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Controlled Release of an Anti-inflammatory Drug Using an Ultrasensitive ROS-Responsive Gas-Generating Carrier for Localized Inflammation Inhibition

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Supporting Information

ABSTRACT: Inflammation is associated with many diseases, in which activated inflammatory cells produce various reactive oxygen species (ROS), including H_2O_2. This work proposes an ultrasensitive ROS-responsive hollow microsphere (HM) carrier that contains an anti-inflammatory drug, an acid precursor consisting of ethanol and $FeCl_2$, and sodium bicarbonate (SBC) as a bubble-generating agent. In cases of inflamed osteoarthritis, the H_2O_2 at low concentration diffuses through the HMs to oxidize their encapsulated ethanol in the presence of Fe^{2+} by the Fenton reaction, establishing an acidic milieu. In acid, SBC decomposes to form CO_2 bubbles, disrupting the shell wall of the HMs and releasing the anti-inflammatory drug to the problematic site, eventually protecting against joint destruction. These results reveal that the proposed HMs may uniquely exploit biologically relevant concentrations of H_2O_2 and thus be used for the site-specific delivery of therapeutics in inflamed tissues.

Inflammation is associated with many pathological disorders, including infections, cancers, atherosclerosis, neurodegenerative diseases, and arthritis. Osteoarthritis (OA) is a common joint disease that causes joint inflammation.[1] The inflammatory responses in the OA joints depend greatly on their activated macrophages, which generate reactive oxygen species (ROS) intracellularly. When formed in excess, these ROS molecules may spill out and exhibit extracellular toxicity, degrading the extracellular matrix (ECM) of the articular cartilage.[2] Dexamethasone sodium phosphate (DEX-P), a synthetic corticosteroid, has been widely used to treat OA, as it relieves inflammation and reduces cartilage ECM loss.[3] Inflammatory responses in OA not only occur in the local areas of the articular joints but also influence their neighboring skeletal muscles.[4] In clinical practice, DEX-P is administered by local injection into the articular cavity[5] or the tibialis anterior muscles,[6] delivering the drug to the site of action to minimize undesired side effects. However, a major challenge in the local injection of a soluble drug such as DEX-P is its rapid clearance, which requires frequent administration of the therapeutic drug (at a rate of, for example, once daily for 3−6 weeks).[7] Therefore, the development of a controlled-release carrier that can deliver a therapeutic drug specifically to inflamed tissues is very desirable.

Poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) has been extensively utilized as a carrier material for drug delivery, but in the absence of a triggering mechanism, the release of a drug from a PLGA-based carrier is typically slow, resulting in a subeffective drug concentration.[8] During OA inflammation, the pH in joints may reach 6.6−7.1, while the local production of ROS is enhanced.[9] Acidification of inflamed joints is caused by the infiltration and activation of inflammatory cells that can actively pump lactic acid.[10] Additionally, the activated inflammatory cells generate various ROS. H_2O_2 is the precursor for the production of most ROS[11] and thus may be an important stimulus target in the design of a triggered drug release system.

Currently, few, if any, polymeric carriers are sufficiently sensitive to deliver bioactive agents selectively at biologically relevant concentrations of H_2O_2 (50−100 μM).[12] This work proposes an ultrasensitive ROS-responsive hollow microsphere (HM) carrier that can be injected and subsequently triggered upon exposure to such low concentrations of H_2O_2 (about 50 μM) to effectively deliver the encapsulated payload to inflamed tissues and protect against arthritis and joint destruction. The HMs proposed herein are fabricated using a microfluidic device in water-in-oil-in-water (W/O/W) double emulsions;[13] each has a shell of PLGA and an aqueous core that contains the anti-inflammatory drug DEX-P, an acid precursor consisting of ethanol and an iron(II) salt ($FeCl_2$), and sodium bicarbonate (SBC) as a bubble-generating agent. Ethanol can be oxidized using Fenton's reagent, which is a system of Fe^{2+} and H_2O_2, to form an acidic (CH_3COOH) solution.[14] Upon reaction with the acid, SBC decomposes to form bubbles of CO_2 gas.[15]

Figure 1 schematically depicts the composition and structure of a proposed ultrasensitive ROS-responsive HM and its mechanism of operation. To establish an OA animal model, an intra-articular injection of monosodium iodoacetate (MIA) through the infrapatellar ligament of the left knee in mice is conducted. The mice exhibit joint inflammation and produce cartilage degeneration with loss of ECM.[16] In the inflammatory environment, a low concentration of H_2O_2 diffuses through the PLGA shell of the HM to oxidize the encapsulated ethanol in

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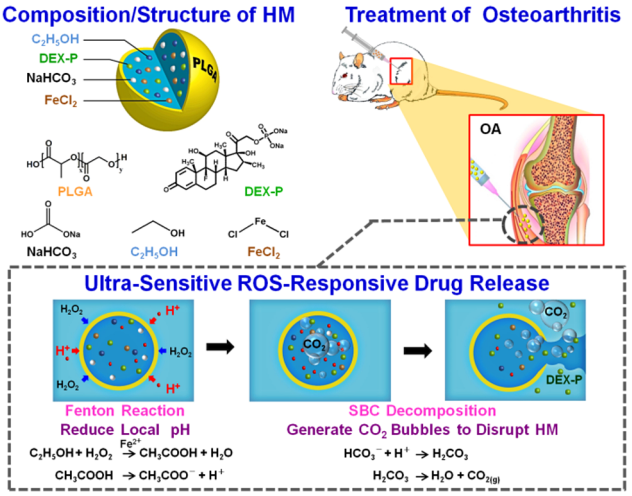

Figure 1. Composition/structure of the ultrasensitive ROS-responsive gas-generating HM developed herein and its mechanism in the treatment of OA.

the presence of Fe^{2+} by the Fenton reaction,[14] forming an acidic milieu (acetic acid). Next, the SBC in the HM reacts with the acid to form CO_2 bubbles, disrupting the PLGA shell and releasing DEX-P, providing a high dosage of the antiarthritic drug to the problematic region. The released DEX-P accumulates in the inflamed tissues in an amount that exceeds the therapeutic threshold, eventually exhibiting its anti-inflammatory activity.

To observe the distribution of the loaded drug in the as-prepared HMs, a hydrophilic fluorescent dye (Cy5) was used as a model drug because DEX-P does not fluoresce. Figure 2a displays a representative fluorescence micrograph of the resultant HMs. As shown, each of the monodispersed particles had a large cavity, with an external diameter of 344.2 ± 10.3 μm and a shell thickness of 20.3 ± 4.2 μm (n = 6 batches). The hydrophilic Cy5 (green fluorescence) was successfully

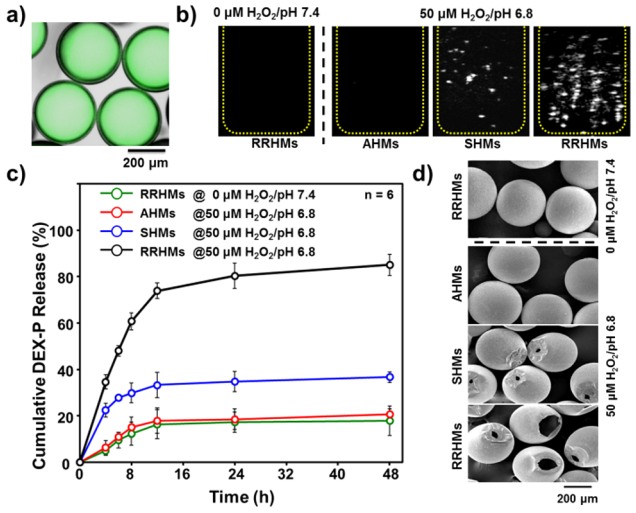

Figure 2. (a) Fluorescence micrograph of as-prepared HMs. (b–d) Characteristics of test HMs that were immersed in PBS at 37 °C in normal (0 μM H_2O_2/pH 7.4) and inflamed (50 μM H_2O_2/pH 6.8) joint environments: (b) ultrasound images showing generation of CO_2 bubbles; (c) release profiles of DEX-P; (d) SEM micrographs of morphologies of test HMs following the experiment.

encapsulated into the aqueous core of the PLGA HMs, suggesting that these particles can be used as a carrier system.

ROS production has been found to increase in cases of OA. The activated inflammatory cells within the inflamed joint can locally generate a low concentration of H_2O_2 (around 50 μM).[9] Moreover, inflamed tissues are frequently characterized by a reduced local pH with a relatively high H^+ concentration. To examine the ROS-responsive controlled release behavior of HMs containing DEX-P, SBC, ethanol, and $FeCl_2$, herein called ROS-responsive HMs (RRHMs), two other HMs were prepared as controls: one contained DEX-P and the acid precursor consisting of ethanol and $FeCl_2$ (AHMs), and the other contained DEX-P + SBC (SHMs). The amounts of DEX-P, SBC, ethanol, and $FeCl_2$ encapsulated in all of the test HMs (if ever present) were 5.2 ± 0.8, 5.7 ± 0.9, 1.3 ± 0.3, and 0.2 ± 0.1 μg/mg, respectively.

The ROS-responsiveness of the test HMs was evaluated in phosphate-buffered saline (PBS) in the presence of 50 μM H_2O_2, which is a prevalent ROS,[12] at an acidic pH of 6.8 and 37 °C to simulate the biological environment in OA. The conditions 0 μM H_2O_2/pH 7.4 were used to mimic the milieu of normal joints.[17] In the OA environment, both H^+ and H_2O_2 infiltrated the PLGA shell of the test HMs. Once the environmental acid (H^+) reached the aqueous core and reacted with the encapsulated SBC, CO_2 bubbles were promptly produced. The reaction of SBC with an acid yields a salt and carbonic acid (H_2CO_3),[8] which readily decomposes to CO_2 and water (Figure 1). Since the pK_a of bicarbonate (HCO_3^-) is 6.4 at 37 °C,[18] the reaction of SBC in acid depends on the pH. At a pH less than the pK_a, the carbonic acid in water is present mostly in its protonated form (H_2CO_3), generating more CO_2 bubbles.[8] However, the infiltrated H_2O_2 oxidizes the loaded ethanol in the presence of Fe^{2+} (Fenton's reagent) to form acetic acid,[14] establishing an acidic milieu.

The ROS-responsive characteristics of the test HMs were elucidated by examining their formation of CO_2 bubbles using an ultrasound imaging system. According to Figure 2b, the AHMs (which did not contain SBC) yielded no gas bubbles in the inflammatory environment (50 μM H_2O_2/pH 6.8), but a few gas bubbles were detected in the sample with the SHMs (which contained SBC). In contrast, many CO_2 bubbles were observed from the RRHMs (which contained SBC and the acid precursor consisting of ethanol and Fe^{2+}) within 1 h of the reaction. This observation was made probably because the environmental H_2O_2 infiltrated the RRHMs and reacted with their loaded acid precursor via the Fenton reaction to generate a more acidic milieu (approximately pH 6.3, which is less than the pK_a of bicarbonate) than that in the SHMs (without the acid precursor, pH 6.8), producing a dramatically larger number of bubbles of CO_2 gas, revealing the ultrasensitive ROS responsiveness of the RRHMs upon exposure to a low concentration of H_2O_2 (50 μM). A colorimetric analysis revealed that the degrees of SBC decomposition were around 20% and 85% at pH 6.8 and pH 6.3, respectively, after the reaction.

Notably, no gas bubbles were formed when the RRHMs were present in the normal tissue milieu (0 μM H_2O_2/pH 7.4). These empirical results suggest that the RRHMs developed herein can distinguish between normal and diseased tissues, as is required to ensure a favorable treatment efficacy.

The effectiveness of each type of test HM for drug delivery was studied. Figures 2c and S1 plot the cumulative DEX-P release profiles from various test HMs in the milieus of normal

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and inflammatory joints. Without CO_2 formation, a relatively small proportion of DEX-P [ca. 21%, equivalent to a local DEX-P concentration of 1.4 μg/mL, which was nontoxic to the cells (Figure S2c)] was released from the AHMs in the inflammatory milieu, indicating that the diffusion of DEX-P through the PLGA shell walls was relatively slow. In contrast, significant amounts of the encapsulated DEX-P were released from the SHMs (ca. 33%; 2.4 μg/mL) and RRHMs (ca. 85%; 6.4 μg/mL), probably triggered by the formation of CO_2 gas within them upon SBC decomposition. When the pressure of CO_2 reached a certain value, the PLGA shells of the test HMs became disrupted and pores were created (Figure 2d), causing the DEX-P to be unloaded locally. Since many more CO_2 bubbles were generated from the RRHMs (induced by both local H_2O_2 and acid) than from the SHMs (triggered by local acid only), the pores created in the former were larger than those in the latter. Therefore, the amount of DEX-P released from the RRHMs greatly exceeded that from the SHMs. The therapeutic concentration of DEX-P for treating OA has been reported to be about 3 μg/mL.[19]

Notably, the percentage of DEX-P released from the RRHMs was lower in the normal milieu (ca. 18%) than in the inflammatory environment (ca. 85%). These experimental results suggest that the RRHMs proposed in this study can distinguish between the environments of normal and inflamed tissues, releasing different amounts of drug accordingly.

Given the promising in vitro results (Figures S2 and S3; see the Supporting Information for a brief discussion), the efficacy of the test HMs in treating OA was evaluated in a mouse model. Three days following the induction of OA, the mice were separated into five groups, and each was treated by direct local injection of one of the following substances; saline (untreated control), free DEX-P (1 mg/kg), AHMs, SHMs, and RRHMs. At the end of the treatments, a bioluminescent probe, L-012, was intravenously administered to detect ROS associated with inflammation in living mice using an in vivo imaging system (IVIS). As shown in Figure 3, a very strong

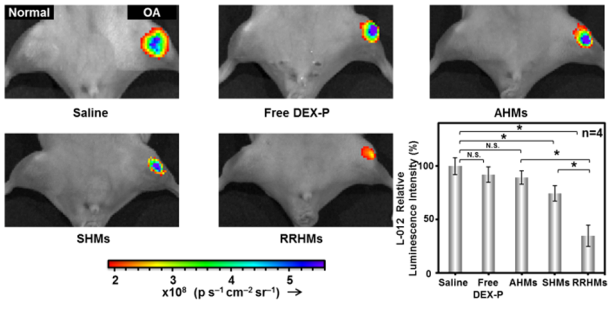

Figure 3. Anti-inflammatory effects of free DEX-P and test HMs in mice with experimentally created OA in their left knees, as shown by IVIS images and relative fluorescence intensities revealing the extent of inflammation in each studied group following treatment. N.S.: not significant. *: statistically significant ($P < 0.05$).

bioluminescent signal from each mouse in the untreated control group (saline) was detected, verifying the induction of the inflamed OA. After the mice with OA had been treated with free DEX-P or AHMs, the reduction of local ROS was insignificant ($P > 0.05$). Following the injection of free DEX-P in solution, the DEX-P rapidly lost its bioactivity because of systemic clearance, while the diffusion of the drug through the AHMs was slow because of the absence of a triggering mechanism (Figure 2c). Some reduction of local ROS, but to a still suboptimal extent, was detected in the group that received the SHMs ($P < 0.05$), probably because the concentration of the drug that was released locally (induced only by the local pH) did not reach the therapeutic threshold. In contrast, the total number of photons emitted from the inflamed knees that were treated with the RRHMs was significantly smaller ($P < 0.05$). This observation suggests that the RRHMs that were injected into the inflamed tissues were induced (by the local H_2O_2 and pH) to release most of their loaded drug (Figure S4) so that the OA inflammation was markedly reduced (Figure S5; see the Supporting Information for a brief discussion).

OA is characterized by the progressive destruction of articular cartilage,[20] which is degraded by the gradual loss of its ECM, including proteoglycan, aggrecan, and collagen type X.[21] As shown in Figure 4, the surface of the normal control

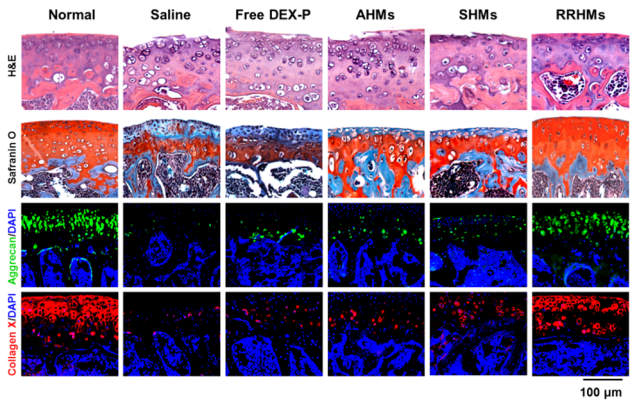

Figure 4. Efficacies of free DEX-P and test HMs in treating OA, as shown by cartilage sections stained with H&E or safranin O and their corresponding immunofluorescence staining sections, revealing intensities of aggrecan and collagen type X.

cartilage was ordered and smooth [hematoxylin−eosin (H&E) staining]; additionally, proteoglycans (safranin O staining)[21] as well as aggrecan and collagen type X (immunofluorescence staining) were uniformly distributed in the cartilage ECM. In the untreated control group, the surface of the articular cartilage was irregular, and proteoglycans, aggrecan, and collagen type X were weakly expressed in the ECM, suggesting the progression of osteoarthritic changes. Following treatment with free DEX-P or test HMs, and in particular with RRHMs, these osteoarthritic changes were reduced. In the group that was treated with these ultrasensitive ROS-responsive HMs, the superficial layer of the cartilage was smooth, and the surface integrity was not disrupted. Furthermore, most of the articular cartilage revealed strong expressions of proteoglycans, aggrecan, and collagen type X, suggesting that the cartilage ECM had not been degraded.

The above results reveal that the RRHMs, which respond well to low concentrations of the biologically relevant ROS, exhibit highly effective local anti-inflammatory activity in the treatment of OA. Like inflammatory diseases, many pathological diseases involve the overproduction of ROS. The ultrasensitive ROS-responsive HMs developed in this work may uniquely exploit the low concentrations of H_2O_2 found in biological environments and be used for the site-specific delivery of therapeutics to diseased tissues.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental details and additional data and figures (PDF)

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Notes

The authors declare no competing financial interest.

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