A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition

GDCh

Chemie

www.angewandte.org

Accepted Article

Title: In Situ Depot for Continuous Evolution of Gaseous H2 Mediated by Magnesium Passivation/Activation Cycle for Treating Osteoarthritis

Authors: Wei-Lin Wan, Yu-Jung Lin, Po-Chien Shih, Yu-Ru Bow, Qinghua Cui, Yen Chang, Wei-Tso Chia, and Hsing-Wen Sung

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201806159
Angew. Chem. 10.1002/ange.201806159

Link to VoR: http://dx.doi.org/10.1002/anie.201806159
http://dx.doi.org/10.1002/ange.201806159

WILEY-VCH

10.1002/anie.201806159

WILEY-VCH

In Situ Depot for Continuous Evolution of Gaseous H_2 Mediated by Magnesium Passivation/Activation Cycle for Treating Osteoarthritis

Wei-Lin Wan[†][a], Yu-Jung Lin[†][a], Po-Chien Shih[a], Yu-Ru Bow[a], Qinghua Cui[a], Yen Chang[b], Wei-Tso Chia*[c], Hsing-Wen Sung*[a]

Abstract: Inflammation is involved in many human pathologies, including osteoarthritis (OA). Hydrogen (H_2) is known to have anti-inflammatory effects; however, the bioavailability of directly administered H_2 gas is typically poor. Herein, a local delivery system that can provide a high therapeutic concentration of gaseous H_2 at inflamed tissues is proposed. The delivery system comprises poly(lactic-co-glycolic acid) microparticles that contain magnesium powder (Mg@PLGA MPs). Mg@PLGA MPs that are intra-muscularly injected close to the OA knee in a mouse model can act as an *in situ* depot that can evolve gaseous H_2 continuously, mediated by the cycle of passivation/activation of Mg in body fluids, at a concentration that exceeds its therapeutic threshold. The analytical data that are obtained in the biochemical and histological studies indicate that the proposed Mg@PLGA MPs can effectively mitigate tissue inflammation and prevent cartilage from destruction, arresting the progression of OA changes.

Inflammation is closely involved in many human pathologies.[1] As an example, it has a critical role in the onset and progression of osteoarthritis (OA), resulting in morphological and histological changes to articular cartilage.[2] Studies have demonstrated that inflammation in OA pathology is significantly related to the activated macrophages, which generate reactive oxygen species (ROS) in excess, causing chondrocyte apoptosis.[3] Moreover, numerous pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are known to be up-regulated during OA progression, enhancing both the mRNA and protein expression levels of matrix metalloproteinases (MMPs), such as MMP-9 and MMP-13, leading to the degradation of cartilage extracellular matrices (ECM).[4]

Hydrogen (H_2) is considered to be a therapeutic medical gas with anti-inflammatory effects.[5] In diseased cells, it selectively reduces highly cytotoxic oxidative radicals, such as hydroxyl radical and peroxynitrite; nevertheless, it neither affects the metabolic oxidation−reduction reactions in normal cells nor disturbs the physiological ROS that participate in cell signaling.[6] H_2 is nontoxic even at high concentrations.[7] The effectiveness of H_2 gas against several inflammatory disorders has been demonstrated in animal models following the direct inhalation of H_2-gas, the oral uptake of H_2-water, or the intravenous injection of H_2-saline.[8] However, its bioavailability in body fluids and at inflammatory sites in these methods is typically poor, because the solubility of H_2 in aqueous solutions is very low (*ca.* 0.8 mM).[9] This limitation may be overcome by using a local delivery system that is able to continuously provide a high therapeutic concentration of gaseous H_2 in diseased tissues.

Owing to their inherent biodegradability and bioabsorbability, magnesium (Mg) and its alloys are extensively used to develop implantable cardiovascular stents and bone-fracture fixation devices.[10] Electrochemically, Mg metal is often used as an anode material, owing to its low standard potential.[11] Mg implants are susceptible to corrosion and can be degraded in aqueous media, evolving H_2 bubbles and forming magnesium hydroxide [$Mg(OH)_2$] on their surfaces, which can then be transformed into soluble $MgCl_2$ by reaction with the chloride (Cl^-) ions in body fluids.[12] This pitting corrosion mechanism promotes the anodic dissolution of Mg, causing continuous evolution of H_2 bubbles, but generating excess OH^- ions, ultimately alkalizing the local environment. The prompt evolution/accumulation of H_2 bubbles may produce gas pockets adjacent to the implants, leading to the necrosis of tissues, whereas the associated alkalization of the local environment may cause the death of cells, reducing the biocompatibility of the Mg implants.

To address the above challenges, a poly(lactic-co-glycolic acid) (PLGA) microparticle system containing Mg powder (Mg@PLGA MPs), which can be injected intra-muscularly close to the OA knee and can serve as an *in situ* depot to evolve H_2 gas with sustained release to inflamed tissues, is proposed herein (Figure 1). The hydrophobic PLGA may impede the infiltration of water into the Mg@PLGA MPs and thereby limit the rate of H_2 evolution. Furthermore, the degradation of PLGA by hydrolysis generates lactic and glycolic acids,[13] which may be used to buffer alkalization upon dissolution of its encapsulated Mg. The cycle of passivation/activation of Mg can evolve

[a] W.-L. Wan, Dr. Y.-J. Lin, P.-C. Shih, Y.-R. Bow, Dr. Q. Cui, Prof. H.-W. Sung
Department of Chemical Engineering and Institute of Biomedical Engineering, Frontier Research Center on Fundamental and Applied Sciences of Matters
National Tsing Hua University
Hsinchu, 30013 Taiwan (ROC)
E-mail: hwsung@mx.nthu.edu.tw
[b] Dr. Y. Chang
Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation and School of Medicine
Tzu Chi University
Hualien, 97004 Taiwan (ROC)
[c] Dr. W.-T. Chia
Department of Orthopaedics
National Taiwan University Hospital, Hsinchu Branch
Hsinchu, 30059 Taiwan (ROC)
E-mail: H56009@hch.gov.tw
[†] W.-L. Wan, Dr. Y.-J. Lin contributed equally to this work.

Supporting information for this article is given via a link at the end of the document.

gaseous H_2 continuously to the OA knee at a high concentration that exceeds the therapeutic threshold, alleviating tissue inflammation and protecting against cartilage destruction.

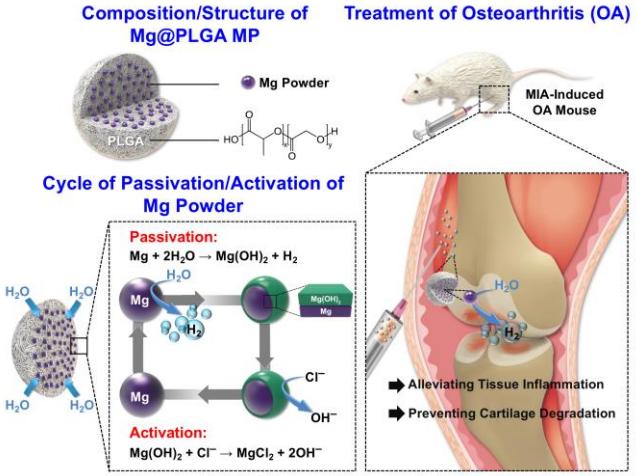


Figure 1. Composition/structure of as-proposed Mg@PLGA MPs and mechanism of their evolution of H_2 gas *in situ*, suppressing tissue inflammation and preventing degradation of cartilage in mice with OA.

The Mg@PLGA MPs herein were fabricated using a microfluidic system in an oil-in-water (O/W) single emulsion, in which the water phase contained poly(vinyl alcohol) (PVA) and the oil phase comprised PLGA and dispersed Mg. The formulation of Mg@PLGA MPs was optimized, with respect to its physiochemical characteristics, by tuning the water-to-oil volumetric ratio. According to Table S1, a higher water-to-oil volumetric ratio favored the production of Mg@PLGA MPs with a greater loading efficiency/content. However, when the water-to-oil volumetric ratio was increased to 1.0:0.1, pores began to form on the surface of the MPs (indicated by red arrowheads in Figure 2a); the porosity of MPs markedly increased the amount of infiltrating water upon exposure to a cell culture medium, which reacted with the encapsulated Mg, eventually greatly increasing the local pH (*ca*. pH 8.0, Figure 2b). Therefore, the MPs that were fabricated at a water-to-oil volumetric ratio of 1.0:0.05, which had a local pH of *ca.* 7.4, were used in the subsequent studies.

The resulting emulsified particles were transparent, as seen in the optical images, clearly revealing their encapsulated Mg (Figure 2c). As the solvent had evaporated off, the emulsified particles solidified; the as-obtained particles were found to be spherical (Figure 2d) with a size of 158.3 ± 9.6 (empty PLGA MPs) and 256.1 ± 24.3 μm (Mg@PLGA MPs).

The dissolution of Mg in cell culture medium increased the local pH, possibly affecting cell viability. The cytotoxicity of free Mg and the MPs that contained equal amounts of Mg (Mg@PLGA MPs) at varying concentrations was evaluated by incubating them with a mouse macrophage cell line (RAW264.7) using a WST-1 assay; the pH variation in the culture medium was monitored concurrently. The control was a cell culture that received no test samples. Notably, a gradual drop in pH in the control medium was observed, likely because of its generation of lactic acid as a by-product of cell metabolism (Figure 2e). However, a significant increase in pH in the medium that contained free Mg was detected, owing to the evolution of gaseous H_2 and the release of OH^- ions (Figure 1). The pH of the medium incubated with Mg@PLGA MPs remained *ca.* 7.5 to 7.0 throughout this study as the acidic by-products of PLGA degradation neutralized the locally alkalized Mg dissolution. The cytotoxicity of free Mg at 6 h exposure was negligible; however, at 24 h, the viability of the cells decreased markedly as the Mg concentration increased ($P < 0.05$). In contrast, Mg@PLGA MPs did not significantly influence cell viability up to a concentration of 10 mg mL^{-1} ($P > 0.05$). Based on these results, a concentration of Mg@PLGA MPs at 10 mg mL^{-1}, which contained 0.8 mg mL^{-1} Mg, was used in subsequent experiments.

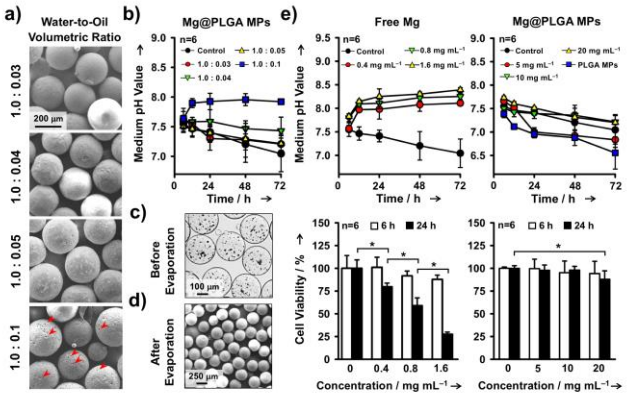


Figure 2. (a) SEM images of Mg@PLGA MPs fabricated with various water-to-oil volumetric ratios and (b) their effects on pH of culture-medium when co-cultured with RAW264.7 cells. (c) Bright field and (d) SEM images of Mg@PLGA MPs before and after solvent evaporation, respectively. (e) Changes in pH of culture-medium and cell viability for RAW264.7 cells that were incubated with free Mg or Mg@PLGA MPs. *$P < 0.05$.

The evolution of H_2 bubbles from the test Mg and Mg@PLGA MPs was examined under an optical microscope by immersing free Mg and the MPs individually in deionized (DI) water or phosphate-buffered saline (PBS), which is a buffer solution that contains sodium chloride (NaCl) and is frequently used in biological research.[14] The profiles of the evolved gaseous H_2 (in % of atmospheric concentration) were obtained by gas chromatography. Upon immersion of free Mg into DI water, an immediate evolution of H_2 bubbles was observed (indicated by red arrowheads); however, the oxide layer that was formed on the surface of Mg subsequently passivated the H_2 evolution (Figure 3a). Conversely, when free Mg or Mg@PLGA MPs were immersed in PBS, the Mg–water reaction was allowed to proceed, mediated by the cycle of passivation/activation of Mg (Figure 1), resulting in continuous evolution of H_2 bubbles. As presented in Figure 3b, free Mg in PBS initially evolved a large amount of H_2, before the rate of evolution rapidly decreased, whereas the Mg@PLGA MPs yielded a sustained H_2-evolution profile, suggesting their therapeutic potential. Owing to its small

molecules, gaseous H_2 can readily permeate cellular membranes to scavenge effectively the ROS and pro-inflammatory cytokines that are overproduced intracellularly.[9]

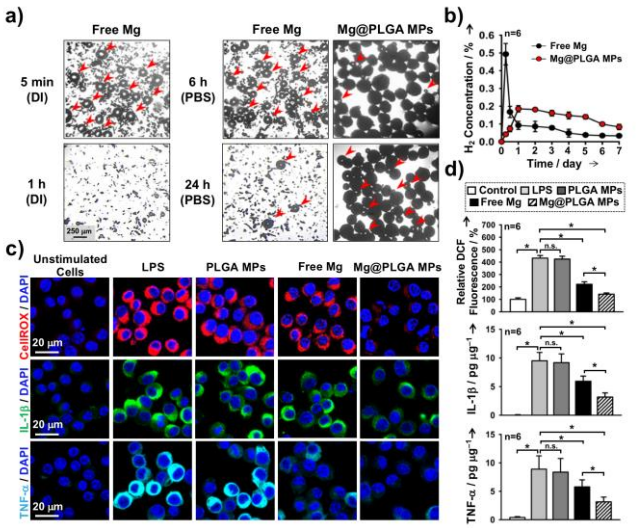


Figure 3. (a) Bright field images of H_2 bubbles evolved from free Mg or Mg@PLGA MPs in DI water or PBS. (b) Profiles of gaseous H_2 evolved from free Mg or Mg@PLGA MPs in PBS. (c) CLSM images of cellular ROS, IL-1β, and TNF-α and (d) their corresponding intensities in LPS-stimulated RAW264.7 cells following various treatments. *P < 0.05; n.s.: not significant.

The therapeutic potential of Mg@PLGA MPs in mitigating the inflammatory responses of RAW264.7 cells stimulated by lipopolysaccharide (LPS) for 6 h, at which time the ROS level peaked, was investigated *in vitro*. The groups that were treated with empty PLGA MPs or free Mg were used as controls. The concentrations of empty PLGA MPs and free Mg that were used in the study were equivalent to those in Mg@PLGA MPs. The production of ROS in LPS-stimulated RAW264.7 cells was qualitatively studied using a cell-permeable fluorogenic probe of ROS and quantitatively measured using DCFH-DA dye.[5] The expressions of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in such cells were measured by enzyme-linked immunosorbent assay (ELISA), while double immunohistochemistry staining versus nucleus was carried out to visualize their intracellular intensities. Photomicrographs were taken by confocal laser scanning microscopy (CLSM).

According to Figures 3c, 3d, and S1, LPS stimulation caused a marked overproduction of ROS and induced the excess expressions of pro-inflammatory cytokines in RAW264.7 cells relative to those in unstimulated cells. The free Mg and Mg@PLGA MPs that evolved H_2 bubbles considerably reduced the LPS-induced overproductions of ROS and pro-inflammatory cytokines (P < 0.05), while empty PLGA MPs failed to do so (P > 0.05). The amounts of ROS and pro-inflammatory cytokines in cells were more significantly reduced by treatment with Mg@PLGA MPs than by treatment with free Mg (P < 0.05). These analytical results indicate that Mg@PLGA MPs effectively suppress the inflammatory responses of LPS-stimulated macrophages, and so may have the potential to treat OA.

The feasibility of using Mg@PLGA MPs to suppress tissue inflammation and protect against cartilage destruction was examined separately in mice with OA that had been experimentally induced by monosodium iodoacetate (MIA) following intra-patellar injection. MIA injection into the mouse knee causes biochemical and histological pathologies that resemble human OA in a biphasic manner with an early (0–7 days) phase and a late (7–14 days) phase.[15] The early phase of the OA is characterized by tissue inflammation at the knee joint and the local overproductions of ROS and pro-inflammatory cytokines, while the late phase involves the apoptosis of chondrocytes and the degradation of cartilage with loss of ECM.[16]

Previous studies have identified robust tissue inflammation on day 1 following the administration of MIA.[17] Accordingly, one day following OA induction, the mice were randomly divided into four groups–the untreated control group (MIA) and three therapy groups (empty PLGA MPs, free Mg, and Mg@PLGA MPs)–for treatment. The early inflammatory responses and histologic changes to MIA-injected joints in each studied group were studied on day 4 following treatment.

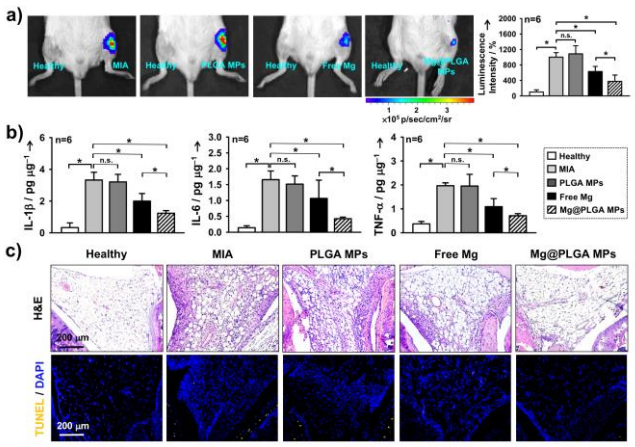


Figure 4. (a) IVIS images and corresponding L-012 intensities of ROS and (b) levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in MIA-stimulated knee joints following various treatments and (c) photomicrographs of their synovial tissue sections stained by H&E or TUNEL. *P < 0.05; n.s.: not significant.

Changes in ROS level in the inflamed joints were evaluated noninvasively by a luminescent probe (L-012) using an *in vivo* imaging system (IVIS). According to Figure 4a, the intensity of the luminescent signals that were emitted from the joints of the untreated control group (MIA) considerably exceeded that of the signals that were emitted from the healthy joints (P < 0.05), verifying the MIA-induced overproduction of ROS. The empty PLGA MPs did not significantly reduce the strength of the luminescent signal that was induced by MIA (P > 0.05). Conversely, the reduction of the detected luminescence intensity in the group that received Mg@PLGA MPs was greater than that in the group received free Mg (P < 0.05).

Figure 4b demonstrates that treatment with Mg@PLGA MPs markedly attenuated the MIA-induced expressions of pro-

inflammatory cytokines (IL-1β, IL-6, and TNF-α) below those following treatment with free Mg ($P < 0.05$). Histological examinations of synovial tissue sections that were stained with hematoxylin–eosin (H&E) also revealed the superiority of Mg@PLGA MPs over free Mg, by revealing lesser infiltration of inflammatory cells (Figure 4c). As indicated by the TUNEL staining, no significant cell apoptosis was detected in any case at this time, suggesting that the localized alkalization that was caused by Mg dissolution may be controlled by the buffering system of the animal body. These experimental results reveal the effectiveness of Mg@PLGA MPs in reducing the excess oxidative stress and pro-inflammatory cytokines in the early phase of OA inflammation.

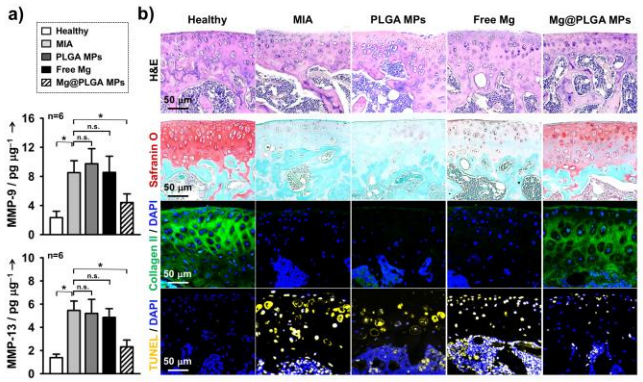


Figure 5. (a) Levels of MMP-9 and MMP-13 in MIA-stimulated knee joints following various treatments and (b) photomicrographs of their cartilage sections stained by H&E, safranin O, collagen type II, or TUNEL. *$P < 0.05$; n.s.: not significant.

To investigate further the therapeutic effects of Mg@PLGA MPs on the protection of articular cartilage against OA inflammation in its late phase, three repeated administrations of each test sample at four-day intervals (day 0, day 4, and day 8) were performed. At the end of the repeated treatments (on day 12), the animals were sacrificed, and their cartilage was retrieved and processed to quantify their expressed MMP levels and analyze histological lesions. In the evaluation of therapeutic efficacy, the expression levels of MMPs reflect the severity of OA.[4]

The expression levels of MMP-9 and MMP-13 in the untreated MIA-induced mice were significantly up-regulated relative to those of healthy mice ($P < 0.05$). Following repeated treatments, only the group that had received Mg@PLGA MPs exhibited significantly attenuated up-regulation of MMP levels ($P < 0.05$, Figure 5a). Histologically, the surface of the articular cartilage in the untreated control group (MIA) was rough, and its proteoglycans and collagen type II were weakly expressed, indicating that its cartilage ECM had been markedly degraded. In contrast, in the group that had received Mg@PLGA MPs, the surface of the cartilage was smooth with strong expression of ECM macromolecules, indicative of arrested progression of OA of the knee. Repeated treatments with free Mg might have overwhelmed the buffering capacity of the physiological environment, resulting in local alkalization, eventually causing broad chondrocyte apoptosis, as indicated by the TUNEL staining.

In summary, the above results clearly demonstrate that the Mg@PLGA MPs that are proposed herein effectively alleviate OA inflammation in mice and prevent the destruction of their cartilage. These encouraging results most likely follow from the fact that the Mg@PLGA MPs act as an *in situ* depot that can continuously evolve gaseous H_2, mediated by the cycle of passivation/activation of Mg, at a concentration that exceeds its therapeutic threshold.

Acknowledgements

This work was supported by the Ministry of Science and Technology (MOST-106-2119-M-007-007) of the Republic of China, Taiwan.

Keywords: osteoarthritis • hydrogen gas • medical gas • tissue inflammation • magnesium

[1] a) H. L. Pu, W. L. Chiang, B. Maiti, Z. X. Liao, Y. C. Ho, M. S. Shim, E. Y. Chuang, Y. Xia, H. W. Sung, *ACS Nano* **2014**, 8, 1213–1221; b) Y. S. Bae, J. H. Lee, S. H. Choi, S. Kim, F. Almazan, J. L. Witztum, Y. I. Miller, *Circ. Res.* **2009**, 104, 210–218.

[2] F. Berenbaum, *Osteoarthritis Cartilage* **2013**, 21, 16–21.

[3] F. J. Blanco, A. M. Valdes, I. Rego-Pérez, *Nat. Rev. Rheumatol.* **2018**, 14, 327–340.

[4] a) P. G. Mitchell, H. A. Magna, L. M. Reeves, L. L. Lopresti-Morrow, S. A. Yocum, P. J. Rosner, K. F. Geoghegan, J. E. Hambor, *J. Clin. Invest.* **1996**, 97, 761–768; b) N. Gerwin, C. Hops, A. Lucke, *Adv. Drug Deliv. Rev.* **2006**, 58, 226–242.

[5] W. L. Wan, Y. J. Lin, H. L. Chen, C. C. Huang, P. C. Shih, Y. R. Bow, W. T. Chia, H. W. Sung, *J. Am. Chem. Soc.* **2017**, 139, 12923–12926.

[6] I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S. Asoh, S. Ohta, *Nat. Med.* **2007**, 13, 688–694.

[7] L. Huang, *Med. Gas Res.* **2016**, 6, 219–222.

[8] a) Y. He, B. Zhang, Y. Chen, Q. Jin, J. Wu, F. Yan, H. Zheng, *ACS Appl. Mater. Interfaces* **2017**, 9, 21190–21199; b) T. Hanaoka, N. Kamimura, T. Yokota, S. Takai, S. Ohta, *Med. Gas Res.* **2011**, 1, 18.

[9] S. Ohta, *Pharmacol. Ther.* **2014**, 144, 1–11.

[10] Y. Xin, T. Hu, P. Chu, *Acta Biomater.* **2011**, 7, 1452–1459.

[11] C. Chen, E. Karshalev, J. Guan, J. Wang, *Small* **2018,** 1704252–1704261.

[12] F. Mou, C. Chen, H. Ma, Y. Yin, Q. Wu, J. Guan, *Angew. Chem. Int. Ed.* **2013**, 52, 7208–7212.

[13] M. M. Pakulska, I. Elliott Donaghue, J. M. Obermeyer, A. Tuladhar, C. K. McLaughlin, T. N. Shendruk, M. S. Shoichet, *Sci. Adv.* **2016**, 2, e1600519– e1600528.

[14] J. Li, P. Angsantikul, W. Liu, B. E.-F. de Ávila, S. Thamphiwatana, M. Xu, E. Sandraz, X. Wang, J. Delezuk, W. Gao, L. F. Zhang, J. Wang, *Angew. Chem. Int. Ed.* **2017**, 56, 2156–2161.

[15] a) R. X. Zhang, K. Ren, R. Dubner, *Osteoarthritis Cartilage* **2013**, 21, 1308–1315; b) A. Sarmanova, M. Hall, J. Moses, M. Doherty, W. Zhang, *Osteoarthritis Cartilage* **2016**, 24, 1376–1383.

[16] P. Maudens, C. A. Seemayer, C. Thauvin, C. Gabay, O. Jordan, E. Allémann, *Small* **2018**, 14, 1703108–1703116.

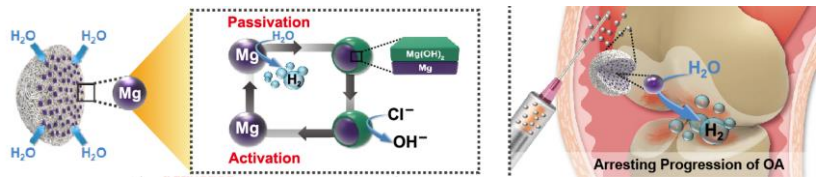
[17] S. E. Bove, S. L. Calcaterra, R. M. Brooker, C. M. Huber, R. E. Guzman, P. L. Juneau, D. J. Schrier, K. S. Kilgore, *Osteoarthritis Cartilage* **2003**, 11, 821–830.

10.1002/anie.201806159

WILEY-VCH

Entry for the Table of Contents (Please choose one layout)

COMMUNICATION



Upon intra-muscular administration, water infiltrates the PLGA microparticles to react with their encapsulated Mg powders, evolving H_2 bubbles and producing $Mg(OH)_2$ on their surfaces, making them unreactive. The passivated Mg can then be activated by the chloride (Cl^-) ions in body fluids. The cycle of passivation/activation of Mg can thus evolve gaseous H_2 continuously to the inflamed cartilage, arresting progression of osteoarthritis.

Wei-Lin Wan, Yu-Jung Lin, Po-Chien Shih, Yu-Ru Bow, Qinghua Cui, Yen Chang, Wei-Tso Chia, Hsing-Wen Sung**

Page No. – Page No.

***In Situ* Depot for Continuous Evolution of Gaseous H_2 Mediated by Magnesium Passivation/Activation Cycle for Treating Osteoarthritis**