Hyaluronic acid Hydrogels Hybridized with Au-Triptolide Nanoparticle for Intra-articular targeted multi-therapy of Rheumatoid Arthritis

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Research Article

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Abstract

Triptolide (TP) is a DMARD highly effective in patients with RA. Hyaluronic acid (HA) hydrogels loaded RGD-attached gold nanoparticles containing TP were synthesized to alleviate the toxicity and increase therapeutic specificity. The hydrogels can be applied for targeted photothermal-chemo therapy, and *in vivo* imaging of RA. Heat was locally generated at the inflammation site after degradation of HA chains due to near-infrared resonance (NIR) irradiation of gold nanoparticles (AuNPs), and TP was released. Administration of the hybrid hydrogels containing low dosage of TP combined with NIR irradiation alleviated arthritic conditions and improved the inflamed joint in collagen-induced arthritis (CIA) mice. *In vitro* effect of the hydrogel was mediated through decrease of phosphorylation of mTOR and its substrate, p70S6K1, thus inhibiting mTOR pathway.

1. Introduction

TP as an epoxide diterpene lactone compound has been used clinically to inhibit the expression of pro-inflammatory cytokine, adhesion molecules and metalloproteinases, thus demonstrating cartilage protective and anti-inflammatory effects on the treatment of RA [1-5]. However, as a DMARD, its anti-arthritic effect has not been fully harnessed in clinics, due to its multi-organ toxicity, poor solubility, low bioavailability, less stability, rapid excretion and non-specific targeting [6, 7]. Therefore, the novel delivery systems to maintain a high concentration of TP within the joint spaces, which is the main area of inflammation, by administration of small dosage of drugs to reduce side effects in accordance with systemic administration of TP, would be an attractive and effective tool for RA treatment.

Recently, the combined use of hydrogels and various NIR nanostructures in a single entity has gained enormous attention as for which are water-swollen and three-dimensional polymer networks that hold potential in the field of tissue regeneration, drug deliver, analgesia, chondroprotection and anti-inflammation [8-11]. And they can provide a favorable microenvironment for the chondrogenic cells to survive, grow and proliferate due to the advantages of moldability and lower invasiveness during delivery [6, 12]. These hydrogels can load bare drug or nanoparticles encapsulating drug molecules, growth factors or any bioactive compounds that can be released in a sustained manner [13]. They also have mechanical and structural properties similar to many tissues and can potentially mimic the native extracellular matrix (ECM) environment especially [12, 14]. HA as an important structural component of ECM is widely used due to its biocompatibility, biodegradability, and non-immunogenicity as biomaterial in clinical settings of RA [6,15,16].

Through chemically modified or physically crosslinked improvement, HA based viscoelastic solutions, in-situ hydrogels and scaffolds have been successfully prepared [6, 12]. However, the large pore sizes and high water content of most hydrogels may cause rapid and uncontrolled release of bare small molecules. Particularly, poorly water-soluble hydrophobic drugs tend to precipitate or to be simply released from the hydrogels in a rapid burst [18]. To overcome these limitations, nanoparticles-based drug delivery systems have been entrapped within hydrogels to form composite hydrogels networks (also called hybrid

hydrogels) [19]. Among various kinds of Au nanostructures, nanocages and nanorods, have attracted a potential interest in the biomedical field such as bio-sensors, drug delivery, imaging, tissue engineering and photothermal therapy [20-22]. Au nanostructures exhibit excellent optical and electronic properties, good biocompatibility and stability in vivo, and the easiness of surface modification [20, 23]. In vitro release studies demonstrated longer sustained delivery of drug from the metallic nanoparticles hybrid higher cross-linked hydrogels [18].

For the treatment of RA, we developed ^{RGD}Au-TP/HA hybrid hydrogels with RGD targeting, are directly intraarticular injected into CIA mice, which may potentially minimize side effects [9]. After the degradation of HA chains, nanoparticles are exposed to the surrounding environment [26]. Due to RGD peptides conjugated to the TP-Au nanoparticles, the accumulation of synthesized nanoparticles is enhanced in the inflamed joints. Upon NIR exposure, heat is generated due to Au and drugs are rapidly released from Poly (DL-lactic-co-glycolic acid) (PLGA)-TP nanoparticles, allowing photothermally controlled drug delivery and release.

To reveal the mechanism of targeted drug delivery in inflamed joints, RA-FLSs for their characteristic pathogenic behavior, such as aggressive phenotype, mediate inflammation and destruction of the joint, are applied for in-depth signaling pathway investigation [27-29]. It indicates that the anti-inflammatory effects of ^{RGD}Au-TP/HA hybrid hydrogels on RA-FLSs are occurred via regulation of the mTOR/p70S6K signal pathway, which is maintained in the abnormal activated state, resulting in high expression of downstream anti-apoptosis genes and a subsequent impact on multiple downstream effector molecules [30, 31]. The present study therefore aimed to investigate the anti-inflammatory effects of ^{RGD}Au-TP/HA hybrid hydrogels in CIA mice and to reveal the potential signaling pathways involved.

2. Material And Methods

2.1 Materials

Monothiol poly (ethylene glycol) with a carboxylic acid group (SH-PEG-COOH, Mw = 5000) and TP were purchased from Shanghai yuanye Bio-Technology Co., Ltd. Hyaluronic acid (HA; from Cockscomb) was purchased from Aladdin. Tyramine hydrochloride (TA), DL-dithiothreitol (DTT) and Sulfo-NHS were purchased from Shanghai yuanye Bio-Technology Co., Ltd. Phosphate buffered saline (PBS, pH 7.4) was purchased from Biological Industries. Pluronic F-127 was purchased from Beyotime biotechnology Co., Ltd. Poly (DL-lactic-co-glycolic acid) (PLGA; L/G molar ratio = 50:50; Mw = 20000) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from the Shanghai Macklin Biochemical Technology Co., Ltd. Horseradish Peroxidase (HRP) was purchased from the Beijing Solarbio Science & Technology Co., Ltd. The cyclic RGD (cyclic Arg-Gly-Asp-D-Tyr-Lys) peptide was purchased from Xi'an Ruixi Biological Technology Co., Ltd. All other chemicals, reagents, and solvents were analytical grade.

2.2 Fabrication of ^{RGD}Au-TP nanoparticles

PLGA (300 mg) and TP (18 mg) were dissolved in dichloroethane (30 ml), and this organic solution (oil phase) was added slowly drop-wise with distilled water (300 ml) containing Pluronic F-127 (300 mg) as a stabilizer under magnetic stirring. After mixing the oil and water phase, the mixture was emulsified by ultrasonication for 1 h (400 W), followed by evaporation of organic solvent (dichloroethane) with stirring for 24h. Then, the TP-PLGA nanoparticles were collected by centrifugation, and re-dispersed in 10 ml of PBS by sonication. TP-PLGA NPs were added to PEI aqueous solution (1 mg mL⁻¹) and stirred for 30 min then centrifuged. After centrifugation, 50 mL AuNPs solution was added to the TP-PLGA-PEI nanoparticles solution and subjected to vigorous stirring for 20 h.

The AuNps are prepared by sodium citrate reduction method [20]. An amount of 64 ml of 0.1 g/L HAuCl₄ solution is heated to boiling. Then the solution stirred strongly at around 120rpm, at the same time, 0.42 ml of 10 g/L sodium citrate are added drop by drop into the solution to keep the reduction time about 6min. After that, the solution is kept boiling until the solution turns red–purple and transferred into a flask and then stored at 4 °C before use.

The TP-PLGA-Au nanoparticles were released into 1wt% SH-PEG-COOH solution from the substrate by sonication and collected by centrifugation at 10000 rpm. The collected carboxylic-acid-terminated TP-PLGA-Au nanoparticles, EDC (8 mg)/NHS (8 mg) and cyclic RGD (6 mg) were dissolved in 18 mL of 0.2 M phosphate buffer (pH 7.4) and stirred at room temperature. During this period, the reaction mixture was then left at room temperature for the RGD peptides to bind covalently to the -COOH group of the SH-PEG-COOH chains adsorbed to Au nanoshells. After 24 h, TP-Au-RGD nanoparticles were collected by centrifugation, the supernatant containing unreacted cyclic RGD was discarded. Finally, the obtained TP-Au-RGD nanoparticles were freeze-dried and stored under 4°C for later use.

2.3 Synthesis of thiol and tyramine modified HA

HA was dissolved in deionized water. EDC and NHS were dissolved in deionized water, and add to the above HA solution, respectively. The mixture was adjusted pH to 5.4 with 1 M HCl and then stirred 0.5 h. After that, TA was added to the mixture and then stirred 24 h. The mixture solution was transferred into a dialysis bag with a cut-off molecular weight of 3.5 k Da, and then dialyzed for 3 days to ensure a complete removal of the residual tyramine hydrochloride and other salts. Finally, the obtained -SH and tyramine modified HA was freeze-dried and stored under 4°C for later use.

2.4 Preparation of ^{RGD}Au-TP /HA hybrid hydrogels

Hybrid hydrogels of a volume of 1 ml were prepared in cylindrically shaped glass vials (volume of 3 ml) as follows. tyramine modified HA (0.5 g) was dissolved in 10 ml of PBS (pH=7.4) at room temperature. After completely dissolved, 10 mg of TP-Au-RGD nanoparticles gently mixed to the previously prepared solutions and ultrasonication for 10 min. Subsequently, HRP was dissolved in PBS (50 µl, 0.02 mg/ml) was added to the previous mixture first, then hydrogen peroxide (H₂O₂) (50 µl, 0.02wt %). The mixture was then stirred gently, which resulted in hydrogels formation. The obtained TP-Au/HA hybrid hydrogels was freeze-dried and stored under 4°C for later use.

2.5 Material characterization

The resulting nanoparticles were characterized using a ^1H NMR spectrometer (Varian Gemini-300, DMSO- d_6). The size and zeta-potential of prepared nanoparticles dispersed in an aqueous medium were measured at 25 °C by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments Ltd.). The elemental mapping of an individual nanoparticles using TEM. The morphology of hybrid hydrogels was analyzed using SEM. The SEM images of modified HA showed smooth surface morphology. The TEM images were evident that nanoparticles were embedded within the hydrogel matrix with homogenous distribution.

2.6 In vitro drug release

Initially, hybrid hydrogels and equivalent nanoparticles were loaded into two separate 1000 Da cutoff membrane dialysis tube. (Tube-O-DIALYZER, G-Biosciences, St. Louis, MO, USA). The tubes were immersed in a transparent vial filled with 10 mL of PBS (pH 7.4, 10 mM) under mild constant shaking (150 rpm). The release experiments were performed with NIR irradiation at the initial time of the experiment at 37°C. The release medium was replaced with fresh PBS at determined intervals to maintain release conditions. The amount of released TP was measured by UV-vis spectrophotometry at 220 nm. All measurements were performed in triplicate.

2.7 RA-FLSs preparation and culturing

RA-FLSs and synoviocyte growth medium were purchased from Cell Applications (Beijing Longyue Biological Technology Development Co., Ltd.). FLS cells obtained from passages 4 to 8 were seeded onto 96-well plates at a density of 1×10^4 cells /mL in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose, 100 IU/ml penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Cells were grown in a humidified 37 °C incubator with ambient oxygen and 5% CO₂.

2.8 CCK-8 assay

A CCK-8 assay was used to measure the effect of TP-Au/HA hybrid hydrogels on RA-FLSs proliferation. In brief, RA-FLSs were collected and seeded into 96-well plates at a density of 2×10^4 cells per well. Then, the cells were plates with different concentration of drug and for different lengths of time (24 h, 48 h). Cells were treated with TP solution (13 and 30µM), hybrid hydrogels with equivalent TP of 13 µM without NIR irradiation, and TP-Au/HA hybrid hydrogels with equivalent TP of 13µM with NIR irradiation. NIR irradiation was conducted 24 h after treatment for 10 min. After the treatments, 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated at 37 °C, and 5% CO₂ for 1 h. The absorbance was determined at 450 nm using an enzyme-linked immunosorbent assay reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's instructions.

2.9 Western blot analysis

mTOR/p70S6K/p-mTOR and p-p70S6K protein expression was evaluated by western blotting. Total protein cell extracts were prepared following treatment using the Cell Lysis Buffer (Cell Signaling, USA) supplemented with protease inhibitors (Pierce, USA). Thirty micrograms of total protein per sample was loaded and separated on a 10% SDS-PAGE gel under reducing conditions. Samples were then transferred onto a nitrocellulose membrane and blocked in 5% nonfat dry milk. The membranes were then incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Primary antibodies used included mTOR/p70S6K/p-mTOR and p-p70S6K (Cell Signaling, USA). Detection was performed using chemiluminescence (Thermo, USA).

2.10 Induction and treatment of collagen induced arthritis

All the mice studies were performed as per the Guiding Principles for the Care and Use of Laboratory Animals according to the Regulations of the People's Republic of China for Administration of Laboratory Animals. All mice were maintained in a specific pathogen-free facility. First, an intradermal injection of 200µg of bovine type II collagen (Chondrex, USA) emulsified in 200µg of complete Freund's adjuvant (Sigma-Aldrich, Shanghai) was given in the base of the tail of male DBA/1J mice (8 weeks old, SLC Inc., Shizuoka, Japan) to induce rheumatoid arthritis. Next, a booster intradermal injection of 100µg of bovine type II collagen in incomplete Freund's (Sigma-Aldrich, Shanghai) was given to mice at 21 days after the primary immunization.

When arthritis was fully developed, saline (group 1), TP solution (group 2), hybrid hydrogels (groups 3 and 4) were intraarticular administered to the mice, and groups 4 were exposed to NIR light for 10 min after intraarticular injection of ^{RGD}Au-TP/HA hybrid hydrogels (n = 6 mice each group). After injection, the mice were monitored twice a week for 4 weeks. The clinical index is the sum of the clinical scores for four paws (maximum scores =16). The evaluated paws were scored from 0 to 4 according to the following scale: 0 = no evidence of erythema and swelling, 1=erythema and mild swelling, 2=erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to metatarsal joints, and 4 = erythema and severe swelling encompassing the ankle, foot, and digits or ankylosis of the limb.

2.11 In vivo NIR imaging

10 mg/kg of ^{RGD}Au-TP-Cy7/HA hybrid hydrogels and Cy7 were intraarticularly administered to CIA mice. IVIS Spectrum (Carestream Health Fx Pro/FX) in vivo fluorescence imaging system was used to observe the mice.

2.12 Histological examination

The mice were sacrificed 28 days after each treatment and the joints were removed from the mice. Joints were removed for histopathological examination and fixed in 10% buffered formalin saline at 4°C for 1 week. The decalcified joints were embedded in paraffin blocks and paraffin sections were sliced (4µm-thick). The joint tissues sections were stained with hematoxylin and eosin (H&E) and scored for changes

in synovial inflammation on a scale of 0-4 [33]. Each score was assessed by two independent observers and the average grades were calculated.

2.13 Biodistribution and clearance

TP-Au/HA hybrid hydrogels (200 μ L, 1mg/ml dispersed in PBS) were administered intraarticularly into CIA mice (n=3). The mice were sacrificed 28 days after injection and the major organs (liver, heart, spleen, kidney, joint, and lung) removed from each mouse. Tissue samples were placed in a mixed acid matrix of aqua regia and heated overnight at 80-90 °C. After additional heating at 130-140 °C for 2 h, the organic compounds were completely removed and only ionized Au remained. This residue was dissolved in 1 mL of 0.5 M HCl and analyzed by ICP-MS (Agilent 7500C).

2.14 Statistical analyses

Data are shown as means $\pm$ standard deviation. Statistical analyses of group differences were performed using the Mann-Whitney U test or ANOVA followed by Tukey's method. For all analyses, P values <0.05 were considered statistically significant.

3. Results And Discussion

3.1 Preparation and characterization of ^{RGD}Au-TP/HA hybrid hydrogels

The preparation route of ^{RGD}Au-TP/HA hybrid hydrogels hybrid hydrogels. For targeted delivery, cyclic RGD peptide, which binds R_v β ₃ integrins expressed on angiogenic vascular endothelial cells at sites of inflammation, was conjugated on the PLGA surface (TP-Au-RGD) [35]. The size of bare TP-Au-RGD nanoparticles was $\sim$ 164.2 nm in diameter. The zeta potential was found to be -23 ± 1.8 .

For the preparation of the modified HA, hydroxylic groups of hyaluronic acid were activated by HCl. This activated polymer was allowed to react with tyramine and cystamine [10]. This process led to the formation of a carbamate bond between the hydroxyl groups of HA and the amine group, and synthesize end-group thiolated HA via reductive amination, as in Fig.2a. ¹H NMR spectra of the obtained modified HA was examined in Fig.3. The newly appeared peaks at 6.69 and 7.2 ppm corresponding to aromatic protons of TA were integrated. Finally, the hydrogels were formed through the oxidative coupling of tyramines and thiol which was catalyzed by H₂O₂ and HRP. The SEM images of modified HA showed smooth surface morphology in Fig.2c. The TEM image was evident that nanoparticles were embedded within the hydrogels matrix with homogenous distribution in Fig.2d, and the SEM image of hydrogels in Fig.2e.

3.3 Drug release and photothermal effects

In vitro release studies, we measured the release of ^{RGD}Au-TP/HA hybrid hydrogels and ^{RGD}Au-TP nanoparticles, which demonstrated longer sustained delivery of drug from the higher cross-linked

hydrogels. The long HA chains with multiple interaction sites form a relatively dense and stable hydrophilic shell as shown in Fig 1a. RGD Au-TP nanoparticles showed that there was a sustained release of TP for 3 days with burst release of over 60% of the drug within 12 h (Fig. 4a). The drug release profile from RGD Au-TP/HA hybrid hydrogels showed the sustained release of TP for 3 days with burst release of around 60% of the drug within 72h and this may be attributed to the entrapment of nanoparticles within hydrogels network. The release from RGD Au-TP/HA hybrid hydrogels was much slower, which might be due to the combined effect of TP-Au-RGD nanoparticles release and coacervate system [40]. The surface of the TP-Au-RGD nanoparticles or the attached molecules is exposed to the surrounding environment after the degradation of HA chains [23, 32]. These temperatures are not high enough to induce irreversible tissue damage [39].

Biodegradable PLGA nanoparticles degrade more rapidly with increasing temperature [42, 43]. In order to study the effect of 10 min NIR irradiation on the TP release rate, we measured the release profile at 37 °C with and without 10 min NIR irradiation (Fig. 4b). The release rate of TP from TP-Au-RGD nanoparticles was nearly constant without NIR irradiation, resulting in a linear release profile. However, 10 min NIR irradiation induced a burst release of TP for 12h, after which the release rate was reduced. These results indicate that the TP release rate from TP-Au-RGD nanoparticles can be controlled by NIR light.

3.4 The proliferation inhibition effect on RA-FLSs in vitro study

In vitro study, we using RA-FLSs confirmed photothermally controlled drug release and anti-arthritis effects of the combination therapy of RGD Au-TP/HA hybrid hydrogels and NIR irradiation. Then, we evaluated the anti-proliferation effect of hybrid hydrogels on RA-FLSs by CCK-8 assay, and the experiment demonstrated that the proliferation of RA-FLSs was significantly inhibited by RGD Au-TP/HA hybrid hydrogels (Fig. 5d). G1 was a FLS control group. G2 and G3 were treated with TP solution of 13 μ M and 30 μ M, respectively. G4 were prepared by culturing the cells with TP-Au/HA hybrid hydrogels (equivalent TP of G2) for 1 day. After that, RA-FLS cells treated with hybrid hydrogels were exposed to NIR for 10 min in G5. As expected, G2 yielded much less cell anti-proliferation than G3 because of the lower TP concentration. The treatment of TP-Au/HA hybrid hydrogels without NIR irradiation (G4) significantly enhance cell anti-proliferation. The combined treatment of TP-Au/HA hybrid hydrogels and NIR irradiation (G5) lead the greatest cell anti-proliferation, although the dosage of TP in G5 was lower than G3. These results demonstrated a synergistic effect of RGD Au-TP/HA hybrid hydrogels combined with NIR irradiation.

3.5 Effects of TP-Au/HA hybrid hydrogels on the mTOR/p70S6K signaling pathway

Synovial inflammation and synovial cell hyperplasia is a distinctive feature of RA [44]. RA-FLSs are a key component of this invasive synovium and have a major role in the initiation and perpetuation of destructive joint inflammation [45]. RA-FLS releases important inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-21, IL-22, and IL-32), chemokines (CXCL1, CXCL5, MCP-1, G-CSF, and IL-8) and Inflammatory mediators (TLR-2, TLR-3, TLR-4, iNOS, and COX-2), which promotes the infiltration of monocytes, macrophages, neutrophils, DCs, T cells, and B cells into joints and results in chronic inflammation and joint destruction

[44]. Studies in recent years have demonstrated that the mammalian target of rapamycin/p70 ribosomal protein S6 kinase (mTOR/p70S6K) signaling pathway is over-activated in RA-FLSs, which plays an important role in regulating cell apoptosis and survival, as in Fig. 5a [41]. mTOR is upregulated in several cancers, regulates cancer cell invasion and its expression also correlates with poor prognosis in cancer [17, 24-25, 27, 36, 45]. The 70 k Da ribosomal S6 kinase p70S6K1 is known to regulate cell growth by inducing protein synthesis components [24]. Considering this background information, we hypothesized that the apoptosis-inducing effect of hybrid hydrogels on RA-FLSs might be associated with the inhibition of the mTOR/p70S6K signaling pathway. Therefore, we analyzed the protein expressions levels of total mTOR/p70S6K/p-mTOR/p-p70S6K in the various groups by western blotting analysis (Fig. 5b and Fig.5c). The results showed that hydrogels-treated RA-FLSs, which were exposed to NIR for 10 min at 0.38 W/cm² after administered, had significantly reduced levels of phosphorylated mTOR (Fig.5b, phospho-mTOR/total median reduction of 54%), as well as reduced levels of a phosphorylated form of one mTOR substrate, p70S6K (Fig.5c, phospho-p70S6K/total median reduction of 38%), confirming the inhibition of this pathway in association with reduced apoptosis. These data suggested that hybrid hydrogels could inhibit the mTOR/p70S6K signaling pathway in RA-FLSs.

3.6 In vivo targeting efficacy and NIR imaging

To evaluate the in vivo targeting efficacy of ^{RGD}Au-TP/HA hybrid hydrogels to inflamed joints, we generated CIA mice. These mice were intraarticular injected with ^{RGD}Au-TP-Cy7/HA hybrid hydrogels solution and Cy7 hybrid hydrogels solution. Because of Au superior capabilities, which will lead to composite systems of hydrogels with unique optical properties [37, 38]. The injected hybrid hydrogels were monitored by measuring in vivo NIR absorbance at 12h and 24h (Fig.6). We noted the color change in the inflamed paw over time, and CIA mice treated with TP-Au/HA hybrid hydrogels exhibited a change in vivo absorbance intensity over time due to localization of TP-Au-RGD nanoparticles in the inflamed paws.

In order to investigate the therapeutic effects of hybrid hydrogel, CIA mice were divided into four groups (n = 6 mice per group), as summarized in Table 1. Fig. 7 shows the clinical index of these groups as a function of time. In the mice treated with TP-Au/HA hybrid hydrogels (0.2 mg/kg) without NIR irradiation (G3), the clinical indices slowly decreased until about day 20 and then increased again, though they were lower than G1. However, when the mice were treated with TP-Au/HA hybrid hydrogels (0.2 mg/kg) and exposed to NIR light for 10 min at 24 h after intraarticular injection (G4), the clinical indices were lower than those of the mice treated with free TP solution four times every week (G2), which might be due to photothermally controlled drug release. For G3, TP was slowly released from hydrogels (Fig. 4b); therefore, the absence of NIR irradiation hampered the sustained release of TP, resulting in the small therapeutic effects. In contrast, the inflamed paw exposed to NIR light, leading to a release of more than 10% of the loaded TP within 12 h (Fig. 4b). This dosage of TP released locally in the inflamed paw was likely above the therapeutic dose, so high therapeutic efficacies were obtained for G4. We noted that the hydrogels injected into G4 contained only 0.2 mg/kg of TP, which is a much lower TP dosage than G2. These results demonstrate that chemo-photothermal treatment using TP-Au/HA hybrid hydrogels is a

good way to maximize therapeutic efficacy and minimize dosage-related TP side effects in the treatment of RA.

3.5 Histopathological evaluation

To confirm the therapeutic effects of the targeted chemo-photothermal treatment, we performed histological examinations of joints 28 days after intraarticular injection (Fig.8a). The joint sections of untreated mice showed severe inflammatory cell infiltration. The histopathological change was significantly reduced in mice treated with free TP solution four times every week (G2) and TP-Au/HA hybrid hydrogels with NIR irradiation (G4). In contrast, no significant differences were observed in G3 mice (Fig. 8b).

3.6 CT imaging

We performed three-dimensional micro-CT to assess bony changes in the paws of CIA mice (Fig. 8c). The paws of saline-treated CIA mice exhibited severe bone destruction. On the other hand, the bony structures were relatively well preserved in the paws of G2 and G4. To determine the extent of bone preservation, the bone volume of the paws of CIA mice was measured. The bone volume in the treated group tended to be better preserved (Fig. 8d). These results suggest that the therapeutic effect of TP-Au/HA hybrid hydrogels with NIR irradiation is comparable to that of conventional TP treatment.

3.7 In vivo toxicity of TP-Au/HA hybrid hydrogels

Histological examinations of major organs (liver, lung, and heart) were also performed at 28 days post-injection to investigate the influence of TP-Au/HA hybrid hydrogels on major organs (Fig. 9). Compared to the hydrogels-free control, G4 showed no apparent tissue damage, implying that the TP-Au-RGD nanoparticles accumulated in major organs did not induce in vivo toxicity.

4. Conclusions

In summary, we demonstrate the synergistic therapeutic effects of RGD Au-TP/HA hybrid hydrogels with chondroprotection, anti-inflammation, chemo-photothermal therapy and in vivo imaging for the treatment of RA. When the hybrid hydrogels were intraarticular injected into the CIA mice, in vivo NIR-absorbance images revealed that the nanoparticles selectively accumulated in the inflamed region. NIR irradiation increased the temperature of the exposed area and accelerated the TP release rate from the hybrid hydrogels, allowing the chemo-photothermal treatment. Compared to conventional single treatment with TP, the RGD Au-TP/HA hybrid hydrogels-based treatment combined with NIR irradiation had greater therapeutic efficacy using a much smaller dosage and low toxicity of TP. Furthermore, the findings suggested that TP-Au/HA hybrid hydrogels inhibited inflammation via suppressing the migration and invasion of FLSs, at least in part, by blocking the phosphorylation of the mTOR/p70S6K pathway. These results demonstrate that the targeted chemo-photothermal treatment using hybrid hydrogels are a useful

and effective strategy for maximizing the therapeutic efficacy and minimizing dosage-related side effects in the treatment of RA.

Declarations

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Biomacromolecules*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Tables

Table 1 is in the supplementary files section.

Figures

Figure 1

The preparation route of ^{RGD}Au-TP/HA hybrid hydrogels.

Figure 2

(a) Synthesis of tyramine modified HA. (b) TEM image of nanoparticles. (c) SEM image of modified HA. (d) TEM image was evident that nanoparticles were embedded within the hydrogels matrix. (e) SEM image of TP-Au/HA hybrid hydrogels.

Figure 3

^1H NMR spectra of modified HA.

Figure 4

(a) Profiles of TP release from TP-Au/HA hybrid hydrogels and TP-Au-RGD nanoparticles with NIR irradiation of 0.53 (54 °C) W/cm² for 10 min at the initial time. (b) Profiles of TP release from TP-Au-RGD nanoparticles with and without NIR irradiation of 0.53 (54 °C) W/cm² for 10 min at the initial time. Data represent mean values for n = 3, and the error bars represent standard deviation of the means (**p* < 0.05).

Figure 5

(a) Schematic illustration of anti-inflammatory effect of TP-Au/HA hybrid hydrogels in mouse CIA model. TP-Au/HA hybrid hydrogels treatment inhibits the phosphorylation of mTOR and mTOR target proteins. (b) TP-Au/HA hybrid hydrogels reduced levels of phospho-mTOR, with a phospho-mTOR/total median reduction of 54% and (c) Levels of phosphorylation of mTOR targets phospho-p70S6K (phospho-p70S6K/total median reduction of 38%, confirming the inhibition of the mTOR pathway. (d) Effect of TP-Au/HA hybrid hydrogels on the anti-proliferation of RA-FLSs. The cells were treated with G1 (a FLS control group), G2 (TP solution of 13Mm) G3 (TP solution of 30Mm) and G4 (TP-Au/HA hybrid hydrogels (equivalent TP of G2)) for 24h or 48 h, and the cell apoptosis rate was measured using the CCK-8 test. The data are expressed as the mean $\pm$ SEM values. By comparison with blank group, **P* < 0.05.

Figure 6

In vivo NIR absorbance images of inflamed paws of CIA mouse after intraarticular injection of ^{RGD}Au-TP/HA bridged hydrogels (the picture below is a ×4 magnification of red box).

3.7 In vivo therapeutic effects of chemo-photothermal treatment in CIA mice

Figure 7

Clinical index versus time for CIA mice injected intraarticularly with saline (G1), TP solution (35 mg/kg×4 times, G2), ^{RGD}Au-TP/HA hybrid hydrogels (0.2 mg/kg) without NIR irradiation (G3), ^{RGD}Au-TP/HA hybrid hydrogels (0.2 mg/kg) and exposed to 1.59 W/cm² NIR light for 10 min at 24 h after intraarticular injection (G4). The clinical index is the sum of the clinical scores for four paws (maximum scores = 16). The evaluated paws were scored from 0 to 4 according to the following scale: 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling, 2 = erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to metatarsal joints, and 4 = erythema and severe swelling encompassing the ankle, foot, and digits or ankylosis of the limb. Error bars represent standard deviation (n = 5). Clinical indices were significantly different among groups (**p* < 0.05).

Figure 8

(a) Histological findings of synovial from a normal mouse (NA) and CIA mice 28 days after each treatment, H&E (synovial inflammation, original magnifications × 100). (b) Semiquantitative analysis of histopathological evaluation (synovial inflammation). The bars represent the standard deviation. Asterisks (*) represent significance compared to untreated mice with **p* < 0.05 (n = 5). (c) Micro-

computed tomography images of hind paws of CIA mice in different group. CIA mice injected intraarticularly with saline (G1), TP solution (35 mg/kg×4 times, G2), ^{RGD}Au-TP/HA hybrid hydrogels (0.2 mg/kg) without NIR irradiation (G3), TP-Au/HA hybrid hydrogels (0.2 mg/kg) and exposed to NIR light for 10 min at 24 h after intraarticular injection (G4). (b) Effects of TP-Au/HA hybrid hydrogels on bone destruction in CIA mice. Bone surface/volume ratio (BS/BV; %). Data are presented as means ± SD for five mice per group. **p* < 0.05 versus G1; ***p* < 0.01 versus G1.

Figure 9

Histological sections of major organs extracted 28 days after intraarticular injection of saline (top) or TP-Au/HA hybrid hydrogels with NIR irradiation (bottom, G4). Images were acquired at 400×magnification.

Supplementary Files

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- [Table1.docx](#)