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pH-sensitive polymeric micelles for targeted delivery to inflamed joints

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ABSTRACT

Effective treatment for rheumatoid arthritis is hindered by the lack of drugs that selectively target inflamed joints. Liposomes, nanoparticles and conventional micelles loaded with limited amounts of drugs may be unstable in the circulation and result in uncontrolled drug release kinetics. Here we developed a new drug delivery system of pH-sensitive polymeric micelles based on an acid-labile hydrazone bond. Amphiphilic conjugates of a PEG-based derivative and the hydrophobic drug prednisolone (PD) self-assembled into PD micelles with a drug loading of 19.29%. When the micelles reached the acidic environment of synovial fluid, the hydrazone bonds hydrolyzed, releasing free PD. Intravenous injection of PD micelles into mice with collagen-induced arthritis led to PD accumulation in affected joint tissues. PD concentrations in plasma and joints of arthritic mice were significantly higher after injection with PD micelles than after injection with free PD. The enhancement effect in joints was 4.63-fold based on the area under the concentration-time curve and 2.50-fold based on the maximum concentration (C_{max}). *In vivo* pharmacodynamics experiments showed PD micelles to have better anti-inflammatory and disease-modifying effects than free PD. Our results indicate the promise of PD micelles for targeted drug delivery in inflammatory disease.

1. Introduction

Rheumatoid arthritis, a chronic autoimmune disease of unknown etiology associated with significant morbidity and mortality, affects approximately 1% of the global population [1–4]. It is a chronic systemic inflammatory disease that destroys diarthrodial joints [5–7]. The inflamed synovium invades and destroys articular bone and cartilage, causing significant pain and disability [8].

Four classes of medications are used to treat rheumatoid arthritis: disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids, non-steroidal anti-inflammatory drug (NSAIDs) and immune-modulators. Glucocorticoids play a particularly important role in treating RA because they can rapidly control the disease and relieve the pains quickly [9,10]. However, most glucocorticoids are hydrophobic, resulting in poor bioavailability, and systemic administration leads them to distribute widely throughout the body, causing significant adverse effects including immune suppression, osteoporosis and diabetes. As a result, overall therapeutic efficacy and patient compliance with treatment can be poor [11].

Delivering glucocorticoids in a targeted way to inflamed tissue might enhance their therapeutic efficacy and reduce systemic side effects. To this end, drug-loaded liposomes, nanoparticles, polymeric micelles and other nanovehicles have been developed [12–14]. Systemically administered nanomedicines have been shown to accumulate selectively in arthritic joints through a process known as ELVIS: extravasation across leaky vasculature followed by sequestration by inflammatory cells. This effect allows encapsulated glucocorticoids to target inflamed tissue, improving efficacy and reducing their systemic toxicity [12,15]. ELVIS is analogous to the classical enhanced permeability and retention (EPR) effect, which allows encapsulated anticancer drugs to accumulate in solid tumors [16–19]. Liposomes are perhaps the most extensively studied nanovehicles for targeted delivery of glucocorticoids to inflamed joints in animal models of rheumatoid arthritis [20]. PEGylated liposomes are particularly attractive because hydrophilic PEG shows good solubility and low toxicity, immunogenicity and antigenicity [21]. However, liposomes have the significant drawback that because the hydrophobic drug is trapped within the liposomal bilayer, it is rapidly released upon lipid bilayer destabilization *in vivo*, leading to uncontrolled drug release [13].

To ensure retention of drug cargo while nanovehicles are in the systemic circulation, researchers have developed ways to attach (pro)drug covalently to the polymeric carrier [22]. The resulting polymer-drug conjugate micelles may present several advantages over liposomes and conventional micelles: they may resist disintegration in the systemic circulation, the micellar building blocks (unimers) may engage in fewer nonspecific interactions with blood components, they can be

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loaded with larger amounts of drug cargo, and they may allow more controlled drug release *in vivo* [18,21].

Although PEG is quite popular as a polymeric carrier, the fact that it has only one functional group on each end severely limits drug loading capacity in polymer-drug conjugates. In addition, PEG inhibits cellular uptake of micelles and liposomes and affects their intracellular trafficking, reducing drug efficacy. Here we designed and synthesized a new type of PEG-based polymeric micelles that would have high drug loading capacity, selectively target inflamed tissue and shed their PEG at the target site in order to allow more efficient intracellular drug accumulation [22]. Our strategy is based on the PEG derivative APN, in which a neutral, linear PEG backbone (3644 Da) provides a hydrophilic surface and hydrazine group pendants on branched chain of the PEG provide multiple, acid-sensitive attachment sites for drug cargo. The multiple drug attachment sites, the number of which can be adjusted based on the number of hydrazine groups present, and the acid-sensitive hydrazine-drug conjugation make APN superior to linear PEG and other polymeric carriers. The pH-sensitivity of the resulting polymer-drug conjugate means that the vehicle should release free drug in the acidic environment of inflamed joints. Local inflammatory reactions in and around joint tissues of patients with rheumatoid arthritis increase metabolic activity, leading to oxygen insufficiency and inducing a shift toward anaerobic glycolysis and lactate formation [8]. This acidifies the microenvironment; for example, pH values as low as 6.0 have been reported in the synovial tissue of patients with rheumatoid arthritis [23,24]. An analogous approach using pH-sensitive hydrazone bonds to load anticancer drugs into polymer-conjugate micelles [25,26] has been used to achieve targeted delivery to the acidic microenvironment of solid tumors [27]. Whether the same process can be effective for treating rheumatoid arthritis has not been reported.

Here we linked the widely used glucorticoid prednisolone (PD) to the hydrazine groups of APN *via* hydrazone bonds, creating the acid-sensitive APN-PD amphiphilic conjugate. This conjugate self-assembled into micelles, which selectively accumulated in the inflamed joints of a mouse model of rheumatoid arthritis after intravenous injection. The micelles released free drug selectively in the acidic environment of synovial fluid, reducing pannus formation and protecting bone to a greater extent than free PD.

2. Materials and methods

2.1. Materials and animals

2.1.1. Materials

Acetonitrile (HPLC grade) was purchased from Kemiou (Tianjin, China). All other chemicals and reagents were obtained commercially and were analytical grade. ^{1}H NMR analyses were performed on an AMX-400 Bruker Spectrometer. DID was obtained from Biotechnology Co. Ltd. (Tianjin, China). Bovine Type II collagen and complete or incomplete Freund's adjuvant were obtained from Chondrex (Washington DC, USA). ELISA kits to assay levels of mouse TNF-α, IL-1β and IL-6 were obtained from eBioscience (San Diego, CA, USA).

2.1.2. Animals

Male DBA/1 mice (6 weeks old, 20 ± 2 g) were purchased from Beijing Huafukang Co. Ltd. (Beijing, China), maintained in a germ-free environment and allowed free access to food and water. All animal experiments were approved by the Animal Ethical Experimentation Committee of Sichuan University and were carried out in accordance with the requirements the National Act on the Use of Experimental Animals (People's Republic of China).

2.2. Biosample preparation and HPLC chromatographic conditions

Samples of plasma, organs and joint tissue homogenates were prepared as described [28]. Methods to assay PD levels in biosamples using HPLC were designed based on the work of Yuan et al. [29]. Analyses were usually performed on a Shimadzu instrument (Chiyoda-Ku, Kyoto, Japan) equipped with a 50-μL injector loop, CTO-10A column thermostat, two LC-10AT pumps and an SPD-10A UV detector. Separations were carried out on a Diamonsil C_{18} reverse phase column (150 × 4.6 mm, 5 μm) protected by a Shimadzu Shim-Pack guard column. The flow rate was 1.0 mL/min, the temperature was 35 °C, and gradient elution was performed using a mobile phase of 50 mM trisodium citrate (adjusted to pH 4.10 with phosphoric acid) from 70% to 30%. The detector was set to monitor the signal at 254 nm, which corresponds to the absorption maximum of PD. The total analytical time was 7.2 min for the entire run. The retention time of PD under these conditions was 5.13 min.

2.3. Preparation of PD micelles

PD micelles were prepared by first generating the pH-sensitive APN-PD polymeric conjugate (5.08 kDa), purifying it on an LH-20 column, and then allowing it to self-assemble. Typically, 30 mg APN-PD was dissolved completely in 7.5 mL ultrapure water in a round flask, which was shaken for 30 min at 25 °C. This typically resulted in a solution of 4 mg/mL PD micelles. The amount of PD conjugated within the micelles (0.772 mg/mL, 19.29 wt%) was determined by HPLC following complete hydrolysis of the hydrazone bonds using 6 N HCl.

2.4. Characterization of PD micelles

The size and zeta potential of PD micelles ($n = 3$) in ddH_2O (pH 7.4) were measured using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, U.K.). PD micelles were also negatively stained and analyzed using transmission electron microscopy. The critical micelle concentration (CMC) was determined using fluorescence spectroscopy and pyrene as the hydrophobic fluorescent probe.

Release of PD from PD micelles was investigated *in vitro* using the dialysis method. Initially, 1 mL PD micelles (4 mg/mL) in quintuplicate were placed into dialysis bags with a molecular weight cut-off of 3500 Da (Sigma, USA). The five dialysis bags were immersed in acetate buffer at one of the following pH values: 5.5, 6.0, 6.5, 6.8 and 7.4. The bags were incubated at 37 °C with constant shaking at 100 rpm. At selected times, 300 μL of the outer dialysis solution was withdrawn for HPLC analysis, and the same amount of fresh buffer was added. Drug release was determined for all samples within 60 h ($n = 3$).

To examine the stability of PD micelles *in vitro*, micelles were stored at 4 or 37 °C over 120 h, then their size and polydispersity index (PDI) were measured. To examine the stability of PD micelles in serum, we adopted the approach of Nelson et al. [30]: first, we encapsulated DID into PD micelles; then we incubated the resulting DID/PD micelles with fresh murine whole blood at 37 °C for 0.5, 1, 1.5, 2, 4 or 24 h. Afterwards, the micelles were centrifuged at 1000 rpm for 10 min to remove any micelle aggregates and red blood cells. The percentage of total DID fluorescence that remained in soluble DID/PD micelles in the supernatant was quantified using a microplate reader (Thermo Scientific Varioskan Flash, Thermo Fisher Scientific, USA).

2.5. Tissue distribution and pharmacokinetics of free PD and PD micelles in a mouse model of collagen-induced arthritis

Male DBA/1 mice were acclimated to their housing for at least 1 week, and then subcutaneously injected at the base of the tail with 100 μL of an emulsion of 2 mL bovine type II collagen (1 mg/mL) in 2 mL of complete Freund's adjuvant (1 mg/mL). On day 21 after the first injection, animals received a booster injection in the tail, followed by subcutaneous injection at the base of the tail with 50 μL of an emulsion of 1 mL bovine type II collagen in 1 mL incomplete Freund's adjuvant. On day 50 after the first set of injections, an articular index (AI) score was calculated for the hind paws of each mouse. Scores were