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Effect of particle size on the biodistribution of nano- and microparticles following intra-articular injection in mice

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ABSTRACT

Intra-articular (IA) injection of extended drug release forms based on biodegradable microparticles holds promise for the treatment of joint diseases. However, the fate of microparticles following intra-articular injection is controversial and has not been thoroughly investigated. The aim of this work was therefore to evaluate the biodistribution of fluorescent poly(lactic acid) particles of different sizes after IA injection in arthritic or healthy mice.

Regardless of the inflammatory status of the joint, 300 nm-nanoparticles leaked from the joint. Due to inflammation and related increase of vascular permeability, 3 μm-microparticles that were retained in the non-inflamed synovial membrane leaked from the inflamed joint. Complete retention of 10 μm-microparticles was observed independently of the joint inflammatory status. Embedding particles in a hyaluronic acid gel prolonged the retention of the formulations only in inflamed joints. Depending on particle's size, formulations were preferentially eliminated by blood vessels or lymphatic pathways. Poly (lactic acid) particles of 3 μm were biocompatible and retained in knee joints at least for 6 weeks.

This work highlights the need to deliver hyaluronic acid-embedded particles of at least 3 μm to guarantee their retention in inflamed joints. These results will contribute to the rational design of long-lasting formulations to treat acute and chronic joint diseases.

1. Introduction

Rheumatic diseases are painful conditions usually caused by inflammation. The inflammatory component can have variable degrees, such as in rheumatoid arthritis, or can be low as encountered in osteoarthritis (OA). Historically, treatments for chronic arthritic diseases were essentially focused on pain relief by oral dosage forms. However, the poor bioavailability of active drug substances in the joints and the major side effects that result from distribution in non-target organs do not allow to efficiently alleviate the symptoms of the patients. Viscosupplementation and delivery of the active drug substance directly to the site of action via intra-articular administration was demonstrated to be a promising strategy. In most studies, rapid drug clearance from the joint requires frequently repeated injections, which are at risk to induce infection (Albert et al., 2006). Thus, maintaining an effective local therapeutic concentration of the drug over longer time periods is needed. This can be achieved using IA delivery of biodegradable particles. Several studies report particles sizes ranging from nanometers (Rothenfluh et al., 2008) to hundreds of micrometers (Liggins et al., 2004) for IA administration (Horisawa et al., 2002a; Liggins et al., 2004; Monkkonen et al., 1995; Pradal et al., 2015; Ratcliffe et al., 1986; Rothenfluh et al., 2008; Setton, 2008). The formulation, technical and physiological parameters have to be taken into account to deliver sufficient therapeutic doses to the site of action. Concerning formulation parameters, administration of large particles may help reduce the amount injected and provide extended release properties. Additionally, drug encapsulation efficiency usually increases with the size of particles, which is an asset. Small particles are easier to inject, but

Abbreviations: AIA, antigen-induced arthritis; CTL, control; DiD, near-red $DiIC_{18}(5)$ fluorescent dye; HA, hyaluronic acid; PLA, poly(D,L)lactide; H&E, hematoxylin and eosin; OA, osteoarthritis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

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might be rapidly cleared from the joint cavity through fenestrations of the synovial membrane. The size of particles was shown to influence their fate in the joint (Horisawa et al., 2002a; Rothenfluh et al., 2008). To target cartilage or synovium, small nanoparticles should be formulated because larger particles would be phagocytized by macrophages (Rothenfluh et al., 2008). Nevertheless, these studies remain unclear about the optimal size of particles to be used for extended residence time in the joints. Specifically, it is still unclear in how far the joint inflammation itself may influence the biodistribution of nano- and microparticles.

In addition to selecting the appropriate size of particles, embedding them in a hyaluronic acid (HA) gel may prolong their persistence in the joints and provide viscosupplementation. Injections of HA gels are frequently done in patients suffering from OA to alleviate pain via a cushioning effect. Although positive results have been reported with the use of HA in OA patients (Bellamy et al., 2006), the long-term clinical efficacy of IA HA remains controversial (Arrich et al., 2005; Jubb et al., 2003).

The aim of the present study was to determine the optimal size of particles that are retained in the mouse joint in the presence or absence of inflammation. Poly(D,L-lactide) (PLA) nano- and microparticles containing DiD as a fluorescent dye were formulated and characterized. The compatibility of the particles with synovial fibroblasts was investigated *in vitro*. Then, particles were injected to antigen-induced arthritis (AIA) mice and to naïve mice. Using intravital fluorescence and fluorescence quantification in organs, persistence and leakage of particles from mice joints could be determined. The optimal size of particles was determined, taking into account the optional dispersion of particles in HA.

2. Materials and methods

2.1. Materials

Poly(D,L)-lactide (PLA, inherent viscosity 0.91 dl g^{-1}; molecular weight: 153 kDa; PI: 1.6 determined by GPC) (Resomer® R 207) was provided by Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). Hyaluronic acid (HA) (Intrinsic viscosity by Ubbelode viscometer: 2.76 m^3 kg^{-1}, molecular weight: 1.8 MDa) was purchased from HTL (Javène-Fougères, France).

DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) was purchased from Life Technologies (Grand Island, New York, USA). Poly(vinyl alcohol) (PVAL) was a gift from Clariant GmbH (Frankfurt, Germany) (Mowiol® 4-88, hydrolysis degree 88%, molecular weight: 26 kDa). Dichloromethane and acetone were of analytical grade. Sodium dodecylsulfate (SDS) and ethyl oleate were provided by Sigma–Aldrich (Saint Louis, Missouri, United States).

Human synovial fibroblasts were provided by the Division of Rheumatology from The University Hospitals of Geneva and were obtained from a 76-year-old patient undergoing joint replacement for osteoarthritis. Samples were obtained after appropriate informed consent, and their use for research was approved by the ethics committee of the University Hospital of Geneva. Cells were cultured in penicillin–streptomycin supplemented with DMEM according to an established protocol (Pradal et al., 2015). All cell experiments were carried out below passage 10. All cells were tested and maintained in mycoplasma-free conditions.

2.2. Formulation of particles

PLA nano- and microparticles were prepared by a solvent-evaporation method in the dark. PLA R207 (20 mg mL^{-1}) and DiD (2% w/w) were first dissolved in dichloromethane and then emulsified (homogenizer T25 Ultra-Turrax, IKA Labortechnik, Staufen, Germany) in a PVAL solution (2% w/v) at an organic/aqueous volume phase ratio of 1:3. Stirring speed and duration of the emulsification were adjusted to obtain particles of 300 nm, 3 μm and 10 μm. The freshly prepared emulsions were then poured in milliQ water and stirred at 500 rpm for 2 h (Eurostar Digital, IKA Labortechnik, Staufen, Germany).

Particles were centrifuged and washed once with PBS supplemented with 0.1% SDS. The particles were centrifuged a second time and washed with milliQ water. Blank particles, *i.e.*, without the fluorescent dye, were also prepared using the same method and parameters and were used as control formulations. HA-embedded particles were prepared by mixing particles suspended in PBS with an appropriate amount of concentrated HA gel at 3% (w/v) with a magnetic stirrer. The final concentration of HA was 0.6% (w/v) in these formulations.

2.3. Characterization of particles

The size of the microparticles was determined by laser light diffraction (Mastersizer S, Malvern Instruments Ltd., Malvern, UK). A Nano ZS (Malvern Instruments Ltd., Malvern, UK) was used to determine the size of nanoparticles. The Nano ZS was also used to assess the zeta potential of micro- and nanoparticles. DiD loading in particles was quantified by spectrofluorometry (Safire, Tecan, Männedorf, Switzerland) after dissolving the particles overnight in acetone.

DiD *in vitro* release kinetics from particles were carried out in sink conditions by suspending 20 mg of particles in 40 mL of PBS containing 0.5 % SDS (m/v), and also in ethyl oleate to mimic a physiologic and cell membrane environment, respectively. Liquid synovial fluid from patients suffering from arthritis was also used as a release medium. The *in vitro* release samples were placed on a see-saw rocking plate (60 rpm) at 25 °C. Homogenization of the vials before sampling was realized by upside down shaking. Aliquots of 50 μL were withdrawn and centrifuged at 26,500 × *g* using an Avanti 30 Centrifuge (Beckman, California, USA) before spectrofluorimetric assay of the supernatant. All experiments were performed in triplicate.

2.4. Viability tests

Toxicity of fluorescent particles was assessed by viability tests performed on synovial fibroblasts from OA patients. For these experiments, cells were deposited in a 96-well plate at a density of 30,000 cells/well. The following day, all aqueous suspensions of dye-loaded or blank particles, as well as particles dispersed in the HA gel, were freshly prepared and adjusted to concentrations of 1 mg/mL of polymer material. Then, 200 μL of the formulations were added to the cells for 24 h at 37 °C. Each experimental condition was replicated 5 times. Then, supernatants were carefully aspirated. Viability was assessed by MTT tests. To perform the tests, 50 μL of 0.1% MTT solution were added to the wells and left in contact for 3 h. Then, 200 μL of dimethyl sulfoxide was added before absorbance was measured at 595 nm.

2.5. Localization of particles in naïve mice and in mice with antigen-induced arthritis

All *in vivo* experiments were performed in compliance with the Swiss Federal Law on the Protection of the Animals. The protocols were accepted by the canton of Geneva Authority (Direction Générale de la Santé, Authorization number GE/4/14).

For the arthritis model, 56 C57Bl/6 male 8–10 week old mice (Charles Rivers, France) were divided into 8 groups of 7 mice each (Table 1). Inflammation was induced as previously described