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Elise Pape, Marianne Parent, Astrid Pinzano, Anne Sapin-Minet, Christel Henrionnet, et al.. Rapamycin-loaded Poly(lactic-co-glycolic) acid nanoparticles: Preparation, characterization, and in vitro toxicity study for potential intra-articular injection. *International Journal of Pharmaceutics*, 2021, 609, pp.121198. 10.1016/j.ijpharm.2021.121198 . hal-03395646

**HAL Id: hal-03395646**

**<https://hal.science/hal-03395646>**

Submitted on 17 Feb 2022

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# International Journal of Pharmaceutics

## Rapamycin-loaded Poly(lactic-co-glycolic) acid nanoparticles: preparation, characterization and in vitro toxicity study for potential intra-articular injection --Manuscript Draft--

<b>Manuscript Number:</b>	IJPHARM-D-21-01297R2
<b>Article Type:</b>	Research Paper
<b>Section/Category:</b>	Pharmaceutical Nanotechnology
<b>Keywords:</b>	rapamycin; Intra-articular injection; Nanoparticles
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<b>Abstract:</b>	<p>Osteoarthritis (OA) is the most common degenerative joint disease. Rapamycin is a potential candidate for OA treatment by increasing the autophagy process implicated in its physiopathology. To optimize Rapamycin profit and avoid systemic side effects, intra-articular (i.a.) administration appeared helpful. However, Rapamycin's highly hydrophobic nature and low bioavailability made it challenging to develop purpose-made drug delivery systems to overcome these limitations. We developed Rapamycin-loaded nanoparticles (NPs) using poly (lactic-co-glycolic acid) by emulsion/evaporation method. We evaluated these NPs' cytocompatibility towards cartilage (chondrocytes) and synovial membrane cells (synoviocytes) for a potential i.a. administration. The in vitro characterization of Rapamycin-loaded NPs had shown a suitable profile for an i.a. administration. In vitro biocompatibility of NPs was highlighted to 10 µM of Rapamycin for both synoviocytes and chondrocytes, but significant toxicity was observed with higher concentrations. Besides, synoviocytes are more sensitive to Rapamycin-loaded NPs than chondrocytes. Finally, we observed in vitro that an adapted formulated Rapamycin-loaded NPs could be safe at suitable i.a. injection concentrations. The toxic effect of Rapamycin encapsulated in these NPs on both articular cells was dose-dependent. After Rapamycin-loaded NPs i.a. administration, local retention, in situ safety, and systemic release should be evaluated with experimental in vivo models.</p>

# Rapamycin-loaded Poly(lactic-co-glycolic) acid nanoparticles: preparation, characterization, and *in vitro* toxicity study for potential intra-articular injection

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## Abbreviations:

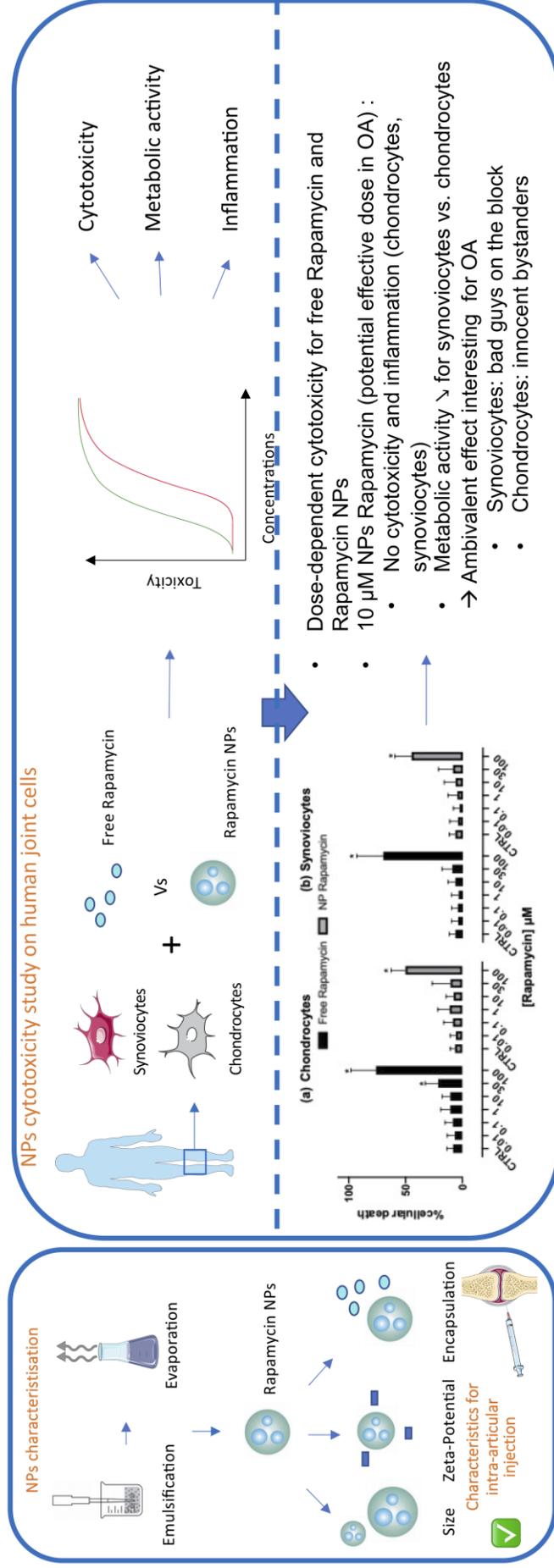
ANOVA: analysis of variance; CO<sub>2</sub>: carbon dioxide; DDS: drug delivery systems; DMSO: dimethyl sulfoxide; EDTA: ethylene diamine tetra acetate; EE: encapsulation efficiency; ELISA: Enzyme-linked immunosorbent assay; HPLC: high-performance liquid chromatography; i.a.: intra-articular; IC<sub>50</sub>: half-maximal inhibitory concentration; LDH: lactate dehydrogenase; mTOR: mammalian target of Rapamycin; MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; NPs: nanoparticles; OA: Osteoarthritis; P: passage; OD: optical density (absorbance); PBS: phosphate buffer saline; PDI: polydispersity index; PGE<sub>2</sub>: prostaglandin E<sub>2</sub>; PLGA: poly(lactic-co-glycolic acid); PVA: polyvinyl alcohol; SD: standard deviation.

## Highlights

- Rapamycin seems a chondroprotective candidate **in experimental rodent osteoarthritis**.
- Articular injection is helpful to optimize its profit and avoid systemic effects.
- We thus developed Rapamycin-loaded nanoparticles by emulsion/evaporation method.
- We evaluated Rapa-PGLA-NPs cytocompatibility towards cartilage and synovium.
- Rapamycin-loaded NPs exert a suitable profile for an i.a. administration (10 $\mu$ M).

# Graphical abstract

## Rapamycin-loaded PLGA nanoparticles (NPs) for potential intra-articular injection in osteoarthritis (OA)



Our Rapamycin NPs were adapted to an intra-articular administration et had dose-dependent cytotoxicity et had dose-dependent cytotoxicity as free Rapamycin. They were responsible for decreasing metabolic activity on synoviocytes but not on chondrocytes, which could benefit OA.



E. Pape et al. (2021)





## Abstract

Osteoarthritis (OA) is the most common degenerative joint disease. Rapamycin is a potential candidate for OA treatment by increasing the autophagy process implicated in its pathophysiology. To optimize Rapamycin profit and avoid systemic side effects, intra-articular (i.a.) administration appeared helpful. However, Rapamycin's highly hydrophobic nature and low bioavailability made it challenging to develop purpose-made drug delivery systems to overcome these limitations. We developed Rapamycin-loaded nanoparticles (NPs) using poly (lactic-co-glycolic acid) by emulsion/evaporation method. We evaluated these NPs' cytocompatibility towards cartilage (chondrocytes) and synovial membrane cells (synoviocytes) for a potential i.a. administration. The *in vitro* characterization of Rapamycin-loaded NPs had shown a suitable profile for an i.a. administration. *In vitro* biocompatibility of NPs was highlighted to 10  $\mu\text{M}$  of Rapamycin for both synoviocytes and chondrocytes, but significant toxicity was observed with higher concentrations. Besides, synoviocytes are more sensitive to Rapamycin-loaded NPs than chondrocytes. Finally, we observed *in vitro* that an adapted formulated Rapamycin-loaded NPs could be safe at suitable i.a. injection concentrations. The toxic effect of Rapamycin encapsulated in these NPs on both articular cells was dose-dependent. After Rapamycin-loaded NPs i.a. administration, local retention, *in situ* safety, and systemic release should be evaluated with experimental *in vivo* models.

**Keywords:** Rapamycin, intra-articular injection, nanoparticles

**Running title:** Rapamycin NPs for intra-articular injections

## 1. Introduction

Osteoarthritis (OA) is a degenerative joint disease that concerns about 300 million people worldwide (Hunter and Bierma-Zeinstra, 2019; Kolasinski et al., 2020). OA induces deterioration and loss of articular cartilage and inflammation of the synovial membrane causing structural and functional disorders in joint and surrounding tissues or bones (Gu et al., 2017; Mobasheri and Batt, 2016). Conventional pharmacological treatments, as analgesics, non-steroidal anti-inflammatory drugs, and so-called chondroprotective drugs, are only symptomatic. However, in few years, advances in OA's pathophysiology have improved and led to identifying new therapeutic targets to delay or decrease OA effects (Latourte et al., 2020). OA is a complex disease, and some metabolic or biological processes are involved in its development as oxidative stress, endoplasmic reticulum stress, or loss of cartilage homeostasis. They should be affected by some growth factors, signaling pathway molecules, or inflammatory cytokines (Zhang et al., 2019).

However, some studies have demonstrated that autophagy is decreased in OA chondrocytes *versus* healthy ones (Carames et al., 2012b) and have observed an improvement in chondrocytes' metabolism by using pharmacological tools, increasing the autophagy process (Cheng et al., 2016; Li et al., 2016; Perl, 2016; Vinatier et al., 2016; Xue et al., 2017). Rapamycin, also known as sirolimus, a recognized antifungal molecule (Li et al., 2014), has demonstrated *in vivo* some chondroprotective potency in rodents' mTOR pathway and is currently already used in few clinical indications as an immunosuppressive and antiproliferative molecule (Li et al., 2014). Therefore, one of the new therapeutic options is to restore autophagy's biologic process in chondrocytes by acting directly into the joint. The mTOR pathway inhibition is a potential therapeutic approach in OA because it induced upregulation of autophagy, notably after systemic (Carames et al., 2012a; Ma et al., 2019; Meckes et al., 2017; Ribeiro et al., 2016), or intra-articular (i.a.) injections in rodents OA models (Bao et al., 2020; Matsuzaki et al., 2014; Takayama et al., 2014). Even if Rapamycin reduces experimental OA development, its various biological effects can induce many side effects after systemic administration. Rapamycin can provoke hematologic/metabolic troubles in clinical practice as anemia, thrombocytopenia, hyperglycemia, and dyslipidemia (Nguyen et al., 2019). Some of these side effects are resolved after treatment discontinuation and are dose-dependent, but some are responsible for increased morbidity and mortality.

Intra-articular injection used as local administration for OA treatment seems to be a proper and adapted drug administration method. Indeed, it presents several benefits: i) using a lower drug dose than an oral administration, ii) reducing a systemic passage while increasing local drug concentration, iii) decreasing potential drug-drug interactions (Evans et al., 2014; Fan et al., 2018). Thus, i.a. administration is of particular interest to OA treatment and is already standard practice for some drugs (e.g., corticoids, hyaluronic acid) (Yu and Hunter, 2016). However, this delivery way is not easy for Rapamycin, a low water solubility ( $\log P = 4.3$ ), and is easily degraded in an aqueous environment by opening the macrolide ring (Nelson et al., 1999; Simamora et al., 2001). Moreover, even if i.a. administration can induce a high dose in the local articular joint, lymphatic clearance is responsible for rapid drug decrease and a short stay in joint space and even more in inflammatory conditions (Dhanabalan et al., 2020; Lima et al., 2019).

45 Drug delivery systems (DDS) such as nanoparticles (NPs), microparticles, liposomes,  
or other vectors might overcome these limitations (Schulze-Tanzil, 2021). Among them, NPs  
seem to be an appropriate system for OA treatment (Kang and Im, 2014). First, these vehicles  
can decrease administrated dose and increase bioavailability and retention time in joints  
(Kavanaugh et al., 2016). Second, formulation into polymeric particles ensures a sustained  
50 release of drugs and decreases the number of i.a. injections facilitating clinical application  
(Mohammadinejad et al., 2020). Biodegradable polymers as synthetic poly(lactic-co-glycolic  
acid) (PLGA) are widely used for DDS production, notably because of their predictable  
degradation in the body that avoids accumulation in the organism (Lima et al., 2019). Then,  
their well-defined structures induce lower risks of toxicity or immunogenicity (Song et al.,  
2018). Therefore, PLGA presents many advantages: demonstrated safety, low toxicity, and  
55 flexibility of chemical properties thanks to the tunable ratio and organization of glycolic and  
lactic monomers (Mirshafiee et al., 2017). PLGA is logically the primary polymer used to  
produce DDS for i.a. purposes in OA treatment (Haeri et al., 2018; Kang and Im, 2014).

Several studies used i.a. (free or loaded) Rapamycin injections to evaluate their interest  
in OA injuries' (Chen et al., 2020; Gu et al., 2017; Matsuzaki et al., 2014; Takayama et al., 2014).  
60 None of them has yet evaluated the cytotoxic effect of Rapamycin on different articular joint  
cells, to the best of our knowledge. Nevertheless, these cells are in direct contact with the drug  
after i.a. administration, and the determination of the potential cytotoxic effect of a drug on  
live chondrocytes and synoviocytes is essential to assess the safety profile of a drug for clinical  
i.a. use (Emami et al., 2018; Sherman et al., 2015). In this **pilot** study, we developed Rapamycin-  
65 loaded nanoparticles adapted to an i.a. administration. To achieve this objective, **we adapted  
a protocol satisfying to the two main specifications**: i) a nanoparticle size taller than 250 nm to  
avoid a fast clearance from joint cavity across blood capillaries (Barua and Mitragotri, 2014;  
Hoshyar et al., 2016), ii) an efficient Rapamycin encapsulation **in the particles (a minimum  
threshold of 70% encapsulation can be established related to published data (Supplementary  
70 data 1))**. We also evaluate NPs *in vitro* cytotoxicity toward human OA chondrocytes and  
synoviocytes.

## 2. Materials and Methods

### 2.1 Chemical and reagents

Rapamycin (purity>99%) was purchased from LC Laboratories (Woburn,  
75 Massachusetts, USA). Ammonium formate, formic acid, poly(lactide-co-glycolic) acid (PLGA  
RG 504 H (50:50, acid terminated)), polyvinyl alcohol (PVA), dimethyl sulfoxide (DMSO), 3-  
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and pronase were  
purchased from Sigma Aldrich (St Louis, Missouri, USA). Methanol (HPLC grade) and  
acetonitrile (HPLC grade) were provided by VWR (Radnor, Pennsylvania, USA). Dulbecco's  
80 modified Eagle's medium Nutrient Mixture F-12, trypsin, glutamine, and penicillin-  
streptomycin were supplied by Fisher Bioblock Scientific (Illkirch-Graffenstaden, France).  
Fetal bovine sera were acquired from Dutscher (Brumath, France), lactate dehydrogenase  
(LDH) assay, collagenase from Roche (Meylan, France), and PGE<sub>2</sub> assay ELISA kit from R&D  
Systems (Minneapolis, USA).

## 85 2.2 Preparation and characterization of nanoparticles (NPs)

### 2.2.1. Preparation of NPs

90 NPs were prepared by using the emulsion/solvent evaporation technique. Briefly, 200 mg of PLGA with or without 20 mg of Rapamycin were dissolved in 2 mL of dichloromethane. Then, this organic phase was emulsified in 20 mL of polyvinyl alcohol (PVA) (0.1%, w/v) by sonication (90 seconds, 30% amplitude) with an ultrasonic homogenizer (Vibracell 72434, Fisher Bioblock Scientific, France). Evaporation of the organic phase was performed to obtain dispersions of Rapamycin.

### 2.2.2. Particle size measurement

95 NPs size (z-average and polydispersity index, PDI) was assessed by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). Results are expressed as mean  $\pm$  standard deviation (SD) of three samples (independent batches of NPs) diluted in sodium chloride 1 mM. The average particle size (z-average) and polydispersity index were obtained with Malvern software.

### 2.2.3. Zeta potential measurement

100 NPs zeta potential was measured by electrophoretic mobility using laser Doppler anemometry in microelectrophoresis cell with Zetasizer (Malvern Instruments, UK). Results are expressed as mean  $\pm$  SD. Experiments were performed with three different batches of NPs.

## 2.3 Drug encapsulation efficiency

105 To evaluate the drug encapsulation efficiency (EE), the free Rapamycin (unloaded) and Rapamycin-loaded NPs (encapsulated) were separated by ultracentrifugation (42,000g, 4°C for 20 minutes). Supernatants were kept, and pelleted NPs were fully dissolved in 5 mL of acetonitrile, sonicated for 5 min, and agitated at 700 rpm for 30 min for a complete release of Rapamycin from the NPs. The same extraction protocol was applied to a similar fraction of the total dispersion. Then, the high-performance liquid chromatography (HPLC)-UV method 110 quantified amounts of Rapamycin (free in supernatant; total after dispersion extraction; inside the particles after pellets extraction).

Briefly, 3.5  $\mu$ L of sample were injected into an HPLC system composed of an InfinityLab Poroshell 120 EC-C18 column (150 x 2.1 mm; 2.7  $\mu$ m, Agilent Technologies) at 60°C. Rapamycin was eluted with gradient of mobile phase A (anhydrous formic acid, methanol, 0.7g/L ammonium formate, acetonitrile (0.05:100:450:450 v/v/v/v)) and mobile 115 phase B (anhydrous formic acid, methanol, 0.7g/L ammonium formate, acetonitrile (0.05:100:330:570 v/v/v/v)) as 100/0/100% mobile phase A at a flow rate of 0.9 mL/min for 14 min.

120 Rapamycin was detected at the wavelength of 278 nm, and the amount was calculated using the calibrator curve of Rapamycin in acetonitrile at different concentrations. This bioanalytical method was developed and fully validated according to our analytical guidelines with a linear relationship from 1 to 20  $\mu$ g/ml and a coefficient of variation at 2.40% and 8.50% for an intra-assay and inter-assay, respectively. The EE was calculated using the formulae EE (%) = (measured amount of Rapamycin after extraction from NPs) / (total amount of Rapamycin in dispersion) x 100 for direct method and EE (%) = (total amount of Rapamycin 125

in dispersion - measured amount of Rapamycin in the supernatant)/(total amount of Rapamycin in dispersion) × 100 for indirect method.

## 2.4 *In vitro* drug release study

130 A known amount of Rapamycin-loaded NPs dispersion was introduced into a flask containing 100 mL of release medium (0.9% saline, isopropanol 90:10 *v/v*) respecting sink conditions and kept at 37°C in a water bath with agitation at 200 rpm on a magnetic stirrer and protected from light. Flasks with Rapamycin solution (similar amount of drug) were used as controls. One milliliter of the medium was removed to measure drug concentration and was replaced by the same fresh medium volume at different time-points (5, 30 minutes, 1, 2, 4, 6, 135 8, 24 hours, 3, 6, 9, 11 days). This dilution was taken into account in the calculation of the results.

Samples were centrifugated (42,000g, 4°C for 20 minutes), and supernatants were collected to determine Rapamycin concentration in the medium with the HPLC method detailed above. The used standard curve (0.05 to 2.50 µg/ml) was established with Rapamycin at different concentrations in the release medium described previously. Release experiments were performed in triplicate for each time point and different batches. The curve showing the percentage of released Rapamycin versus time was built. Release experiments were performed in triplicate (independent batches of NPs), and results were expressed as cumulative % of Rapamycin in the medium *versus* time.

## 145 2.5. Stability studies

Rapamycin-loaded NPs dispersion was kept at +4°C and protected from light for several days until three weeks. Determination of NPs size and Rapamycin encapsulation efficiency was performed to evaluate these conditions' stability using the previously described method. Three batches were evaluated.

## 150 2.6. Analysis of Rapamycin effect on articular cells

### 2.6.1. Cell culture

155 Synoviocytes and chondrocytes were isolated from human knees obtained from OA patients undergoing total knee replacement surgery. All patients have been informed of this research protocol, approved by the Institutional Committee on Human Research (DC-2014-2148), and have given informed consent. Briefly, synovial membrane and small cartilage pieces were individually digested using collagenase (1.5 mg/mL) overnight at 37°C. For chondrocytes isolation, we performed a pre-digestion using pronase (2 mg/mL) for one hour at 37°C.

160 After digestion, synoviocytes and chondrocytes were suspended in Dulbecco's modified Eagle's medium with F12, 10% of fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin and were cultured in 55 cm<sup>2</sup> dishes with 25,000 cells/cm<sup>2</sup>. Cells were cultured at 37°C with 5% CO<sub>2</sub> in normoxia (20% O<sub>2</sub>). The medium was changed every three days until cells reached 80% confluence in Passage (P) 1 and P2. Monolayer cells were washed with Phosphate Buffer Saline (PBS), and 2 mL 0.05% trypsin-EDTA was added. Then cells were resuspended in an entirely fresh medium and seeded into culture plates at adapted cellular density for Rapamycin treatment and their effect on mitochondrial activity, cell viability, and 165 an archetypal pro-inflammatory mediator production like Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

### 2.6.2. Rapamycin treatment

170 Synoviocytes and chondrocytes were individually treated for 24 hours with either Rapamycin-loaded NPs or free Rapamycin. Rapamycin solution (10 nM – 100  $\mu$ M) or particles containing equivalent amounts of Rapamycin were individually incubated with synoviocytes or chondrocytes 24 hours. Rapamycin was previously dissolved in dimethyl sulfoxide (DMSO, due to its low water solubility) before dilution in a culture medium for the solution. The maximum final DMSO concentration was 0.5%, and this condition was tested as a control. Cytocompatibility of blank NPs (equivalent polymer amount) was also determined as an additional control.

### 2.6.3. Mitochondrial activity by MTT assay

180 Mitochondrial activity was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay in P2. Cells were cultured in 96-wells plates with cell density at 5,000 cells/well with 5% CO<sub>2</sub> at 37°C to allow cell adherence, then were treated as described previously. Briefly, after washing with fresh media, 25  $\mu$ L of MTT solution (5 mg/mL in PBS) were added to each well. After incubation at 37°C with 5% CO<sub>2</sub> for 4 hours, the medium was removed, and 100  $\mu$ L of sodium dodecyl sulfate with dimethylformamide were added, allowing to dissolve formazan derivate during incubation at 37°C with 5% CO<sub>2</sub> for 24 hours. The absorbance (OD) was measured at 580 nm with Varioskan (Thermofischer). The absolute half-maximal inhibitory concentration (IC<sub>50</sub>) was calculated with GraphPad Prism version 9.00, GraphPad Software (La Jolla California, USA) nonlinear regression curve fitting with a response for each type of cells (chondrocytes and synoviocytes) and type of Rapamycin (free Rapamycin and Rapamycin-loaded NPs).

### 2.6.4. Cell viability by LDH assay

190 Cells were cultured in 24-wells plates with cell density at 10,000 cells/well with 5% CO<sub>2</sub> at 37°C, then were treated as described previously. Cell viability was performed with the lactate dehydrogenase (LDH) assay according to the manufacturer's protocol on cell culture supernatants. Briefly, LDH is released in culture supernatant as a result of membrane damage. The amount of enzyme is proportional to the amount of formazan formed by the reduction of tetrazolium. Absorbance was measured at 490 nm by Varioskan (Thermo Fischer), and the percentage of cellular death was calculated with the formula: ((experimental value - low control)/(high control - low control))  $\times$  100. For normalization, we computed cells treated with Triton (X-100, 1%, Sigma) for high control (100%) and medium free for common control (0%).

### 2.6.5. Level of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)

200 The level of PGE<sub>2</sub> was determined using a PGE<sub>2</sub> assay ELISA kit according to the manufacturer's protocol (R&D Systems). Chondrocytes and synoviocytes were cultured with cell density at 10,000 cells/well with 5% CO<sub>2</sub> at 37°C in 24-well plates. The assay was performed on cell culture supernatants of chondrocytes and synoviocytes after treatment previously described for 24 hours. The supernatants were then frozen at -20°C until analysis, and after thawing, each sample was diluted with calibrator diluent, as advised in the protocol. Afterwards, 150  $\mu$ L of diluted supernatants were transferred into a 96-well plate coated with a goat anti-mouse polyclonal antibody, and 50 $\mu$ L of mouse monoclonal antibody to PGE<sub>2</sub> were added and incubated for 1 hour at room temperature with shaking at 500 rpm. A second incubation for two hours was performed after adding 50 $\mu$ L of PGE<sub>2</sub> conjugated to horseradish

210 peroxidase. After four washes, 200 $\mu$ L of substrate were added and incubated for 30 min,  
protected from light. The reaction was stopped with 100 $\mu$ L of the stop solution, and  
absorbance was measured at 450 nm by Varioskan (ThermoFischer). Each optical density was  
215 subtracted with the nonspecific binding optical density obtained from a well without  
monoclonal antibody to PGE<sub>2</sub>. PGE<sub>2</sub> concentration of samples was calculated using a standard  
curve absorbance versus concentration (from 39 pg/mL to 2500 pg/mL) obtained in the same  
experimental conditions. Calibrator diluent was used as the zero standard. Each condition was  
tested in triplicates, and results were expressed as mean  $\pm$  SD.

## 2.7 Statistical analysis

220 Statistical differences between treatment groups were determined with a one-way  
analysis of variance (ANOVA) followed by Dunnet's post hoc test (vs. controls) using  
GraphPad Prism version 9.00, GraphPad® Software (La Jolla California, USA). Significance  
was considered as a "p" value less than 0.05. The experiments were performed in triplicate  
using cells from three different OA patients.

## 3. Results

### 225 3.1 Preparation and characterization of nanoparticles

*Size, PDI, zeta potential, and EE.* The averages of size and PDI were respectively  
393.8 nm ( $\pm$  33.7) and 0.173 ( $\pm$  0.099) for Rapamycin-loaded NPs and 398.0 nm ( $\pm$  22.8) and  
0.269 ( $\pm$  0.048) for blank NPs. The averages of zeta potential were -9.02 mV ( $\pm$  2.41) and -6.73  
230 mV ( $\pm$  1.41) for Rapamycin-loaded NPs and blank NPs, respectively. **Table I** summarizes the  
characterization of these NPs after preparation. NPs were obtained with a  $61.0 \pm 3.0\%$  process  
recovery yield (n=9). Subsequently, EE was determined by both direct and indirect  
methodologies, with coherent results:  $100 \pm 7.9\%$  and  $92.5 \pm 1.7\%$ , respectively. During the  
short-time stability study, Rapamycin-loaded NPs were stored as dispersions at +4°C: size,  
zeta potential, and EE remained stable over the whole period considered, as shown in **Figures**  
235 **1a and 1b**.

### 3.2 In vitro drug release kinetic.

We studied the drug release kinetic from Rapamycin-loaded NPs and the free  
Rapamycin dispersion concentration, and the results are represented in **Figure 2**.  
Approximately 36% of Rapamycin was released in the medium by NPs within the first 5 min.  
240 After this burst, cumulative release stabilized around 40% after one hour and was maintained  
around these values during the two following weeks. For Rapamycin solution, only 50% of  
the initial amount was quantified until 24 h, and a drastic decrease of the concentration was  
observed within time (for example, 32 and 13% of the initial amount after 72 h and 16 days,  
respectively).

### 245 3.3 Cytotoxicity.

*Effects on chondrocytes and synoviocytes metabolic activity (MTT assay)* were  
determined for different Rapamycin concentrations for both free and loaded Rapamycin  
(**Figures 3a and 3b**). We observed that the highest tested Rapamycin concentration (100  $\mu$ M)  
induced a sharp, significant decrease in metabolic activity on chondrocytes and synoviocytes  
250 for both free and loaded Rapamycin. Similarly, with 30  $\mu$ M, synoviocytes experienced a  
significant decrease of metabolic activity with both free and NPs loaded-Rapamycin,

contrasting with the effect on chondrocytes, for which only loaded Rapamycin decreased metabolic activity. Concerning synoviocytes, concentrations from 100 nM for free Rapamycin and 10 nM for loaded Rapamycin induced a significant decrease in metabolic activity, whereas we did not detect any effect on chondrocytes for these Rapamycin concentrations. Calculated  $IC_{50}$  from metabolic activity showed the same trend with lower values for rapamycin-loaded NPs than free Rapamycin: 30.2  $\mu$ M and 39.3  $\mu$ M for chondrocytes and 19.0  $\mu$ M and 30.2  $\mu$ M for synoviocytes, respectively. Values  $IC_{50}$  also showed a more acute sensitivity for synoviocytes than chondrocytes.

**Concerning cytotoxicity**,  $IC_{50}$  was quite similar for free and Rapamycin-loaded NPs on both types of cells: 38.3  $\mu$ M and 39.8  $\mu$ M on chondrocytes and 38.1  $\mu$ M and 37.7  $\mu$ M on synoviocytes, respectively. The highest concentration of free and loaded Rapamycin (100  $\mu$ M) induced an increase in cell death than the control for both kinds of cells (**Fig 4.a and 4.b**). No cellular death was observed with blank NPs (data not shown).

**Phlogistic reaction.** The supernatant's PGE<sub>2</sub> concentration increased after treatment with free Rapamycin concentration as 30  $\mu$ M for chondrocytes and synoviocytes. Data were quite similar for loaded Rapamycin on synoviocytes with a significant effect of 30  $\mu$ M on both cells (**Figure 5**). With the highest concentration, all the cells died and, of course, did not respond to both free and loaded Rapamycin.

## 4. Discussion

This study is the first to evaluate Rapamycin-loaded PLGA NPs' toxicity towards main cellular types of articular joint (i.e., chondrocytes and synoviocytes) i.a. application. This administration way involves direct exposure of cartilage and synovium to the injected drug (McLean et al., 2019). With this in mind, we prepared Rapamycin-loaded NPs and demonstrated their suitability for i.a. administration by characterizing their physicochemical properties and assessing their biocompatibility towards chondrocytes and synoviocytes in comparison with free Rapamycin. To choose the various concentrations studied herein, we considered Takayama et al., demonstrating the positive effect of i.a. administration of Rapamycin (10  $\mu$ M) on murine OA lesions *in vivo* (Takayama et al., 2014). They performed this *in vivo* with the dose of Rapamycin (10  $\mu$ M) previously used as an autophagy activator in human chondrocytes *in vitro* (Sasaki et al., 2012). However, this concentration appears to have been selected empirically. Indeed, previous studies demonstrated the induction of autophagy at different concentrations and on various cell types (e.g., 0.2  $\mu$ g/mL on yeast (Noda and Ohsumi, 1998), 3  $\mu$ M on human neuronal cells (Pan et al., 2009), and 1  $\mu$ M on bovine cartilage explants (Caramès et al., 2012b)). Only one cytotoxicity study of Rapamycin on cell joint was performed by Caramès (Caramès et al., 2012b), which showed no effect on cell viability at 1, 5, and 10  $\mu$ M in bovine chondrocytes. We explored the cytotoxic effects of Rapamycin simultaneously on the two main human joint cell types at concentrations of potential interest in OA. We also evaluated their concomitant pro-inflammatory effect.

### 4.1. Size and distribution.

Rapamycin-loaded NPs used in other studies were generally smaller in size (100-250 nm) than ours (average 393.8 nm) (Boada et al., 2020; Holyoak et al., 2016; Li et al., 2017; Shi et al., 2014; Shirasu et al., 2016; Zhang et al., 2014; Zou et al., 2011). However, they were designed for different clinical uses with intratumoral, intravenous, or retroorbital administrations, for

295 which a minimum size is needed. On the contrary, to avoid free escape from the joint cavity  
after i.a. administration, particles must be more significant than 250 nm to reduce synovial  
capillaries clearance (Evans et al., 2014). Systemic diffusion from the joint is also delayed if  
resident macrophages phagocytose particles and NPs are usually more phagocytosed than  
300 microparticles (Burt et al., 2009; Butoescu et al., 2009). As a result, for an NP, i.a. delivery  
system, the size range is considered a vital property (Burt et al., 2009), and NPs described in  
the literature for i.a. injection of various drugs are in the 300-500 nm range (Edwards, 2011),  
so our NPs fully matched this size target. The PDI value  $< 0.2$  indicated that particles also had  
a narrow size distribution which is a critical characteristic to guarantee consistent drug release  
profiles (Butoescu et al., 2009; Horisawa et al., 2002; Shin et al., 2012; Zou et al., 2011).

#### 305 **4.2. Electric charging.**

As expected with PLGA polymers, the surface charge of our NPs (both blank and  
Rapamycin-loaded) is slightly negative (Boada et al., 2020; Li et al., 2017). Anionic NPs  
generally show a shorter joint retention time than cationic ones because this latter can establish  
electrostatic interactions with the highly anionic glycosaminoglycans present in healthy  
310 cartilage. Despite this, anionic NPs have several advantages for i.a. administration, as i) they  
accumulate in the superficial zone of cartilage, the first zone of OA degeneration, ii) they are  
not modified (size, charge) by synovial fluid, and iii) they are sufficiently retained in cartilage  
affected by OA, because of loss of negatively charged proteoglycans and ancillary decrease of  
electrostatic repulsions (Brown et al., 2019; Fan et al., 2018).

#### 315 **4.3. Entrapment efficiency.**

Rapamycin encapsulation in hydrophobic polymeric NPs is generally high ( $> 70\%$ , e.g.,  
(Butoescu et al., 2009; Shi et al., 2014; Zou et al., 2011)). Rapamycin was encapsulated with high  
efficiency with our process, and we demonstrated a good correlation between EE results  
obtained with the direct and indirect methods (EE of  $100.0 \pm 7.9\%$  and  $92.0 \pm 2.0\%$ ,  
320 respectively).

#### **4.4. Stability study.**

A three-week stability study was performed on NPs dispersions obtained after  
production: neither Rapamycin content nor size nor zeta potential were affected by storage at  
 $+4^{\circ}\text{C}$  (protected from light). It demonstrated that PVA 0.1% w/v is sufficient to stabilize NPs  
325 as dispersions. Nevertheless, attempts to freeze or to lyophilization, our NPs resulted in an  
essential variation of their size and PDI (data not shown), probably due to the irreversible  
aggregation of NPs. Therefore, this low PVA concentration might not be sufficient to maintain  
NPs characteristics when exposed to freezing and/or drying stresses. For example, Shi et al.  
(Shi et al., 2014) did not report similar issues with their Rapamycin-loaded PLGA NPs after  
330 lyophilization, but they used a much higher PVA concentration (3%), which also resulted in  
smaller particles (250 nm) used for periadventitial application (Vandervoort and Ludwig,  
2002; Zou et al., 2011). As explained above, such a small size will, however, not be ideal for i.a.  
administration. We proved that our NPs could be stored up to 3 weeks before *in vivo*  
application.

#### 335 **4.5. In vitro drug release kinetic.**

The results showed that rapamycin can diffuse out of PLGA particles in an artificial  
medium. This choice of medium permitted to ensure the solubility and the stability of the

active ingredient and to meet the analytical constraints for this *in vitro* assay but does not strictly correspond to the natural joint environment. *In vitro* release, experiments are generally carried out in PBS, pH 7.4. However, Rapamycin is highly unstable in aqueous media due to the alkaline hydrolysis of the lactam ring. Naseerali et al. suggested 9:1 (v/v) normal saline: isopropanol to be the best medium for release experiments (Naseerali et al., 2010). Besides limiting the drug degradation, this mixture also improves Rapamycin solubilization (experimental solubility 4.2 µg/mL), facilitating respect of sink conditions during the experiment. Then, this release medium was also used in another study (Katiyar et al., 2016). Haddadi's study performed a release kinetic with phosphate buffer ethanol mixture (9:1 v/v) for release medium. However, no verification for Rapamycin stability in this release medium was mentioned (Haddadi et al., 2008). The drug release study from Rapamycin NPs showed a burst effect in the first five minutes with 36% released and then a low sustained release until the end of the experiment after two weeks. This profile (initial massive release, then sustained release for days or weeks) is expected with degradation-controlled releasing polymers such as PLGA (Dinarvand et al., 2011). In our case, the burst could be explained by the presence of Rapamycin on NPs surface or by the isopropanol-containing medium. Others in the literature reported lower bursts, less early bursts (Jhunjhunwala et al., 2009; Tyler et al., 2011; Zou et al., 2011), or even no bursts at all (Fan et al., 2017) for various Rapamycin PLGA nano- or microparticles. Although the variety of experimental set-ups renders the results difficult to compare, these studies were all conducted in small volumes of aqueous media. Haddadi et al. reported a very gradual release (around 5% after one day) of Rapamycin from PLGA NPs (280 nm) in PBS: ethanol (9:1), but the experiment was conducted in a minimal volume (1.5 mL) without changing the medium, therefore probably not in sink conditions (Haddadi et al., 2008). Interestingly, Katiyar et al. tested their PLGA NPs (150 nm) in PBS: isopropanol (9:1) and observed a 30-40% burst similar to our results but delayed at 3h (Katiyar et al., 2016). This can be explained by using a dialysis membrane in their experiment, which might delay the apparition of released Rapamycin in the medium. To conclude, burst effects observed *in vitro* might be helpful to compare formulations tested in similar conditions but cannot be fully extrapolated to *in vivo* conditions because the duration of release in i.a. conditions could be modified (more limited diffusion associated with erosion of particles).

After the burst, the cumulative release from our NPs remained steady at around 40% during the two weeks of the experiment. This relatively flat curve is not the most common profile in the literature but can be explained by an equilibrium between Rapamycin release from the particles and its degradation once in the medium. The control with free Rapamycin solution highlighted this intense degradation, with values dropping from 50% at 5 min to 13% after 16 days. However, we determined the amount of residual drug in the NPs after 16 days of incubation to be substantial (around 40% of the initial loading, data not shown), indicating i) that the drug release could continue after two weeks and ii) that the sum of total released Rapamycin in the medium and residual in the NPs covered 80% of the initial amount. This indicated that Rapamycin in the NPs had been protected from *in vitro* hydrolysis, contrary to free Rapamycin, as previously observed by others (Fan et al., 2017). Rapamycin is a drug with a narrow therapeutic index: the ability to preserve the drug in the environment after administration is, therefore, critical for a DDS to ensure successful clinical application (Fan et al., 2017). Moreover, their gradual release makes our NPs especially relevant for i.a. administration, with reduced frequency of administrations (Dhanabalan et al., 2020).

#### 4.6. Cytotoxicity.

Human chondrocytes were used for cytotoxicity studies to evaluate the potential chondrotoxicity inherent to a higher drug concentration in articular cartilage after an i.a. injection (Evans et al., 2014). Previously, Busse et al. studied *in vitro* cytotoxicity on human chondrocytes cultures and performed cell viability assays to conclude the dose-dependent toxicity of classical i.a. drugs (Busse et al., 2019). With this in mind, we tested different Rapamycin concentrations with monolayer chondrocytes. Within the same concept, we concomitantly tested it on synoviocytes (Jung et al., 2020). To the best of our knowledge, there were no previous data about such concomitant cytotoxicity reports of Rapamycin towards chondrocytes and synoviocytes. The studies assessing toxic effects on synoviocytes were less common than those on chondrocytes, even for other drugs commonly used (Bolam et al., 2021). Some authors have already shown *in vivo* the potential usefulness of i.a. Rapamycin injection to delay OA cartilage damage, but they did not evaluate its cytotoxicity on resident cells simultaneously (Matsuzaki et al., 2014; Takayama et al., 2014). However, these cytotoxic data could seem exciting to highlight potential delayed clinical damages after i.a. drug injection(s). For example, cytotoxicity of i.a. anesthetic drugs could warn about the risk of shoulder chondrolysis (Stueber et al., 2014). Ambivalent properties of steroids, beneficial on OA symptomatology but possibly detrimental on cartilage, have also been suggested (Black and Grodzinsky, 2019; Kompel et al., 2019; McAlindon et al., 2017; Zeng et al., 2019). Our data showed that Rapamycin has dose-dependent toxicity on chondrocytes and synoviocytes. The higher concentration of 100  $\mu\text{M}$  was toxic because both Rapamycin and Rapamycin-loaded NPs induced high cellular death on chondrocytes and synoviocytes (71% and 62 % with free Rapamycin and 58% and 50% with Rapamycin NPs) at this concentration.

$\text{IC}_{50}$  values, similar for cells (chondrocytes and synoviocytes) and Rapamycin (free and nanoparticles), reflected that nanoparticles were not more deleterious for neither chondrocyte (38.3  $\mu\text{M}$  and 39.8  $\mu\text{M}$  for free and loaded Rapamycin respectively) nor synoviocyte (38.1  $\mu\text{M}$  and 37.7  $\mu\text{M}$  for free and loaded Rapamycin respectively). Poly(lactide-co-glycolide) (PLGA) is biocompatible, biodegradable, and approved by EMA and FDA authorities. It has been extensively studied for the development of devices for the controlled delivery of macromolecules. PLGA particles loading Rapamycin were already described (Alarcin et al., 2020; Butoescu et al., 2009; Gerwin et al., 2006; Haeri et al., 2018; Jung et al., 2020; Kapoor et al., 2015; Riffault et al., 2015; Zerrillo et al., 2019; Zhang and Huang, 2012). For example, Dhanabalan et al. (Dhanabalan et al., 2020) described Rapamycin microparticles' cytocompatibility on the human chondrocyte cell line (C28/I2, used as a model cell line for studying normal and pathological cartilage repair mechanisms related to chondrocyte biology and physiology). After 24 hours, no cytotoxic effect was observed using 100 even 500  $\mu\text{g}/\text{mL}$  Rapamycin delivered by microparticles. Our study on clinical articular OA chondrocytes and synoviocytes did not affect both cells' viability observed with blank NPs (according to LDH and MTT assays; data not shown). Similar results were observed with other PLGA particles in contact with chondrocytes (Yan et al., 2016). However, we observed a critical death rate at 100  $\mu\text{M}$  of Rapamycin delivered by NPs, with a significant increase of LDH assay and a cellular death for both cell types, as mentioned above with free Rapamycin. This is probably due to an extensive exchange area offered by NPs in the cell culture medium, which cannot limit Rapamycin exposure to cells at this concentration. It could probably be different from *in vivo* studies because monolayer cell models should be more sensitive to a cytotoxic effect than cells

organize in an extracellular matrix or a three-dimension scaffold (Bolam et al., 2021; Busse et al., 2019; Sherman et al., 2015)

#### 430 **4.7. Mitochondrial activity.**

About cell viability assay, we also demonstrated dose-dependent toxic effects for both types of cells. Indeed, we observed at 100  $\mu\text{M}$  a significantly decreased mitochondrial activity compared to DMSO control. For the effects of other concentrations on mitochondrial activity, synoviocytes were more sensitive to Rapamycin than chondrocytes since a decrease of metabolic activity was observed from the low concentration of 10  $\mu\text{M}$  of Rapamycin NPs. In  
435 comparison, we observed only this effect at 30  $\mu\text{M}$  of Rapamycin-loaded NPs on chondrocytes. The synoviocytes seemed to be more sensitive, particularly for loaded Rapamycin (**Fig 3.b**) as reflected by  $\text{IC}_{50}$  values of 30.3  $\mu\text{M}$  and 19.0  $\mu\text{M}$  for free and loaded Rapamycin respectively compared to chondrocytes (39.3  $\mu\text{M}$  and 30.2  $\mu\text{M}$  respectively). A few previous data about  
440 Rapamycin toxicity have been demonstrated towards human breast cancer cells with similar results (Katiyar et al., 2016) with an  $\text{IC}_{50}$  of 20.3  $\mu\text{M}$  and 11.4  $\mu\text{M}$  for free Rapamycin **and** Rapamycin-loaded NPs (metabolic activity assay). On the other hand, Katiyar et al. had concluded that higher efficacy of Rapamycin loaded NPs with an  $\text{IC}_{50}$  lower than free Rapamycin solution in breast cancer cells for the expected antiproliferative effect (Katiyar et al., 2016). Nevertheless, we must keep in mind that Rapamycin cytotoxicity should depend on  
445 the kind of tested cells, notably for 10  $\mu\text{M}$ , which has been described as chondroprotective in experimental OA. Cheng et al. have already shown that 100 ng/ml, one of the lowest tested concentrations, induced a significant cell viability decrease on human lung cancer cell lines, whereas we did not observe this cytotoxicity onto synoviocytes or chondrocytes (Takayama et al., 2014). Other authors have described essential cellular growth inhibition with the same concentration range as in our study on other cell lines (rodent gliosarcoma cell lines) (Tyler et al., 2011).

However, these results' metabolic activity are not harmful to Rapamycin-loaded NPs compared to Rapamycin solution for several reasons: i) detrimental effects as cellular death  
455 was observed only with the highest concentration (100  $\mu\text{M}$ ) like for Rapamycin solution as explained previously; ii) chondrocytes could be protected *in vivo* thanks to 3D conformation and extracellular matrix production; iii) effect on synoviocytes could be compared to synoviorthesis (medical/chemical synovectomy) that have demonstrated beneficial effects in the past for inflammatory synovitis (Karavida and Notopoulos, 2010), preeminent in OA flares  
460 (Lambert et al., 2020) and during its early stages (Mathiessen and Conaghan, 2017; Thoenen et al., 2021). According to our data, we finally confirmed 10  $\mu\text{M}$  Rapamycin's safety for the i.a. injections used in Takayama's study and described it as delaying OA effects (Takayama et al., 2014). However, we must keep in mind that Rapamycin at 10  $\mu\text{M}$ , 4-fold less than  $\text{IC}_{50}$ , in i.a. administration with nanoparticle form still presents a narrow therapeutic range, and long-  
465 term toxicity could be evaluated.

#### **4.8. PGE<sub>2</sub>.**

PGE<sub>2</sub> concentration measurement revealed that PLGA NPs did not induce an inflammatory response for both cell types than control treatment after contact for 24 hours. Even if Nicolette et al. suggested that components of particles inside cells could increase the  
470 inflammatory response (Nicolette et al., 2011), Riffault et al. showed that NPs using PLGA polymer could be responsible for a reversible inflammatory response NP contact with

chondrocytes and synoviocytes but could not affect their biocompatibility. They thus could be used for i.a. administration (Riffault et al., 2015). We can confirm the PLGA biocompatible hypothesis because of the lack of cytotoxicity with our unloaded NPs in cells surrounding the joint.

#### 4.9. Limitations of the study.

We have to consider that this original study was performed in 2D culture conditions. We can believe that it is a real-life condition for synoviocytes considered during OA as "the bad guys on the block," directly irrigated by the synovial fluid, into the joint, without a biological barrier concomitantly releasing pro-inflammatory signals into the synovial fluid. Consequently, a deleterious or anti-inflammatory (even pro-apoptotic) influence of Rapamycin on OA-related synovitis will be beneficial, all the more so the biological half-life is sustained within the joint with NPs, prone to diffuse into inflamed cells. On the contrary, chondrocyte, "the innocent bystander," is relatively protected by the "matrix effect" (three-dimensionally cross-linked networks of extracellular matrix compounds capable of limiting drug diffusion between their polymeric chains) (Brushi, 2015). With this in mind, we can expect that 10  $\mu$ M Rapamycin concentration will exert an ambivalent effect locally, simultaneously detrimental on synoviocytes and beneficial on chondrocytes. The high concentration used by Ma et al. i.a. (100  $\mu$ M) in mice, as judged histologically (Ma et al., 2019), is reassuring in this way. Further pharmacokinetic/pharmacodynamic works on explants and rodents injected, i.a. with NPs -loaded Rapamycin is yet to come.

Finally, we must consider that NP's size is a critical characteristic for biocompatibility and tolerability after i.a. administration. For example, Burt et al. described hyperplasia of the synovial membrane due to PLGA NPs' phagocytosis but not with PLGA microspheres. The homogeneous size of prepared NPs in our study was in the range of usual PLGA NPs (Burt et al., 2009). One hypothesis could explain the different metabolic profiles between chondrocytes and synoviocytes, the latter being more prone to phagocyte NP. In contrast, Horisawa et al. showed that both micro and nanospheres are well tolerated after i.a. injection in rats (Horisawa et al., 2002). As Zhang and Huang (Zhang and Huang, 2012) conclude, the PLGA particle-matrix is biocompatible and can be used *in vivo*, avoiding local side effects after i.a. administration.

## 5. Conclusion

OA is a frequent, severe, and disabling disease, thus constituting a significant health issue. Among possible novel treatments, Rapamycin has been shown experimentally to delay OA evolution. However, to fully develop its clinical potential as OA treatment, a form of Rapamycin ensures good drug retention in joint space after i.a. administration is still needed. We developed biodegradable Rapamycin-loaded NPs that achieve this aim: size adapted to i.a. administration, high encapsulation efficiency, and sustained release of Rapamycin with reproducible results. Moreover, our drug delivery system preserved Rapamycin from degradation upon contact with an aqueous environment for several days. Our NPs were then tested on the two cell types directly exposed to Rapamycin after i.a. administration, i.e., chondrocytes and synoviocytes obtained from OA patients. Our study's significant results are biocompatibility on articular tissues for potential concentration used for OA treatment and the dose-dependent toxicity of Rapamycin toward both relevant cell types, namely human OA

515 chondrocytes and synoviocytes. This study provided beneficial results for developing a Rapamycin-controlled release system in local articular joint treatment, notably in an OA animal model.

### **Acknowledgements:**

520 We thank Morad BENSIDHOUM, PhD and Véronique REGNAULT, PhD, for their scientific advice, as designated referees for the thesis follow-up committee of the Doctoral School of the University of Lorraine; this work is a part of EP's PhD work. We also acknowledge the nursing staff of Chirurgie Orthopédique, Traumatologique & Arthroscopique, and Centre Emile GALLÉ, CHRU NANCY, for their assistance in the provision of clinical material.

### **Funding**

525 Dedicated funding from “Université de Lorraine, Appels à Projets internes BMS, Projets Incitatifs 2019” supported this work.

### **Competing interests**

The authors declare that they have no competing interests.

### **Ethical approval and consent to participate**

530 The Ethical Committee approved our university hospital's clinical protocol # 223 (File DC-2019-3387, actualized 14 January 2019, initially registered DC-2008-459).

### **Data Statement**

The datasets taken during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Table

**Table I:** Characterization of Rapamycin-loaded and blank nanoparticles (NPs)

	<b>Rapamycin-loaded NPs</b>	<b>Blank NPs</b>
Size Z-average (d.nm)	393.0 ± 33.7	398.0 ± 22.8
PDI	0.173 ± 0.099	0.269 ± 0.048
Zeta potential (mV)	-9.02 ± 2.41	-6.73 ± 1.41
<b>Direct EE (%)</b>	<b>100.0 ± 7.9</b>	<b>ND</b>

Values expressed as mean ± SD. n=10 batches for Rapamycin-loaded NPs, n=5 for blank NPs.

PDI: polydispersity index.

EE: entrapment efficiency.

ND: not detectable

## Legends of the Figures

### Figure 1: Characterization of nanoparticles.

Entrapment efficacy (%) (a) and size (Z-average in nm) (b) for Rapamycin-loaded nanoparticles after different storage times at +4°C. Values expressed as means  $\pm$  SD, n=3 in 3 independent samples; ns: non-significant, one-way ANOVA between all groups.

### Figure 2: Cumulative % of Rapamycin release in the medium.

*In vitro* release profile from 100  $\mu$ g Rapamycin-loaded nanoparticles (equivalent to 100  $\mu$ g of Rapamycin) in 50 mL of release **medium** (standard saline solution 0.9% and isopropanol (9:1, v/v) (grey curve with circled spots). Results (means  $\pm$  SD, n=3-6 experiments) are expressed in a proportion of released Rapamycin amount among initially loaded Rapamycin (theoretical value 0%). In parallel, we observed the degradation profile for 100  $\mu$ g of Rapamycin solution in 50 mL in the same **medium** (black curve with squared spots, initial theoretical value 100%). Please note that X-axis is in two segments (linear scale but different units: the first part in hours (H), the second part in days (D)). Time-points studied: 0 (theoretical 0 and 100%), 5, 30 minutes, 1, 2, 4, 6, 8, 24 hours, and 3, 6, 9, 11 days)

### Figure 3. Articular cell metabolism assessed by MTT assay.

- MTT assay on human OA chondrocytes with different concentrations of free Rapamycin and Rapamycin-loaded nanoparticles. The free **Rapamycin** and Rapamycin-loaded nanoparticles were compared with DMSO (0.5%) used as control. Results are expressed as mean  $\pm$  SD (\*,  $p < .05$ , one-way ANOVA followed by a Dunnet's post hoc test vs. controls, CTRL).
- Same experiment performed on human OA synoviocytes.

### Figure 4. Articular cell viability with LDH assay.

- LDH assay on human OA chondrocytes with different concentrations of free Rapamycin and Rapamycin-loaded nanoparticles. The percentage of cellular death for different concentrations of free and loaded Rapamycin was compared to DMSO 0.5% used as control. Results are expressed as mean  $\pm$  SD (\*,  $p < .05$ , one-way ANOVA followed by a Dunnet's post hoc test vs. controls, CTRL).
- Same experiment performed on human OA synoviocytes.

### Figure 5. Phlogistic reaction to Rapamycin.

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) measurements were assessed in the supernatant of chondrocytes (a) and synoviocytes (b) treated **24 h** with different concentrations of free Rapamycin and Rapamycin-loaded NPs. (\*,  $p < .05$ , one-way ANOVA followed by a Dunnet's post hoc test vs. controls, CTRL). Results are expressed in mean  $\pm$  SD for three independent experimentations in three OA human articular cells.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

## Credit author statement

Elise PAPE contributed to experimental design, preparation, and characterization of nanoparticles, analysis of Rapamycin effect on human cells, statistical analyses, and manuscript preparation, visualization of the data.

Marianne PARENT contributed to the preparation and characterization of nanoparticles, analysis of the data, and editing of the manuscript, visualization of the data.

Astrid PINZANO contributed to experimental design, analysis of Rapamycin effect on cells, and manuscript preparation/editing, visualization of the data.

Anne SAPIN MINET contributed to the experimental design, manuscript editing, preparation, and characterization of nanoparticles, analysis, visualization and validation of the data.

Christel HENRIONNET contributed to the analysis of Rapamycin's effect on cells, conditioning of human material, anonymization, experimental design, validation of the data, and manuscript preparation.

Pierre GILLET, Julien SCALA BERTOLA, and Nicolas GAMBIER supervised study conception, experimental design and statistical analyses, and manuscript revision and editing, visualization of the data.

Julien SCALA BERTOLA obtained funding from our University.

All authors read and approved the revised manuscript.

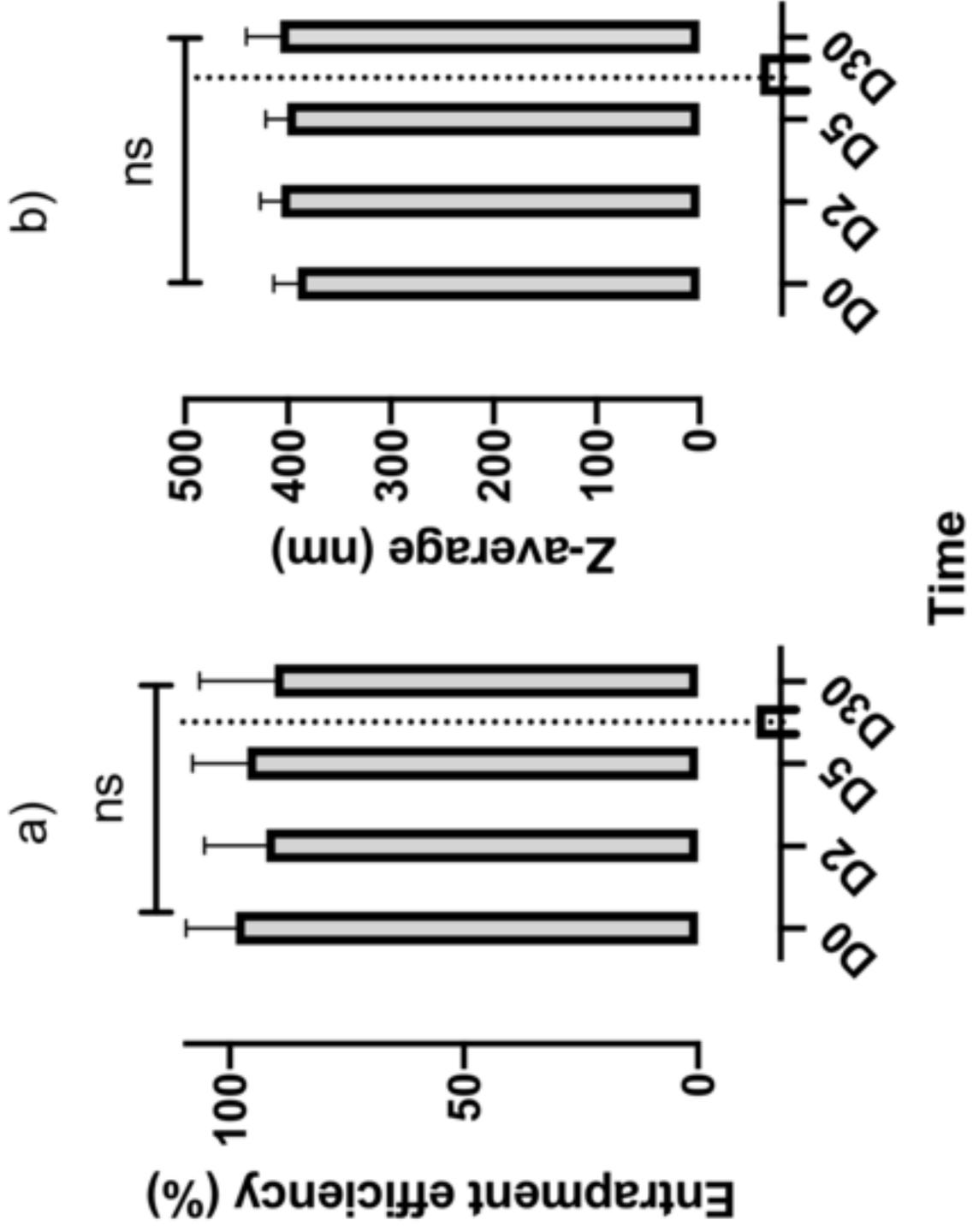


Figure 1: Characterization of nanoparticles.

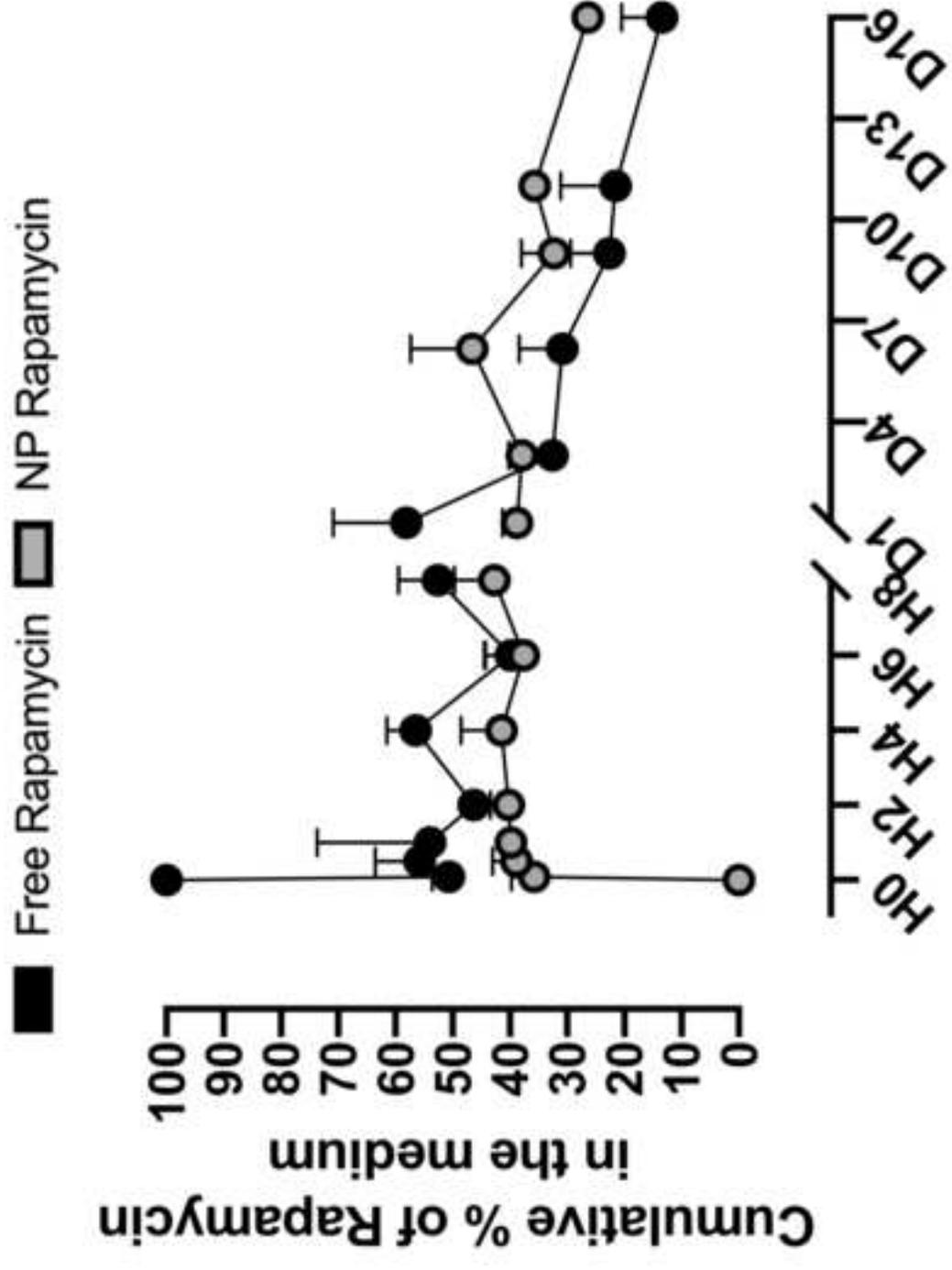


Figure 2: Cumulative % of Rapamycin release in the medium.

Figure(s)

**(a) Chondrocytes**

**(b) Synoviocytes**

■ Free Rapamycin    ▨ NP Rapamycin

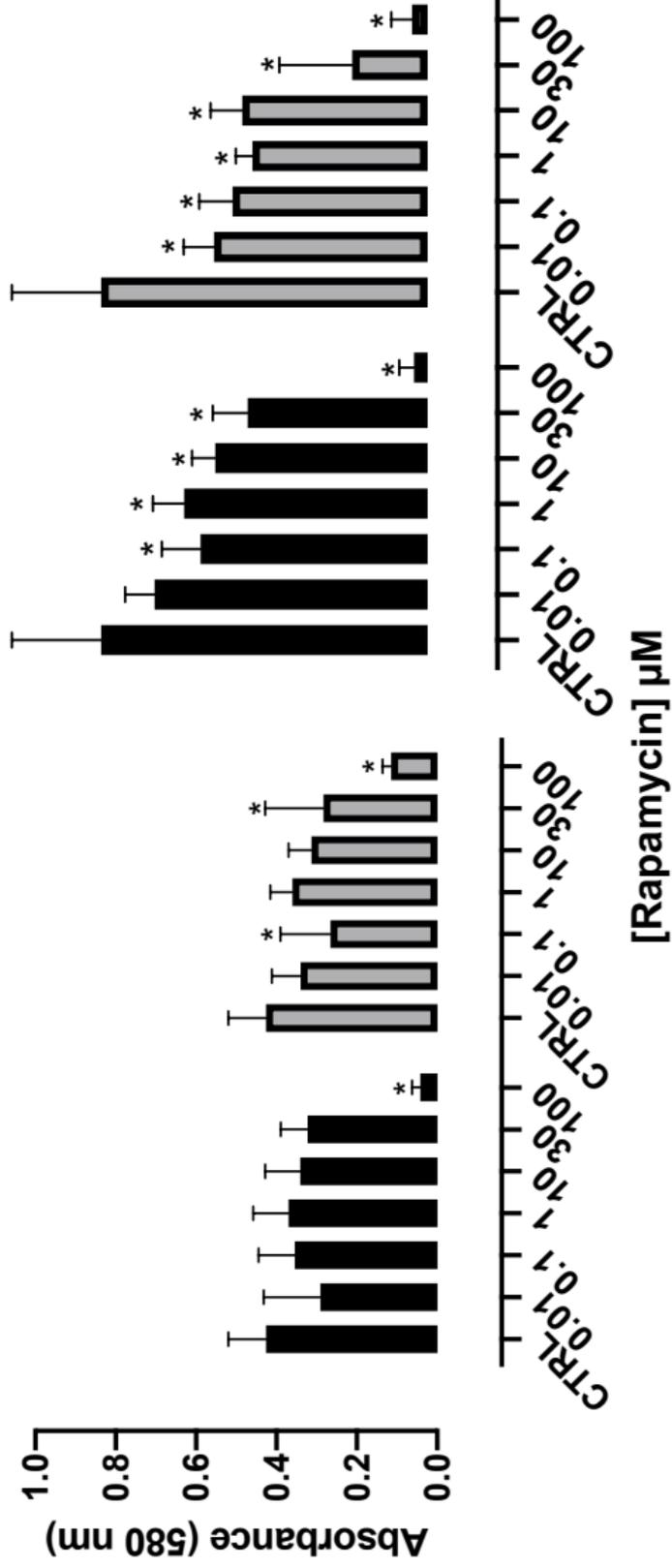


Figure 3. Articular cell metabolism assessed by MTT assay.

Figure(s)

(a) Chondrocytes

(b) Synoviocytes

■ Free Rapamycin    ■ NP Rapamycin

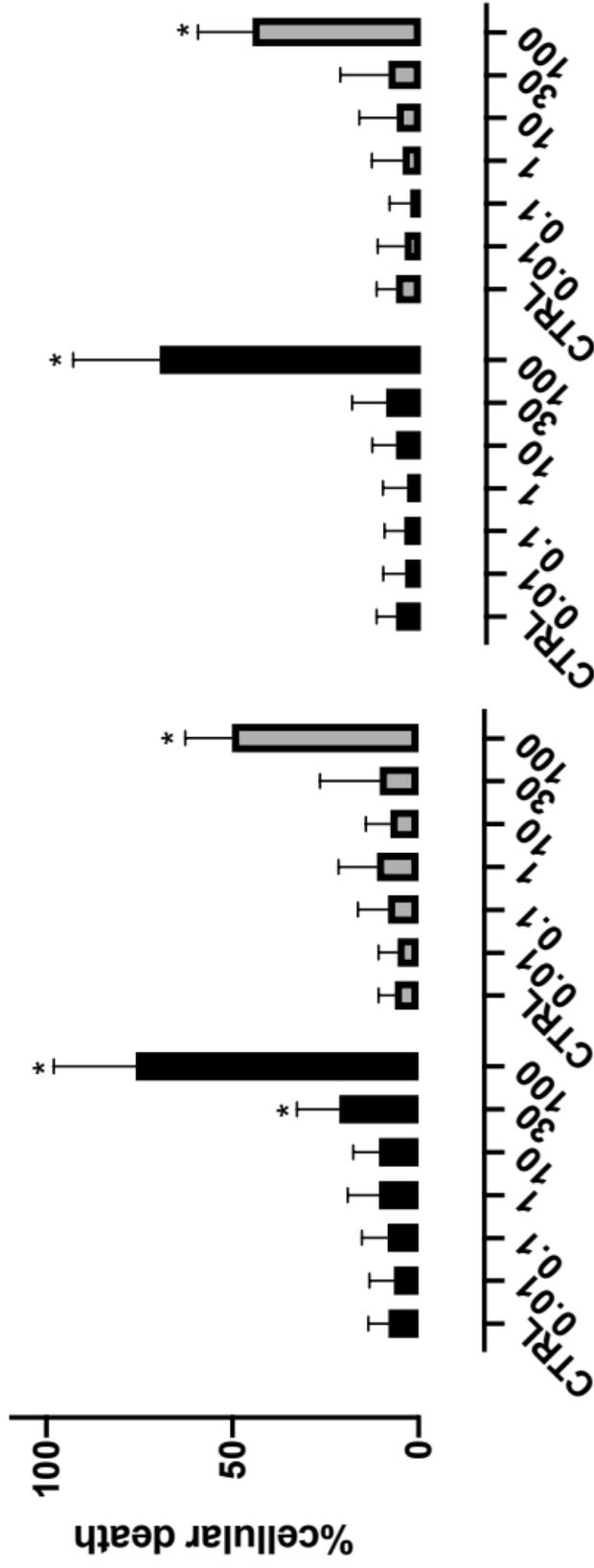


Figure 4. Articular cell viability with LDH assay.

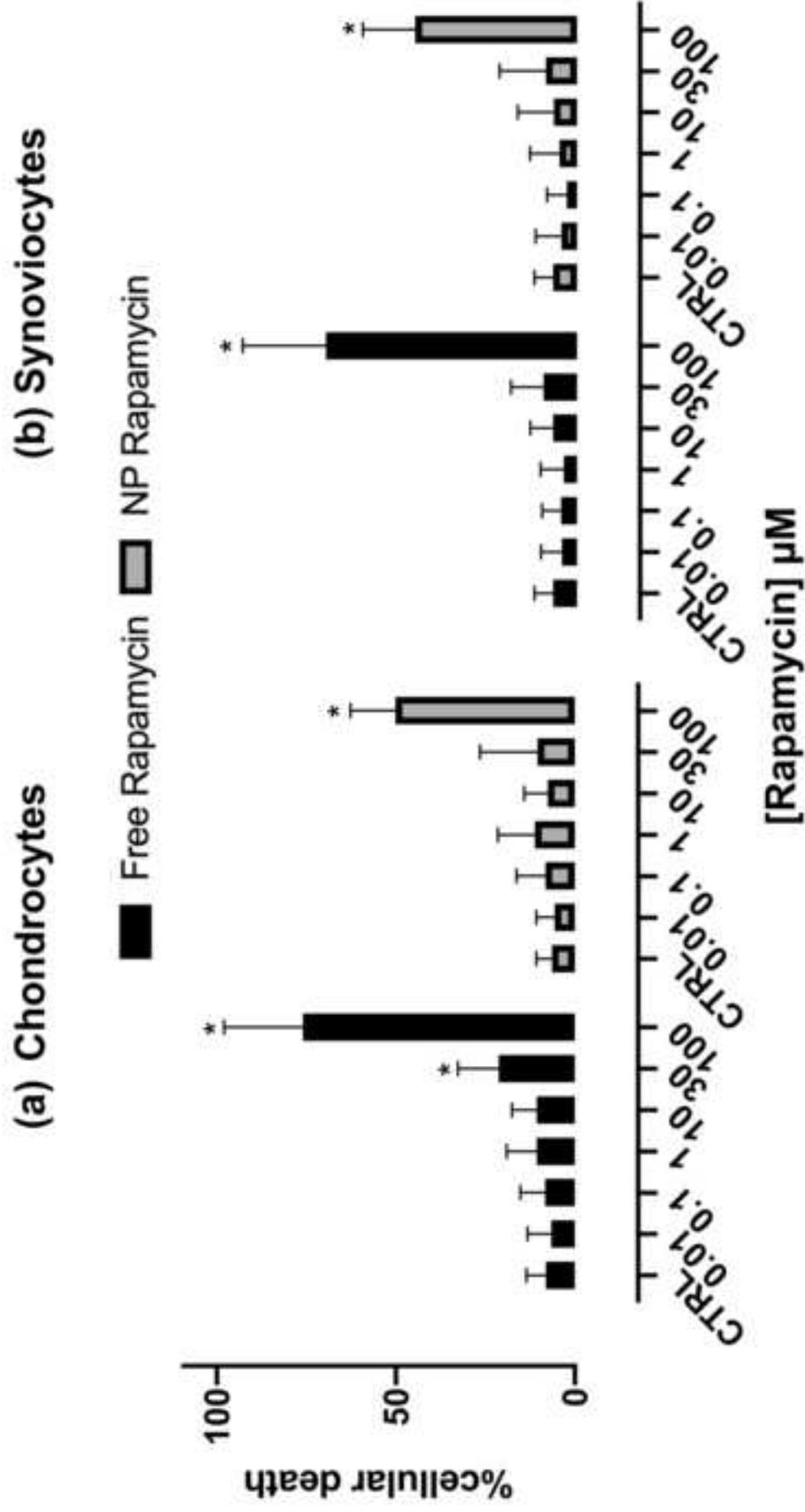


Figure 4. Articular cell viability with LDH assay.

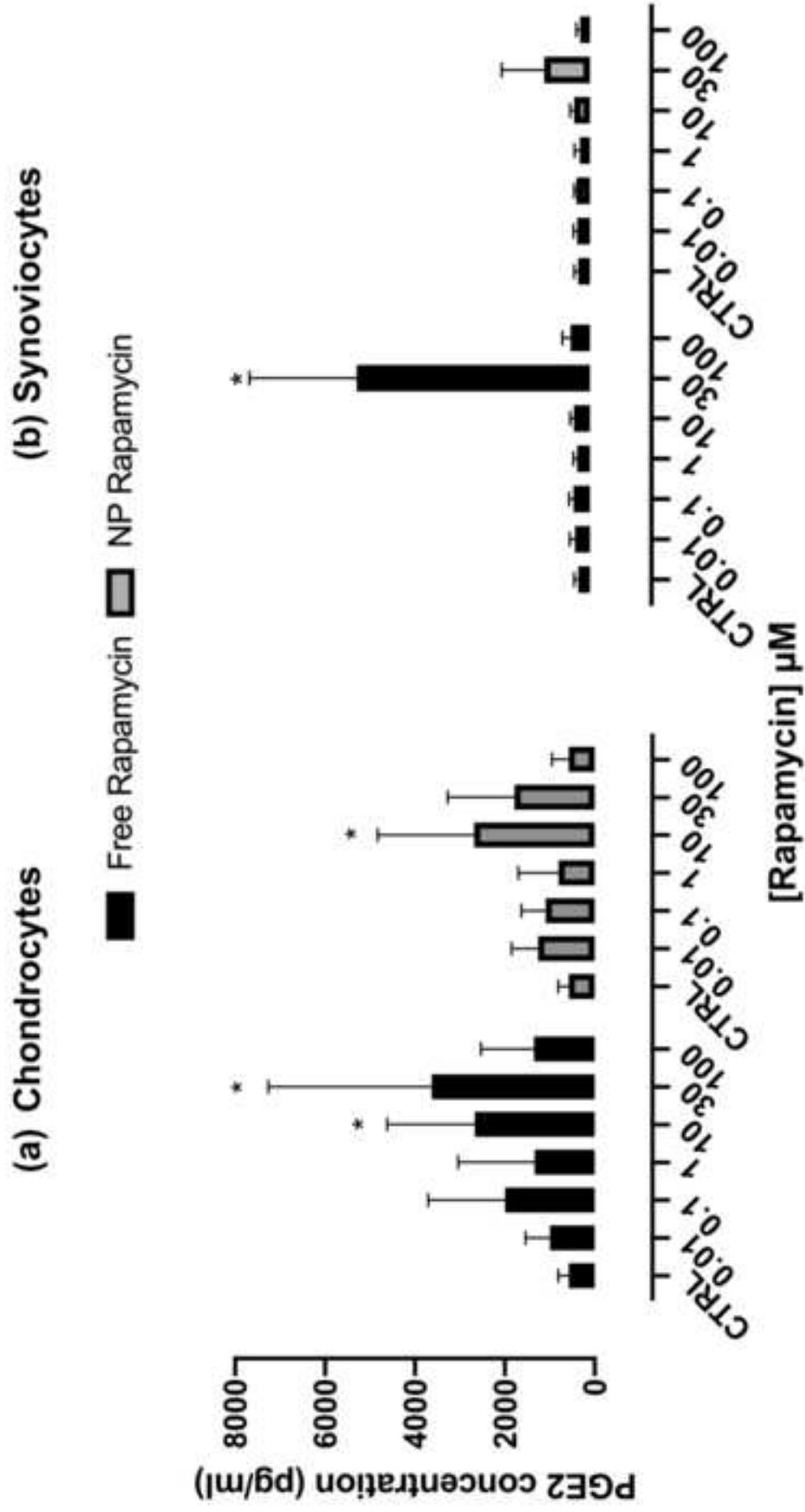


Figure 5. Phlogistic reaction to Rapamycin.

**Supplementary data 1: Non-exhaustive table summarizing preparation and characteristics about Rapamycin (RAPA)-loaded nanoparticles**

(Authors)	Preparation method	Size	Encapsulation efficiency	RAPA amount	Polymer (amount)	Clinical application
(Jhunjhunwala et al., 2009)	emulsion/solvent evaporation	3.4 ± 1.6 µm	73.9 ± 5.4%	1 mg	PLGA (200 mg)	Dendritic cell-based therapeutics
(Katiyar et al., 2016)	nanoprecipitation	151.6 nm	70.0%	1 mg	PLGA (10 mg)	Breast cancer
(Li et al., 2017)	nanoprecipitation	129 nm	63.7%	2 mg	PLGA acetonitrile solution (1mg/mL)	Hemangiomas
(Othman et al., 2016)	antisolvent precipitation	189±4 nm (drug/excipient 0.1); 218 ± 5 nm (drug/excipient 0.5)	98.8%	NA	PCL	NA
(Zhao et al., 2020)	emulsion/solvent evaporation	132.6±13.5 nm	EE% increased when polymer/rapamycin was 100:1: 77.9%	1 mg	PEG-PLGA (20 mg)	Hepatic steatosis, liver injury
(Segura-Ibarra et al., 2017)	emulsion/solvent evaporation	17.1±2.8nm	54.0±1.9%	NA	PEG-PCL,	Diseased lung vasculature ; PAH
(Shi et al., 2014)	emulsion/solvent evaporation	250 nm	69.1%	12 mg	PLGA (65 mg)	Vascular Stenosis
(Xie et al., 2019)	phase separation method	190 nm	86.6±2.4%	NA	NA	NA
(Zhang et al., 2014)	emulsion/solvent evaporation	169.3±1.2 (Drug to polymer feed ratio was 5%) ; 180.3 ± 3.6 (drug to polymer feed ratio 15%)	84.1±4.3 % (Drug to polymer feed ratio was 5%); 99.7±1.81 % (drug to polymer feed ratio was 15%)	NA	PCEC	Corneal transplantation
(Zou et al., 2009)	solvent displacement technique	256±11 nm	78.6 %	50 mg	PLGA (100 mg)	Restenosis

NA: not accessible

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