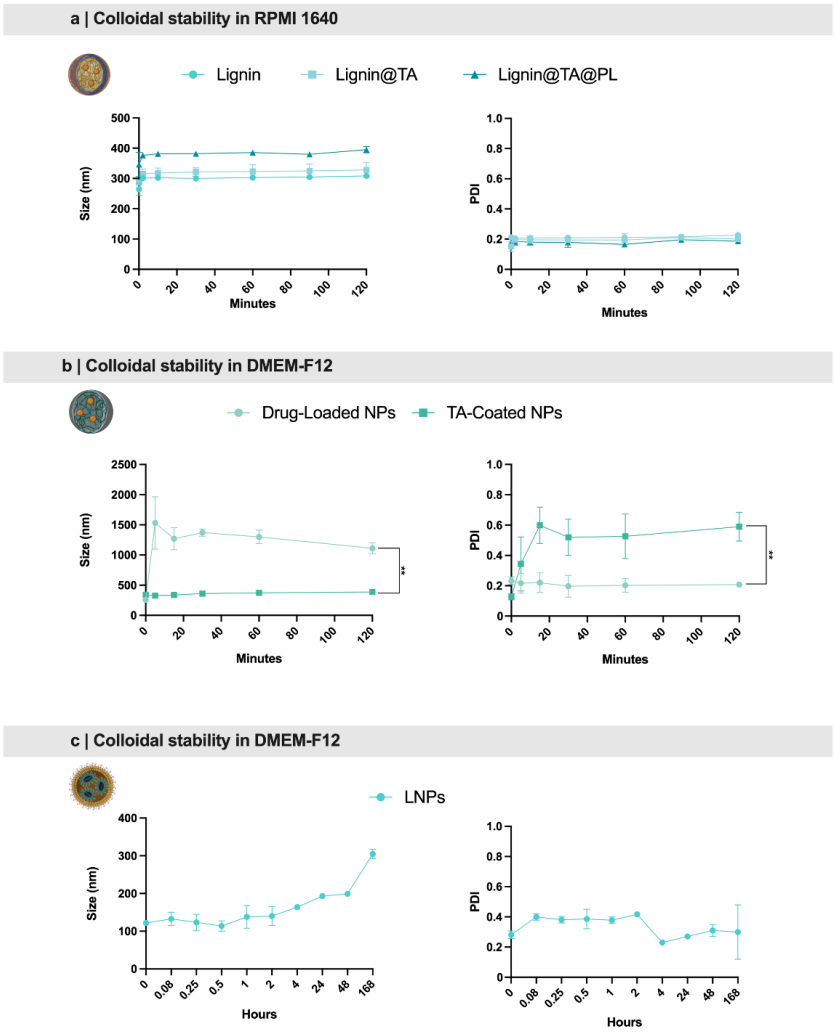


Figure 12. Colloidal stability of the developed nanosystems in terms of size and PDI. Results are presented as mean ± s.d. ($n \geq 3$ independent batches of particles prepared in different days). A paired Student's *t*-test was used for statistical analysis of b, and *p* values were set at probabilities $*p < 0.05$ and $**p < 0.01$, comparing the profile size vs time or PDI vs time for both samples.

In conclusion, the 3 nanosystems showed good colloidal stability in the respectively cell media, and in publication **II,** the TA-coating improved the stability of the overall system.

5.1.2.Morphological analysis: TEM, Cryo-EM and SEM (I-III)

The morphological characteristics of the polymeric nanosystems were investigated by TEM and SEM microscopy, to further confirm the shape and the presence of the TA-coating over the polymeric particles (**Figure 13**). The TA-iron complex can be seen on the surface of the Lignin NPs, resulting irregular, while the surface after the platelet cloaking is smoother. Moreover, the SEM images confirmed the deposition of the TA-iron complex on the surface of the NPs, as well as the mechanism of particles formation; in some parts of the NP-structure there is a partial collapse due to the complete exchange of the organic solvent.[243] The TEM picture of the TA-Coated NPs shows a clear irregular and blunt surface when compared with the AcDEX one, which results regular and smoother. Furthermore, the SEM picture clear show the presence of the coating on the TA-Coated NPs compared with the AcDEX ones.

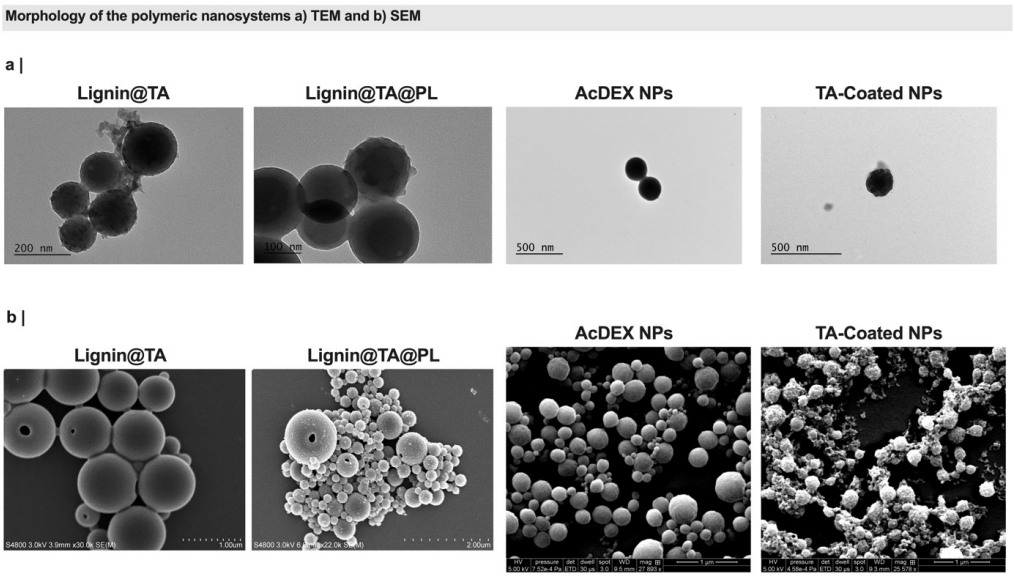


Figure 13. (a) TEM and (b) SEM images displaying changes in the morphological structure of the polymeric nanosystems. Scale bars are reported in each image.

In publication **III**, Cryo-EM analysis were conducted to further analyse the shape of LNPs. **Figure 14** displays that both Empty and Dual-loaded LNPs have the typical appearance of lipid particles, as already reported by others.[152,244] The images show no aggregation, verifying that using microfluidics technique and adding PEG in the lipid formulation ensure the stability of these particles, confirming the acceptable size homogeneity for local delivery.[245] Moreover, Cryo-EM images confirmed also the results obtained by DLS and NTA, showing some particles above or below the average size.

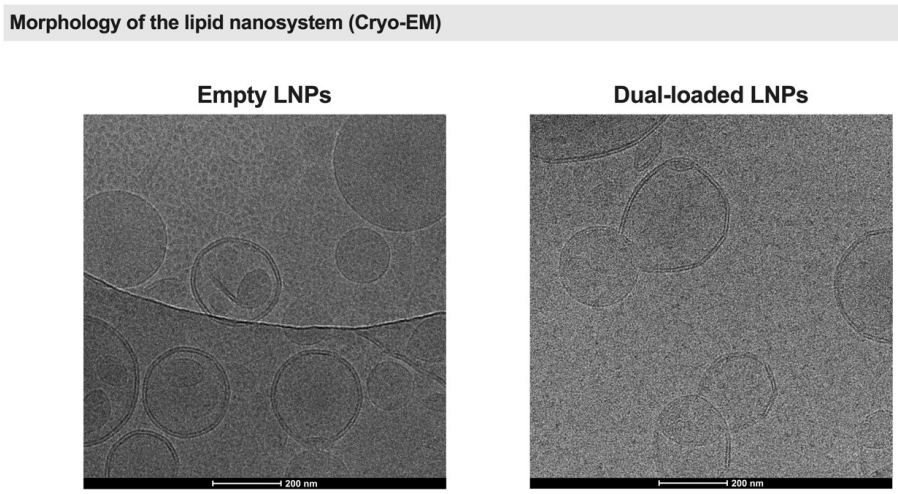


Figure 14. Cryo-EM images of Empty and Dual-loaded LNPs. Scale bar is 200 nm in each image.

Overall, TEM and SEM analysis confirmed the development of NPs with narrow size distributions and, for the polymeric nanosystems, the images confirmed that coating with TA was successful.

5.2. *In vitro* drug release studies (I-II)

The LD of the different polymeric formulations was calculated before starting the release studies, to determine the amount of drug associated with the NPs, that could be released. After quantification by HPLC, the release studies were performed in the respective buffers, at 37°C for 24 h.

In publication **I** (**Figure 15a**), the release was assessed at pH 7.4 and 5.5 to simulate the condition in the bloodstream post IV administration, and the inflammatory environment of the atherosclerotic plaque. At both pH, the release of curcumin was rapid, reaching more than 90% within the first 2h. This fast release can be attributed to the pH-sensitive solubility of lignin and the presence of 1% of Tween-80 in the buffer solution.[246] Moreover, the presence of any coating did not significantly altered the release profile, and curcumin was released rapidly at pH 7.4 for the final formulation of Curc@Lignin@TA@PL than at pH 5.5. However, the release profile exhibited by lignin NPs may be unsuitable for intravenous administration, as the particles would first be distributed systemically and subsequently interact with atherosclerotic plaques.

In publication **II** (**Figure 15b**), the release of curcumin was assessed in a synovial-mimicking fluid,[238,247] at pH 7.4 and 5.5, to simulate the physiological and pro-inflammatory environment in tendon. At pH 5.5, both Drug-Loaded and TA-Coated NPs exhibited a rapid release within the first 2h, releasing the 80% and 75% of curcumin, respectively. Following this initial phase, the release rate stabilised and reached a plateau from 6h up to 24h. At the acidic pH the release of curcumin from the NPs was faster due to the pH-dependent degradation of AcDEX, in which the acetal group is hydrolysed in acidic conditions.[100] Additionally, the TA-Fe^{3+} complex disassembled at pH 5.5, as the hydroxyl groups present in TA become protonated, leading to the breakdown of the coating.[91,235] As shown in **Figure 15b**, the release at pH 7.4 had a similar trend of the one at pH 5.5, but the % of the release was lower when the curves are compared.

Overall, in both case, the presence of 1% Tween in the synovial mimicking fluid, added to maintain curcumin stability under sink conditions, further increases the drug released from the nanosystems. Furthermore, the developed system showed preferential release under pro-inflammatory conditions, considering the properties of the polymer used, making it suitable for drug delivery in tendon inflammation.

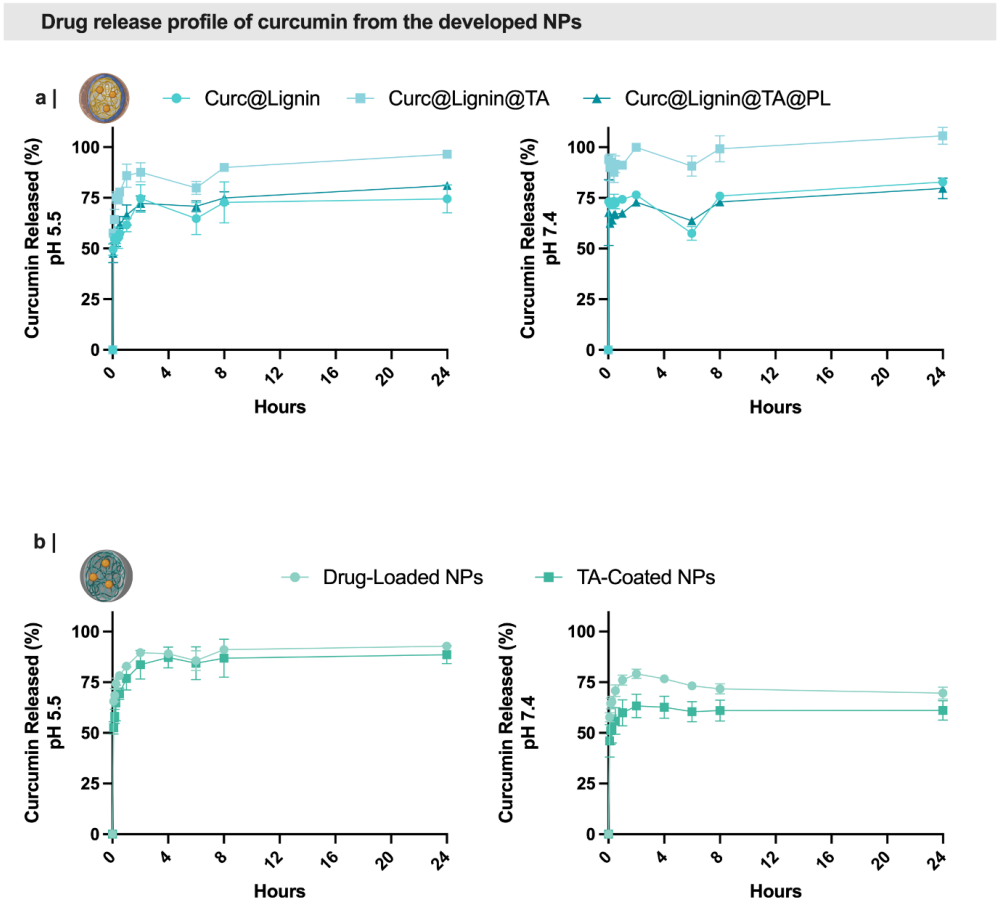


Figure 15. Evaluation of drug release in sink conditions of curcumin from (a) Lignin NPs and (b) AcDEX NPs, at pH 5.5 and 7.4, kept at 37°C under stirring (300 rpm). The results are presented as mean ± s.d. (*n* ≥3 independent replicates, each constituted by three technical replicates).

5.3. Cell-cytocompatibility studies of the polymeric and the lipid-based nanoparticles (I-III)

In vitro studies are indispensable in order to assess the safety of the nanosystems and the interactions that the cells have with the developed NPs. In this thesis, specific *in vitro* assays were performed, to analyse the behaviour of the NPs in contact with endothelial cells of coronary arteries and the immune system (**I**) and with primary human tenocytes (**II-III**). The first study performed was the cytocompatibility assay, evaluating the effect of particles on the cellular viability using the adenosine triphosphate (ATP)-luciferase assay.

The different cells lines were incubated with different NPs' concentrations, at established time points, in order to evaluate if the toxicity was dose-dependent and to evaluate the highest safe concentration to use in all the other *in vitro* studies.

In publication **I**, HCAEC were used as a model for the endothelial cells, KG-1 for macrophages and PBMC for monocytes. In HCAEC, the Curc@Lignin@TA@PL nanoparticles (NPs) were safe up to a concentration of 50 µg/mL after 24 h of incubation, as were all intermediate NPs (**Figure 16a**). Additionally, curcumin, tested at concentrations equivalent to its loading percentage in Curc@Lignin@TA@PL, was safe at all tested levels. Previous studies indicated that HCAEC cells are more sensitive to metal oxide NPs than other endothelial cell types, with the highest tested concentration being 200 µg/mL.[238] The addition of the TA-iron layer and platelet membrane coating slightly reduced compatibility; however, at concentrations above 50 µg/mL, lignin and Curc@Lignin NPs maintained good cytocompatibility, whereas coated NPs showed a decrease, though not significantly.

For immune cells (KG-1 and PBMC), both the final NPs and the intermediates were safe up to 50 µg/mL. Unlike endothelial cells, the presence of curcumin in the loaded NPs significantly increased toxicity in KG-1 and PBMC compared to empty NPs and intermediates at the same concentration. Curcumin inhibits several pathways in immune cells, such as NF-κb and STAT.[248] In KG-1 and PBMC cells, curcumin-induced toxicity is thought to occur through reduced Bcl-2 expression, thereby increasing apoptosis.[249,250] No significant differences in cell viability were observed among particles at different stages of surface modification. Overall, the final NPs and their intermediate were cytocompatible up to 50 µg/mL across all cell types. In HCAEC curcumin provided protection against particle-induced toxicity, while in immune cells increased the toxicity of the NPs.

In the publication **II**, the cytocompatibility was tested in primary human tenocytes, isolated from a sample derived from a 58-year-old patient due to the rupture of the extensor *indicis proprius*. Cell viability was measured after 24h of incubation with the NPs, coinciding with the maximum release of curcumin. Negative and positive controls were cells in DMEM-F12 and 1% Triton-X, respectively. The concentrations of NPs tested were from 50 to 500 µg/mL to evaluate the impact of the concentration on cell viability. As shown in **Figure 16b**, AcDEX NPs reduced the cytocompatibility at the highest concentration of 500 µg/mL and TA-Coated NPs maintained around the 80% of the viability, showing no significant concentration-related toxicity. The cell viability studies were also conducted in KG-1 cells, a model for the macrophages that may be present during tendon inflammation,[251] and the toxicity due to curcumin and TA-coating was relevant for concentrations above 100 µg/mL. This can be explained by the activity of curcumin, which increases apoptosis reducing Bcl-2 expression and by the downregulation of P3K-AKT, promoted by TA.[252] P3K-AKT is an important pathway for the proliferation of KG-1, tumor cells involved in acute myelogenous leukemia,[253] for these reasons the decreasing in cell viability is due to the synergistic effects of both compounds. Following the results obtained from the cell viability assay, further *in vitro* tests were conducted with the concentration of 100 µg/mL for TA-Coated NPs, excluding higher concentrations that might cause cell toxicity.

In publication **III**, the safety profile of LNPs, SMAD-3 LNPs, COL1A1 LNPs, dual-loaded LNPs and mix LNPs was evaluated in human primary tenocytes, extracted from a sample derived from the *human flexor digitorum profundus* of a 25-year-old patient. The concentrations tested were 25, 50, 100, 250, and 500 µg/mL with incubation times of 24 h and 48 h (**Figure 16c**). Tenocytes in cell media were used as a negative control to establish the 100% cell viability reference. All formulations showed good cell viability with no toxicity up to the highest concentration, and an increasing above the 100% was observed when

tenocytes were treated with Dual-loaded LNPs. This phenomenon, previously observed, may result from interactions between the assay and the LNPs, as well as the assay variability.[89] In this study, the concentration of 100 µg/mL was selected to conduct further *in vitro* studies.

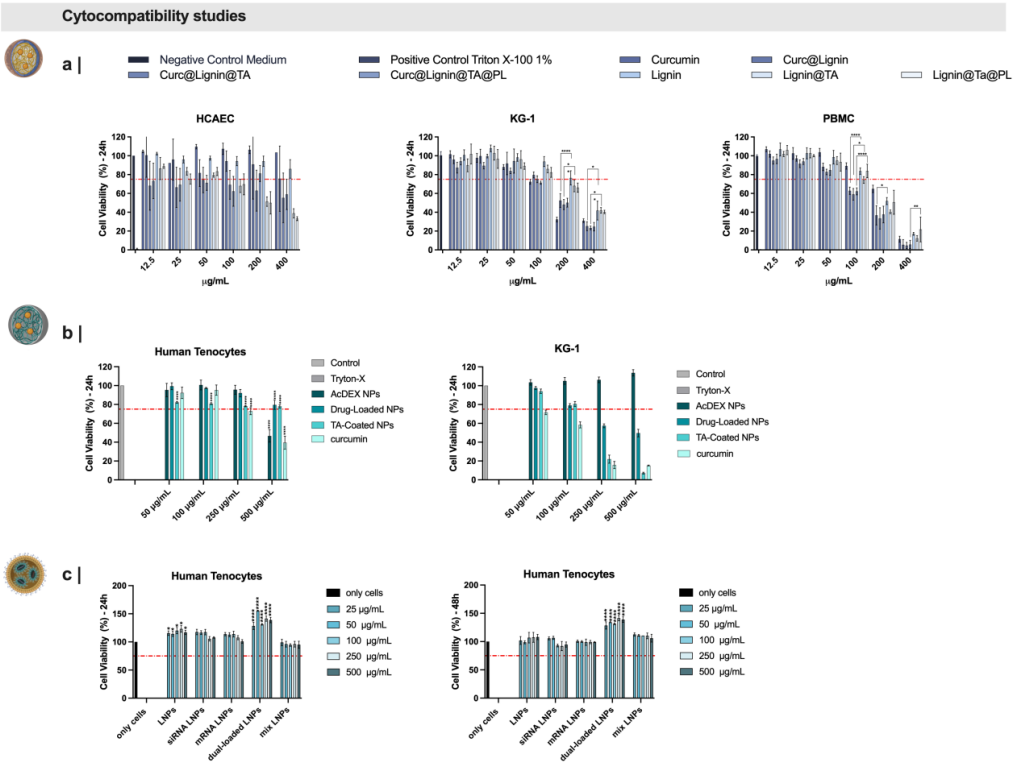


Figure 16. Cell viability studies of (a) Lignin NPs in HCAEC, KG-1 and PBMC after 24h of incubation; (b) AcDEX NPs after 24h of incubation in human tenocytes, and (c) LNPs after 24 and 48h of incubation in human tenocytes. Results are presented as mean ± SD ($n \geq 3$), and the samples were analysed with ordinary two-way ANOVA, followed by a Dunnett post-hoc test (b-c) and a Tukey's post-test (a), setting the probabilities $*p < 0.05$, $** p < 0.01$, $***p < 0.001$ and $****p < 0.0001$, comparing all the samples *vs.* only cells.

5.4. Quantification of the transfection efficiency of the lipid-based nanosystems (III)

The efficacy of this formulation in delivering functional RNAs was assessed by evaluating the particle uptake and endosomal release of mRNA and siRNA from LNPs, leading to gene expression and silencing, respectively. RAW 264.7 and RAW 264.7 cells expressing eGFP were used due to their common *in vitro* use for early stage transfection efficiency studies of LNPs and the lack of an eGFP-expressing tenocyte model.[254] For the evaluation of transfection efficiency, RAW 264.7 cells were used with LNPs loaded with the model eGFP-mRNA and eGFP-RAW 264.7 cells were used with LNPs loaded with the model eGFP-siRNA. **Figure 17a** shows that 100 µg/mL of LNPs resulted in an approximately 80% increase in eGFP expression compared to control cells, comparable to the positive control with lipofectamine. Furthermore, **Figure 17b** shows that 100 µg/mL LNPs achieved almost

40% silencing of eGFP in eGFP-RAW 264.7, outperforming the lipofectamine positive control with eGFP siRNA.

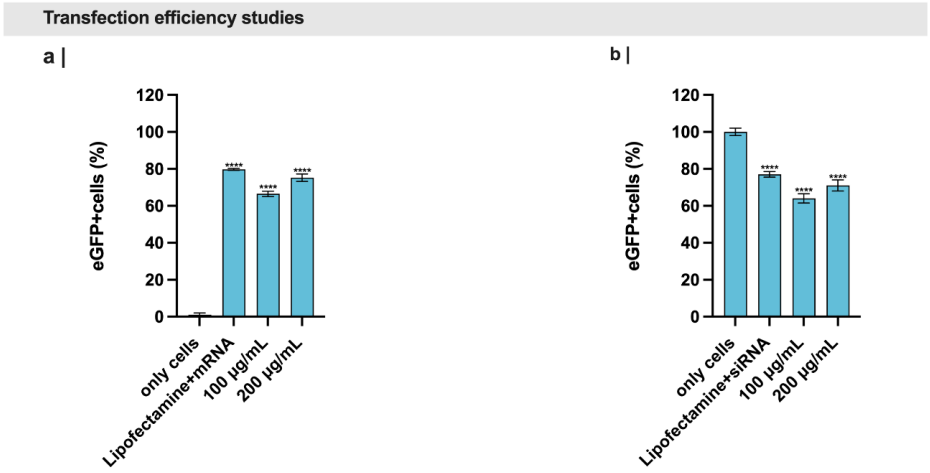


Figure 17. Transfection efficiency of eGFP-mRNA LNPs in RAW 264.7 cells (a) and in eGFP-expressing RAW 264.7 cells (b). Particles were incubated for 48 h and the percentages of $eGFP^+$ cells were quantified by flow cytometry analysis. Results are represented as percentage of $eGFP^+$ cells ± s.d. (n = 3). Results are presented as mean ± SD (n ≥ 3), and the samples were analysed with ordinary one-way ANOVA, followed by a Dunnett post-hoc test, setting the probability at ****$p < 0.0001$, comparing all the samples *vs.* only cells.)

These results demonstrate the suitability of this formulations for the delivery of siRNA and mRNA. Considering the results of the cell viability and the transfection efficiency, the concentration of 100 µg/mL (equivalent to 0.3 µg/mL of the respective RNAs) was selected for further mechanistic and in vitro efficacy studies, as this is a safe concentration with potential therapeutic efficacy.

5.5. Immunological profile of the empty nanosystem (I)

The lignin nanosystem developed in study **I** aims to reduce inflammation in atherosclerotic plaques, in particular by targeting activated macrophages within the plaque. Therefore, it is crucial that the NPs and their individual components do not induce a pro-inflammatory response or increase an existing inflammation. To assess this, the expression of CD86, an activation marker, was examined, in both KG-1 macrophages and PBMC.

As showed in **Figure 18**, there was no statistically significant increase in the mean fluorescence intensity (MFI) of CD86 between the negative control and the samples at two NPs' concentration (25 and 100 µg/mL), in both healthy and LPS-activated cells. Activation with LPS for 24 h led to an increase in CD86 expression in all samples, with a significant increase compared to the corresponding healthy samples. Furthermore, the increase in NPs' concentration from a safe low level (25 µg/mL) to the maximum safe concentration (100 µg/mL) did not lead to increased CD86 expression. Similarly, in PBMCs, there were no significant differences in CD86 expression between samples and controls, nor between healthy and LPS-activated conditions.

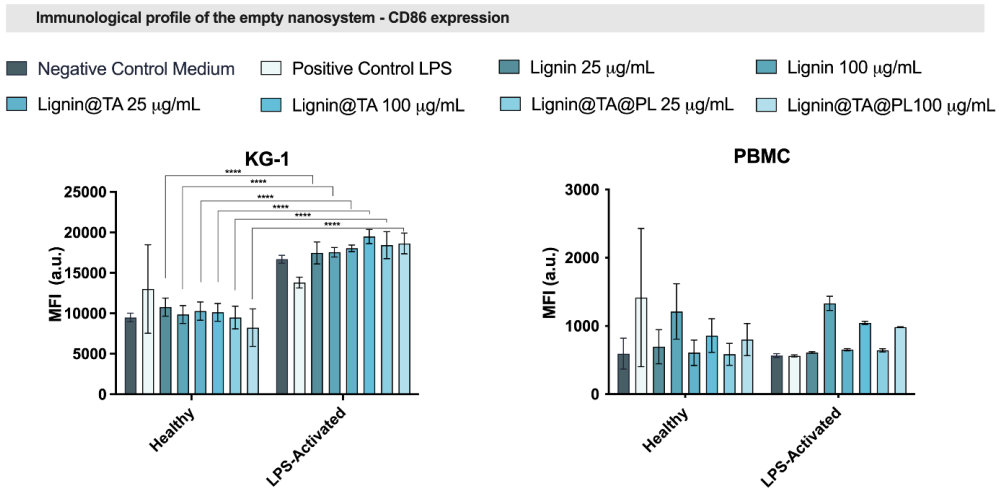


Figure 18. MFI of APC anti-human CD86 antibody in KG-1 cells and PBMC after 48 h incubation. The cells were incubated with the final empty NP (Lignin@TA@PL) or intermediates for 48 h before being stained for CD86 expression. The results are presented as mean ± s.d. (n = 3 biological replicates each constituted of three independent technical replicates). The data were analysed with two-way ANOVA, followed by Tukey's post-test comparing all the samples to the control within the same group (healthy or LPS-activated) or comparing the same sample between the different groups (lignin in healthy cells *vs* lignin in LPS-activated cells). The level of significance was set at the probability of ****$p<0.0001$.

Overall, incubation with the NP's did not raise the inflammatory profile of the immune cells at any of the concentrations tested.

5.6. Qualitative and quantitative uptake studies (I-III)

In publication **I**, the interaction between the NPs and cells was evaluated quantitatively, using flow cytometry, and qualitatively with confocal microscopy images. The interaction between negatively charged NPs and cell membranes is generally lower compared to positively charged NPs. However, surface modifications on lignin NPs can enhance their interaction with cells. For instance, the platelet-derived membrane fragments have increased interactions with atherosclerotic plaques, damaged vasculature, cancer cells, and pathogens.[250,255] One key interaction examined was with endothelial cells, both in their healthy state and after inflammation, to determine if the particles could interact with damaged vasculature. As shown in **Figure 19a-b**, each surface modification significantly increased the interaction of NPs with the cells at a concentration of 50 μg/mL, in both healthy and LPS-activated conditions, as measured by flow cytometry. The only notable difference between healthy and inflamed conditions was a significant increase in interaction between TA-iron-coated NPs and LPS-activated cells. This enhanced interaction in inflamed conditions was also observed qualitatively in confocal microscope images (**Figure 10c**). Macrophages, particularly inflamed ones, are the target cells within atherosclerotic plaques.[256] Therefore, the interaction between the particles and KG-1 cells, a macrophage-like immortal cell line, was evaluated both in their normal state and after activation with LPS to an M1-like phenotype.

As shown in **Figure 19b**, the surface modifications with the TA-iron complex and additional cloaking with cell membrane fragments significantly increased the fraction of

particles interacting with the cells (from approximately 20% with Curc@Lignin to 30-40% with Curc@Lignin@TA, and to ≥60% with Curc@Lignin@TA@PL). However, no significant differences in uptake were observed between healthy and LPS-activated cells, indicating that the interaction is not dependent on the presence of LPS as an inflammatory signal. Previous studies have shown that cloaking with platelet membranes reduces interaction with macrophages differentiated from THP-1 cells, but increases interaction with foam cells derived from RAW 264.7 cells.[139]

Overall, the surface modifications on lignin NPs enhanced their interaction with both endothelial cells and macrophages. The TA-iron complex effectively increased the number of particles in contact with activated human coronary artery endothelial cells (HCAECs), while cloaking with platelet-derived cell membrane fragments resulted in the highest interaction with both cell types, regardless of healthy or LPS-induced inflammatory conditions.

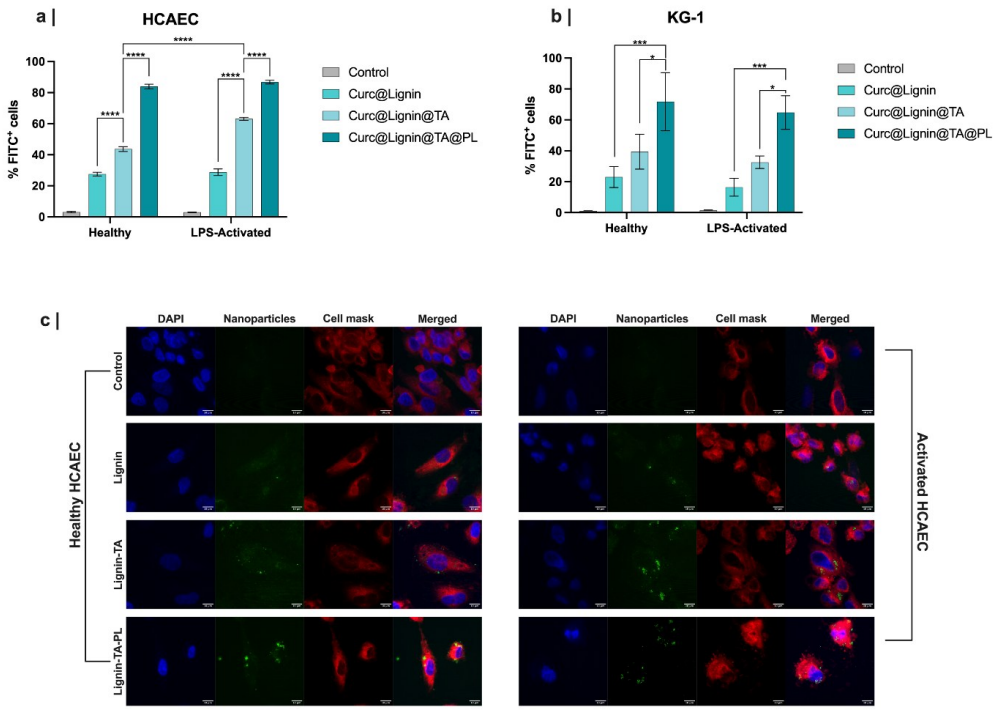


Figure 19. Percentage (%) of FITC⁺ (a) HCAEC or (b) KG-1 cells after interaction with Curc@Lignin@TA@PL and intermediate samples for (a) 1 h and (b) 30 min at +37 °C. The results are presented as mean ± s.d. (n = 3 biological replicates, each constituted of 3 technical replicates). The results were analysed with two-way ANOVA followed by Tukey's post-test. The percentage of FITC+ cells in each sample was first compared to the other samples within the same type of cells (healthy *vs.* healthy, *e.g.*, Curc@Lignin vs Curc@Lignin@TA), and then compared with the respective particle in the activated cells (*e.g.*, Curc@Lignin in healthy cells *vs.* Curc@Lignin in activated cells). The levels of probability were set at the probabilities of * $p<0.05$, *** $p<0.001$, *and* **** $p<0.0001$. (c) Qualitative uptake of Curc@Lignin@TA NPs and the intermediates in HCAEC cells in steady state or after activation with LPS for 24 h by confocal microscopy. The uptake was evaluated after incubation with the NPs for 1 h at +37 °C. The curcumin loaded in the NPs was imaged in the FITC channel (Nanoparticles, NPs), while the cell nuclei were stained with DAPI (blue

channel) and the cell membranes with Cell Mask Deep Red (red channel). Scale bar is 10 μm in all the images.

The interaction between the AcDEX NPs and primary human tenocytes was quantitatively assessed in publication **II**. Cells were treated with curcumin, drug-loaded NPs and TA-coated NPs for 3 hours and uptake was measured by flow cytometry. Fluorescence of curcumin in the FITC channel was used to quantify uptake. As shown in **Figure 20a**, about 55% of the tenocytes were FITC-positive for curcumin and drug-loaded NPs, while 90% were FITC-positive for TA-coated NPs, indicating significantly higher uptake.

Endocytosis is a primary pathway for the entry of NPs into cells.[35] To understand the mechanism of NPs' internalisation, human tenocytes were pre-treated with different endocytosis inhibitors before NPs were added. Genistein, an isoflavone, inhibits tyrosine kinase, disrupting the actin network and caveolae-dependent endocytosis.[257] Clathrin-mediated uptake was inhibited by sucrose, which prevents clathrin recycling in the plasmatic membrane.[258] Sodium azide, an active transport inhibitor, interferes with ATP production by inhibiting cytochrome C oxidase.[259] Nocodazole suppresses microtubule polymerisation,[260] while methyl-β-cyclodextrin disrupts lipid rafts by reducing cholesterol.[261] **Figure 20b** shows that nocodazole and sodium azide significantly inhibit the uptake of Drug-Loaded NPs, reducing it by more than 90%. Genistein and sucrose also decrease NP internalisation, suggesting that multiple endocytosis processes are involved in NP-cell interactions. **Figure 20c** shows that sucrose and nocodazole predominantly inhibit the internalisation of TA-Coated NPs, suggesting that clathrin-mediated endocytosis and actin polymerisation are the main uptake mechanisms. Furthermore, energy-dependent pathways and caveolin-mediated endocytosis reduce TA-coated NP uptake by about 50%, indicating their role in NPs' internalisation. The quantitative uptake of the AcDEX nanosystems was also investigated in KG-1 macrophages, at the state of M0 and LPS-activated M1 (data shown in publication **II**). In this case, both Drug-Loaded and TA-Coated NPs were preferentially uptaken by M1 macrophages, with a difference around 50% when compared to M0. These data confirmed the property of M1-activated macrophages to have higher phagocytosis capacity when compared with M0 cells.[262]

Qualitative uptake studies were performed on human tenocytes using confocal microscopy. Cells were incubated with drug-loaded, TA-coated NPs for 1 h at 37°C to assess NPs-cell interaction. Untreated cells were used as a negative control. After incubation, the cell membrane was stained with CellMask Deep Red, the cells were fixed with PFA and the nuclei were stained with DAPI. As shown in **Figure 20c**, the TA-Coated NPs had a greater interaction with the tenocytes than the drug-loaded NPs, which remained mostly outside the cells. This observation is in line with the affinity of TA for collagen I,[263] a key component of the ECM produced by tenocytes. Therefore, it is likely that TA-Coated NPs achieve greater internalisation in these cells, consistent with flow cytometry data showing a higher uptake rate for TA-coated NPs than for drug-loaded NPs. Furthermore, **Figure 20c** suggests that Drug-Loaded NPs can aggregate outside the cells, which supports the data on the stability of these particles in tenocyte media (**Figure 12b**).

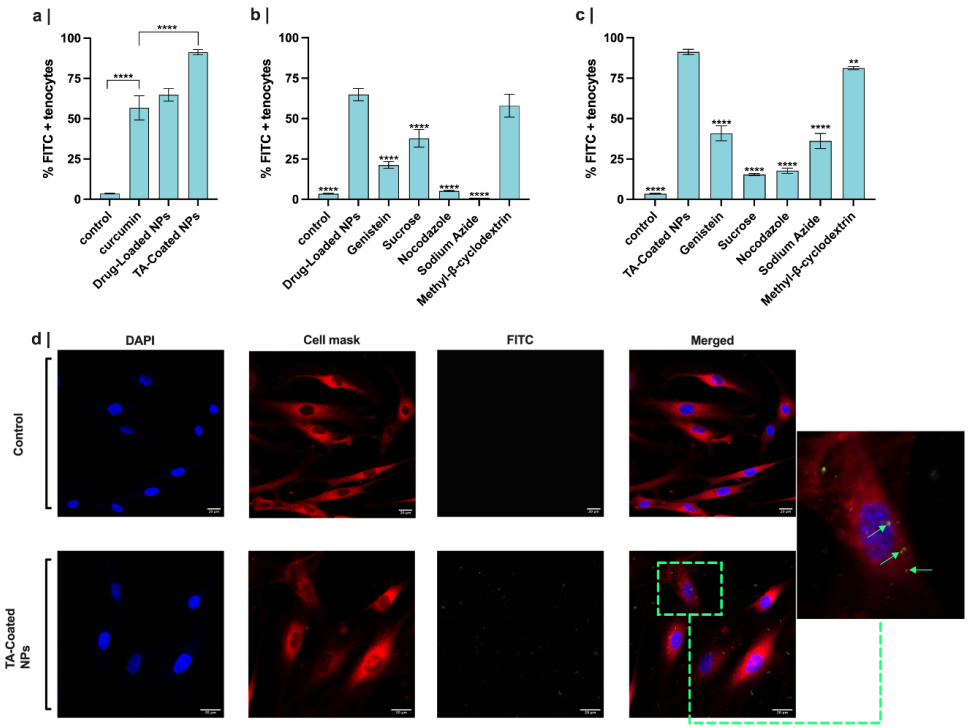


Figure 20. Quantitative cell uptake and cell uptake mechanism studies on human primary tenocytes using a flow cytometer. (a) Cells were incubated for 3 h with curcumin, Drug-Loaded, and TA-Coated NPs. (b) Cells were pre-treated with different inhibitors and then incubated for 3 h with drug-loaded or **c|** TA-coated NPs to study the mechanism of endocytosis by a flow cytometer. Results are presented as mean ± SD ($n \geq 3$), and the samples were analysed with ordinary one-way ANOVA, followed by a Dunnett post-hoc test, setting the probabilities $^{**}p < 0.01$ and $^{****}p < 0.0001$, comparing in (a) all the samples vs curcumin, (b) all the samples vs the drug-loaded NPs, and in (c) all the samples *vs.* the TA-coated NPs. (d) Qualitative uptake studies of NPs in human tenocytes. The cell uptake was evaluated by confocal fluorescence microscopy after incubation with the NPs for 1 h at 37 °C. The NPs, loaded with curcumin, were visualized in FITC (green channel), while cells were stained with DAPI (nuclei, blue channel) and Cell Mask (cell membrane, red channel). Scale bars are shown in each image.

In publication **III**, the interaction between cells and LNPs was evaluated both quantitatively and qualitatively, in human primary tenocytes, using 100 µg/mL of LNPs prepared with FITC-labelled PEG, which emits fluorescence when excited by a 488 nm laser. For the quantitative uptake studies (**Figure 21a**), tenocytes were incubated with FITC-labelled LNPs for 1, 3, 6 and 12 hours and analysed by flow cytometry. As shown in **Figure 21a**, a time-dependent uptake was observed; more than 80% of the FITC-labelled LNPs were internalised after 6 h and the uptake stabilised around 90% at 12 h. The low percentage of uptake at 1 h is in line with the colloidal stability of LNPs in the tenocyte medium (**Figure 12c**), indicating that LNPs remain suspended in the medium without aggregating, thus taking time to interact with adherent tenocytes. However, the positive charge of the LNPs facilitates electrostatic interactions with the negatively charged cell membrane, leading to efficient internalisation at later time points (6 and 12h).[25]

Qualitative uptake was also performed in human primary tenocytes using confocal microscopy after a 6h incubation with FITC-labelled LNPs. A single cell was selected for better visualisation of LNPs' internalisation. **Figure 21b** shows that the FITC-labelled LNPs were internalised by the cells, supporting the flow cytometry uptake results.

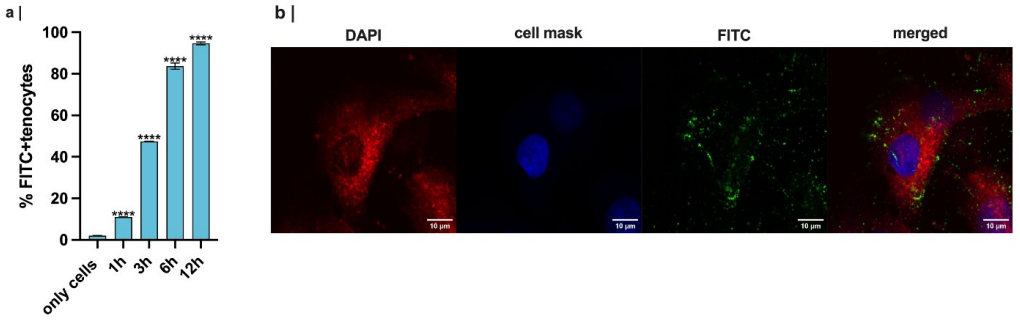


Figure 21. (a) Quantitative cell uptake on human primary tenocytes using flow cytometry. Tenocytes were incubated with 50 µg/mL of FITC-labelled LNPs for 1, 3, 6 and 12 h. Results are presented as mean ± SD (n ⩾ 3), and the samples were analysed using ordinary one-way ANOVA, followed by a Dunnett post-hoc test, setting the probability at ****$p < 0.0001$, comparing in all the samples *vs.* only cells. (b) Qualitative uptake studies of LNPs in human tenocytes. The cell uptake was evaluated using confocal microscopy after incubation with the FITC-labelled LNPs for 6h at 37°C. The LNPs were visualized in FITC (green channel), while cells were stained with DAPI (nuclei, blue channel) and CellMask (cell membrane, red channel). Scale bars are shown in each image.

5.7. *In vitro* studies of the anti-inflammatory and anti-fibrotic activity of the developed nanosystems (I-III)

A crucial step in developing a nanoformulation for clinical application is the preliminary evaluation of its proposed activity *in vitro*. In order to validate the anti-inflammatory and anti-fibrotic activity of the developed nanosystems, RT-qPCR studies were conducted for publications **I-III** and ELISA tests for publication **I**, to quantify the production of two pro-inflammatory cytokines.

In study **I**, the Curc@Lignin@TA@PL NPs were designed to reduce inflammation at the site of atherosclerotic plaques; therefore, their efficacy in reducing Nf-κb expression and inflammation in other types of cells involved in plaque formation, such as endothelial cells, needs to be evaluated. To test this, HCAEC cell inflammation was induced for 24 hours with LPS, followed by a 24-h incubation with curcumin or NPs. This time point was chosen based on maximum time for curcumin release. The expression of NFKB1 and TGFB1 was then quantified by RT-qPCR, with the 18S housekeeping gene as control. As shown in **Figure 22a**, inflammation in HCAECs was confirmed by increased gene expression in the positive control. Incubation with empty NPs and intermediate formulations did not reduce Nf-κb gene expression. In contrast, both curcumin and curcumin-loaded NPs significantly reduced Nf-κb expression to levels comparable to or below the negative control. This progressive decrease in gene expression with each additional coating layer is likely due to increased uptake of Curc@Lignin@TA and Curc@Lignin@TA@PL by HCAECs, under inflammatory conditions (**Figure 19a**). Moreover, the TA-coating also reduced TGFB1 expression, suggesting an antifibrotic effect, particularly in samples coated with platelet cell membranes. Thereafter, the impact of incubation with NP on the secretion

of pro-inflammatory cytokines from LPS-activated HCAECs was assessed. **Figures 22b** show that curcumin reduces the secretion of IL-6 and IL-1β. Curcumin-loaded NPs (Curc@Lignin@TA@PL) were found to be more effective than curcumin alone in reducing cytokine secretion, potentially due to a higher interaction with cells and a higher effect on the NFKB signalling pathway.[264] The strongest inhibition of IL-1β secretion was observed with Curc@Lignin particles, which are smaller and do not contain the TA-coating. In contrast, the final system (Curc@Lignin@TA@PL) was the least effective of all curcumin-loaded NPs.[265-267] Empty NPs showed an opposite trend, indicating a lack of synergistic effect when curcumin is administered with the final NP system.

Overall, curcumin inhibited gene expression of *NFKB1* and *TGFB1* in HCAEC cells, and Curc@Lignin@TA@PL significantly reduced the secretion of pro-inflammatory cytokines from LPS-inflamed HCAECs, with a more pronounced effect on IL-6 than on IL-1β.

In study **II**, the anti-inflammatory properties of curcumin and the antifibrotic effects of TA were evaluated using RT-qPCR. This study explored the synergistic impact of these compounds on the reduction of pro-inflammatory NF-κb and pro-fibrotic TGF-β gene expression. Furthermore, the expression of MMP-3 and MMP-9 was also investigated, as these enzymes are crucial in the degradation of the tendon matrix, leading to structural changes and contributing to the pain and decreased function commonly associated with tendinopathy.[180] Human tenocytes were pre-treated with 250 ng/mL LPS for 24 h, followed by a 24-h treatment with various formulations: AcDEX, AcDEX TA-coated, drug-loaded and TA-Coated NPs, curcumin, TA and DMEM-F12 as control. The 18S gene served as the control housekeeping gene. NF-κb is a critical regulator of pro-inflammatory cytokine production and apoptosis,[268,269] while TGF-β is a key mediator of fibrosis, both of which are upregulated in tendonitis.[270] As displayed in **Figure 22c**, TA-coated NP-treated cells showed a significant reduction in the expression of *NFKB1, TGFB1, MMP3* and *MMP9* compared to the positive control. In particular, the expression of NF-κb and TGF-β decreased approximately two-fold with TA-coated NP treatment. Drug-loaded NPs also showed a significant reduction in NF-κb expression, which was mainly attributed to the role of curcumin.[269] AcDEX TA-coated NPs contributed similarly to the reduction in *NFBK1* and *TGFB1* expression due to the presence of TA on their surface. The 1.5-fold decrease in MMP3 and 2-fold decrease in MMP9 by TA-coated NPs further validated curcumin's role in down-regulating metalloproteinase expression through its mechanism of NF-κb inhibition.[271,272] These results indicate that TA and curcumin, delivered through NPs, can effectively inhibit the expression of pro-inflammatory and pro-fibrotic genes *in vitro*, suggesting their potential in repairing inflamed tissues.

In study **III**, human primary tenocytes were treated for 48 h with siRNA LNPs, mRNA LNPs, dual-loaded LNPs (concentration of 100 µg/mL, equivalent to 0.3 µg/mL of siRNA and mRNA, respectively), mix LNPs (100 µg/mL of siRNA LNPs + 100 µg/mL of mRNA LNPs, equivalent to 0.3 µg/mL of siRNA and mRNA, respectively), and controls (SMAD3 siRNA, COL1A1 mRNA, empty LNPs, and DMEM-F12 medium). The antifibrotic and the pro-regenerative effects of the developed nanosystem were assessed by measuring the gene expression levels of *SMAD3, BMP2, COL3A1* and *TGFB1* by RT-qPCR. The TGF-β/Smad2/3 signalling pathway is crucial in the regulation of fibrotic tissue formation in injured tendons.[273] Previous research has shown that knockdown of SMAD3 can contribute to scarless tendon remodelling, in a late stage of tendon repair, by decreasing collagen III levels, modulating matrix anabolism and reducing proliferation, migration and differentiation of tenogenic stem cells.[274-276] Bone morphogenetic protein 2 (BMP-2), an important

mediator of the TGF-β/Smad2/3 signalling pathway, plays a crucial role in ECM synthesis and tendon healing. Enhancement of BMP-2 has been proposed as a strategy for tendon healing and prevention of fibrosis.[277,278] Therefore, these genes were selected to better investigate the activity of LNPs in human tenocytes.

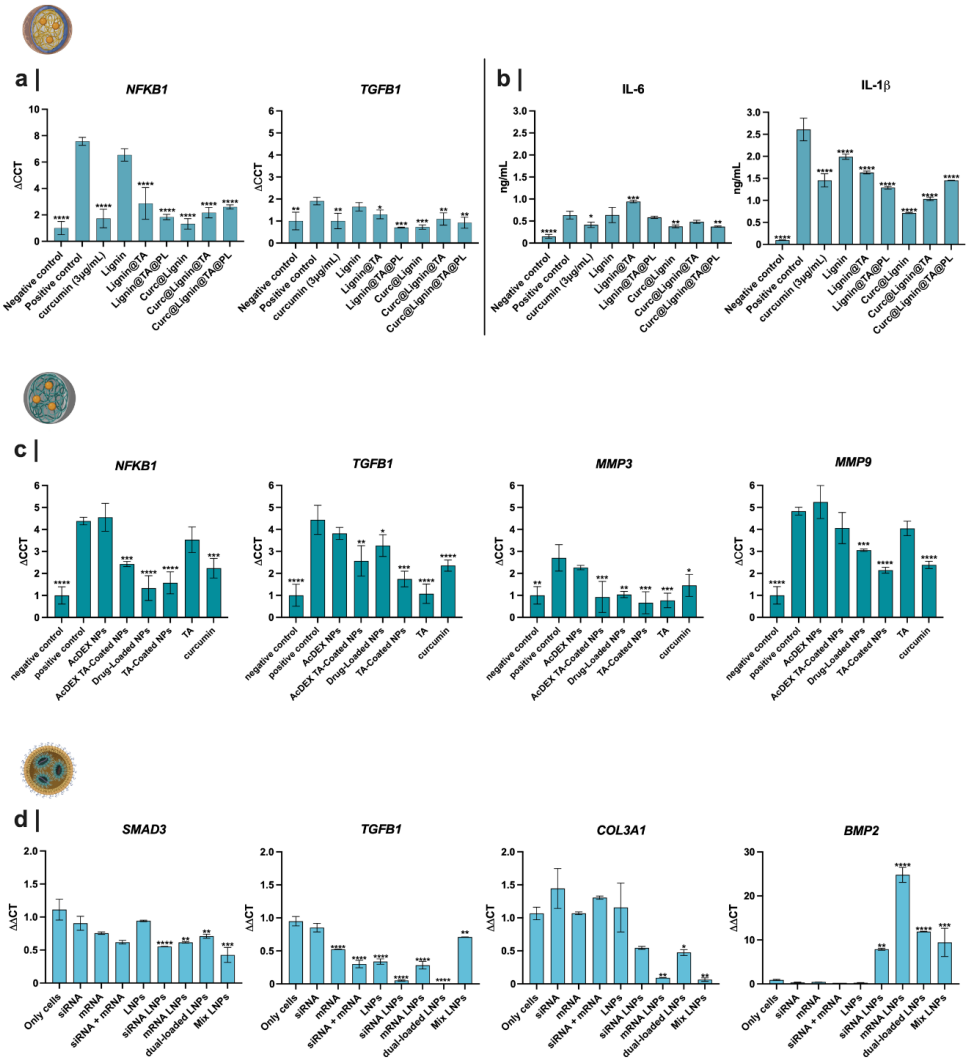


Figure 22. (a) Evaluation of gene expression of NFKB1 and TGFB1 in HCAEC cells after inflammation with LPS and incubation with curcumin-loaded or empty NPs and relative intermediates. The ΔCCT values were normalized to the housekeeping gene *18s*, and are further normalized by the negative control. The results are presented as mean ± s.d. ($n \geq 3$ independent samples) and were analysed by paired One way ANOVA versus the positive control LPS. (b) IL-6 and IL-1β quantification in the media of HCAEC cells after 24h incubation with the samples. The results are presented as mean ± s.d. ($n \geq 3$ independent samples) and were analysed by paired One way ANOVA *vs.* the positive control LPS. (c) Evaluation of the expression of pro-inflammatory and pro-fibrotic genes in human primary tenocytes by RT-qPCR. The properties of curcumin and TA have been evaluated by quantification of the gene expression of *NFKB1*, *TGFB1*, *MMP3*, and *MMP9*. Results are represented as fold increase values compared to the control ± s.d. ($n \geq 3$). An

ordinary one-way ANOVA followed by a Dunnett post-hoc test was used for the statistical analysis. (d) Evaluation of the expression of *SMAD3*, *TGFB1*, *COL3A1*, and BMP2 in human primary tenocytes using RT-qPCR. Results are represented as fold increase values compared to the only cells control ± s.d. ($n \geq 3$). An ordinary one-way ANOVA followed by a Dunnett post-hoc test was used for the statistical analysis. The significance levels of the differences for all the studies were set at the probabilities of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Figure 22d shows that SMAD3 gene expression was significantly reduced by siRNA, mRNA, dual-loaded and mix LNPs by almost twofold compared to the control. The reduction of SMAD3 in LNPs-treated samples is significant because the tenocytes used were characterised by a pro-fibrotic profile, due to the isolation from tissues from patients with advanced tendinopathy. Significantly, mRNA-loaded LNPs also reduced SMAD3 expression, but dual-loaded LNPs did not further reduce SMAD3 expression compared to siRNA-loaded LNPs, indicating that complete silencing of SMAD3 is difficult due to multiple activating factors.[278-280] BMP-2 expression increased significantly when cells were treated with siRNA, mRNA, dual-loaded and mix LNPs. The interaction between BMP-2 and SMAD3 is bidirectional and BMP-2 can promote both Smad and non-Smad signalling pathways, which justifies the simultaneous activation of SMAD3 by BMP-2.[278] This increase in BMP-2 expression is advantageous, as BMP-2 is considered a potential therapeutic target to prevent fibrosis and promote tissue remodelling.[281,282] In accordance with the silencing of SMAD3, COL3A1 expression was reduced by siRNAs, mRNAs, dual-loaded and mix LNPs, underlining the reduction in collagen production and in favour of a higher ratio of collagen I to collagen III, beneficial for ECM symmetry.[280,282] Finally, TGF-β expression was reduced by control RNAs, with even more pronounced effects in cells treated with siRNA and dual-loaded LNPs, further indicating that silencing of SMAD3 contributes to the reduction of fibrotic markers.[276]

Overall, SMAD3 siRNA delivery via LNPs effectively modulates gene expression in human tenocytes. Synergistic effects of SMAD3 siRNA and COL1A1 mRNA delivered by LNPs were observed, highlighting the complex interaction within the TGF-β/Smad2/3 signalling pathway, consistent with previous studies.[277,278,283]

5.8. *In vitro* efficacy of the lipid-based nanosystems in the tendon repair process: anti-fibrotic and pro-regenerative effects (III)

Further experiments to quantify the extracellular production of COL1A1 and the intracellular production of SMAD3 were conducted, since the changes in gene expression not always coincide with changes at protein level.[284] Therefore, immunostaining of collagen I and intracellular staining of SMAD3 were performed. **Figure 23a** shows the qualitative images of collagen I immunostaining, highlighting that in tenocytes treated with mRNA-LNPs, dual-loaded LNPs and mix LNPs is induced the production of more collagen fibres around the cell cytoplasm compared with the untreated cells or with cells treated with COL1A1 mRNA alone. These qualitative observations are supported by the quantitative analysis of collagen I production shown in **Figure 23b**. The results indicate a significant increase in collagen I production when tenocytes were treated with mRNA-LNPs, dual-loaded LNPs and mix LNPs. This increase is statistically significant when these results are compared to the treatment with COL1A1 mRNA alone, demonstrating the advantages to use LNPs as delivery system. Furthermore, the increase in collagen I production due to the mRNA containing LNPs is also statistically significant compared to the positive control (TGF-β treated cells). This suggest that dual-loaded and mix LNPs can compensate the inhibitory

effect of SMAD3 on collagen production, supporting the therapeutic RNAs combination used in this study.[281,285] Furthermore, **Figure 23c** shows a reduction in the intracellular production of SMAD3, when tenocytes were treated with siRNA LNPs, dual-loaded LNPs and mix LNPs, when compared with the negative control. Moreover, the siRNA alone was not reducing the expression of SMAD3, highlighting the relevance of LNPs for effective RNA delivery.

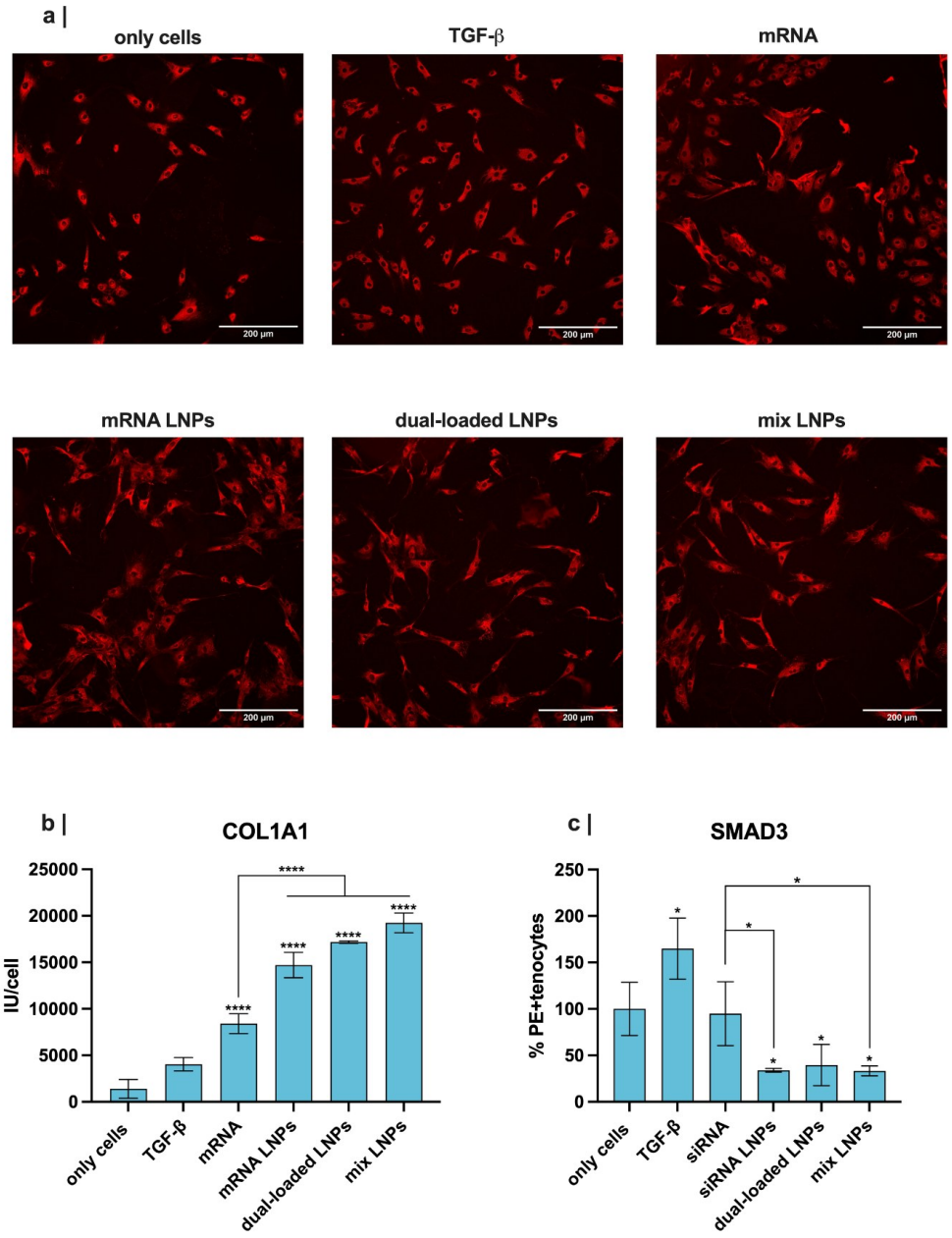


Figure 23. (a) Immunofluorescence staining of collagen I in a monolayer culture of human tenocytes visualized by MolecularDevices Nano Instrument. Collagen I was quantified 48h after the treatment with COL1A1 mRNA, mRNA LNPs, dual-loaded LNPs and mix LNPs using an anti-collagen type I antibody. Cells treated with TGF-β (20 ng/mL) were used as a positive control. 32 images were taken per well. (b) Quantitative analysis of the collagen I production using MetaXpress software. The results represent the sum of the intensity values of Texas Red divided by the count of cells. The 32 images were analyzed per well and per sample. (c) Intracellular staining of SMAD3 expression analysed by flow cytometry. An ordinary one-way ANOVA followed by a Dunnett post-hoc test was used for the statistical analysis in a-b. The significance levels of the differences were set at the probabilities of $*p < 0.05$, and $****p < 0.0001$ for comparison with only cells(a-b), mRNA (a) and siRNA (b).

Overall, the results confirm the efficient delivery of COL1A1 mRNA and SMAD3 siRNA by dual-loaded and mix LNPs, demonstrating the therapeutic potential of this nanoplatform in primary human tenocytes *in vitro.*

6. Conclusions

Nanomedicine is an evolving field that integrates biology with advanced technologies to address the limitations of conventional therapies. The application of NPs has opened up new possibilities for nanomedicine-based treatment and diagnosis of various diseases. The development of advanced nanoscale drug delivery systems offers a promising, non-invasive, and effective alternative for patients suffering from tendinopathy, potentially improving their treatment options.

In this thesis, three different formulations were developed for the delivery of different compound, to treat atherosclerosis, a possible risk factor for tendinopathies, and to treat tendon diseases at different stages.

Firstly, in **publication I**, biomimetic NPs were developed to deliver the anti-inflammatory compound curcumin. Curcumin was loaded into lignin, a wood-derived biopolymer, and the surface was modified with a TA-iron complex, followed by a coating with platelet-derived cell membrane fragments. The resulting NPs showed homogeneity in size, negative charge and stability for up to 7 days at +4 °C in ultrapure water, with excellent stability for up to 2 h in the cell medium at +37 °C under strong mixing conditions. The payload release was characterised by an initial burst of up to 75% in the first 2 h at pH 7.4 and up to 50% at pH 5.5, followed by sustained release for 24 h. The particles were cytocompatible with both endothelial and smooth muscle cells. However, the presence of curcumin caused toxicity at high concentrations in selected immune cell models. Surface modifications enhanced the interaction between NPs and endothelial cells under both steady-state and LPS-inflamed conditions, with similar results observed in macrophage cells. The developed NPs did not increase the expression of the co-stimulatory signal in KG-1 cells. The efficacy of NPs in reducing inflammation was demonstrated in LPS-inflamed endothelial cells by RT-qPCR and ELISA, significantly reducing gene expression of NFKB1, TGFB1 and the levels of IL-1 and IL1-β cytokines secreated, compared to the positive control.

In **publication II**, the microfluidic technique was employed to develop AcDEX-based NPs loaded with curcumin and coated with tannic acid (TA). Our proof-of-concept investigation aimed to evaluate the therapeutic potential of these TA-coated NPs in reducing inflammation and fibrosis. The NPs showed homogeneity in size and stability in cell culture media, with higher stability observed when coated with TA. The TA-coated NPs showed a burst release profile within the first 2 h in synovial-mimicking fluid, promoting curcumin release under inflammatory conditions. These NPs were biocompatible at low concentrations in KG-1 cells and at concentrations between 100 and 500 µg/mL in human tenocytes, demonstrating good biosafety. Efficacy was confirmed by RT-qPCR, where curcumin-loaded NPs showed significant anti-inflammatory properties, reducing NF-κb expression by more than 60%. Furthermore, the TA-coating demonstrated antifibrotic activity, reducing TGF-β gene expression by more than half. Due to these synergistic anti-inflammatory and antifibrotic effects, this nanosystem shows promise for the treatment of tendonitis. Further research is needed to fully explore its benefits, limitations and efficacy in human patients with tendinopathies.

In **publication III**, a novel lipid nanoparticle platform was developed using microfluidic technology for the co-delivery of SMAD3 siRNA and COL1A1 mRNA, as a potential therapeutic approach for the treatment of tendon diseases. The LNPs had homogenous size and good stability in various media, demonstrating high cytocompatibility in human tenocytes up to the concentration of 500 µg/mL. The LNPs showed high internalisation in

human primary tenocytes, with the highest peak at 12h after incubation. *In vitro* efficacy studies confirmed a decreasing in the expression of SMAD3 and an increasing in the expression of collagen I production, as well as modulation of other factors related to fibrosis and tendon remodelling. The complex interaction between factors of the TGF-β/Smad2/3 signalling pathway was also observed with LNPs' treatment. This study highlights the potential advantages of combinatorial therapeutic approaches to target complex diseases such as tendinopathy. The RNA-loaded nanoplatform is proposed as a promising candidate for the development of dual therapeutic strategies to prevent fibrosis and promote simultaneously tissue remodelling in late-stage tendon diseases.

Nonetheless, in the future, *ex-vivo* studies would be necessary to evaluate the penetration of the NPs into the tendon tissue, in order to assess whether the particles are actually effecting their activity in the tissue, as well as *in vitro*. Furthermore, all three nanosystems developed in this thesis will need to be tested *in vivo*, in specific models representing the pathologies of atherosclerosis and tendinopathies, and this will be a crucial step to confirm the activity, and better evaluate the response of the animal model to the administration of the NPs. Moreover, in the future, collaborations with industry partners will be crucial in order to establish a manufacturing process for NPs in accordance with GMP rules, to achieve scalability and approval for human clinical trials.

In conclusion, the present work provides new strategies in the treatment of atherosclerosis and tendon diseases. The nanosystems herein developed showed a great potential as platforms for drug delivery, opening up the possibility of innovative new treatment modalities in patient with hypercholesterolemia and tendinopathies.

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