Intra-articular injection of PLGA/polydopamine core-shell nanoparticle attenuates osteoarthritis progression

Lujie Zong  
The First People's Hospital of Changzhou, Soochow University

Qing Wang  
The First Affiliated Hospital of Soochow University, Soochow University

Houyi Sun  
Qilu Hospital of Shandong University

Qian Wu  
The First Affiliated Hospital of Soochow University, Soochow University

Yaozeng Xu  
The First Affiliated Hospital of Soochow University, Soochow University

Huilin Yang  
The First Affiliated Hospital of Soochow University, Soochow University

Shujun Lv  
Hai’an People's Hospital

Liang Zhang  
Capital Medical University

Dechun Geng  
szgengdc@suda.edu.cn

The First Affiliated Hospital of Soochow University, Soochow University

Research Article

Keywords: osteoarthritis, polydopamine, targeted delivery, chondrogenesis

Posted Date: April 21st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2812832/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at ACS Applied Materials & Interfaces on April 22nd, 2024. See the published version at https://doi.org/10.1021/acsami.3c18464.
Abstract

Osteoarthritis (OA) is a common joint disease characterized by progressive cartilage degeneration, which is regulated by oxidative stress, and there is currently no clinical drug to alleviate its development. Kartogenin (KGN) was found to treat cartilage damage in early OA, but its application is limited by the rapid clearance from synovial fluid. This study synthesized a KGN-loaded nanocarrier based on PLGA/polydopamine core/shell structure to treat OA. The prepared KGN@PLGA/PDA-PEG-E7 nanoparticles could stay in the joint cavity for more than four weeks, ensuring the long-term sustained release of KGN after a single intra-articular injection. Moreover, the polyphenolic structure of PDA makes it effective in scavenging reactive oxygen species (ROS), so the KGN@PLGA/PDA-PEG-E7 NPs could promote chondrogenic differentiation even under oxidative stress conditions. In addition, the BMSCs-targeting peptide E7(EPLQLKM) conferred effective BMSCs affinity to KGN@PLGA/PDA-PEG-E7 NPs, which enhanced the efficacy of inducing cartilage in vitro and in vivo. As a result, the KGN@PLGA/PDA-PEG-E7 nanoparticles could effectively protect cartilage and subchondral bone in a rat ACLT model. In summary, KGN@PLGA/PDA-PEG-E7 nanoparticles can be used for intra-articular injection to effectively alleviate OA progression. This therapeutic strategy can also be extended to the delivery of other drugs or targeting other tissues to treat joint diseases.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease that starts with articular cartilage, although cartilage damage is fundamental, it progresses to degeneration of the entire joint eventually, including cartilage degeneration, synovial inflammation, subchondral sclerosis, and osteophytes[1]. The prevalence of OA continues to increase as the population ages. By 2017, there were 61.2 million OA patients in China[2]. Unfortunately, current treatment strategies for OA focus on symptom relief, clinically available drugs are mainly non-steroidal anti-inflammatory drugs (NSAIDs), yet up to 78% of OA patients in China still suffer from chronic knee pain[3]. Therefore, there is an urgent need to develop the disease-modifying osteoarthritis drugs (DMOADs) that can slow OA progression[1, 4]. At the same time, since articular cartilage has no blood vessels, how to deliver the drugs to their target and exert their effects is an important challenge.

Recently, the development of nanomaterials in targeted drug delivery provides an attractive therapeutic option for OA. Intra-articular administration may be an ideal drug delivery method for the treatment of OA, because it can distribute the drug directly to the joint capsule, which can improve local drug concentration, reduce systemic side effects, and reduce the administered dose [5]. However, there are two main problems that need to be addressed. On the one hand, most drugs will be cleared from the joint due to the rich capillary network in the synovial surface[6]. On the other hand, the emerging, tissue-specific or cell-specific drugs need to act on specific tissues or cells within the joint, and the off-target effects of drugs cannot be ignored[7]. Targeted delivery of nanomaterials provides an attractive therapeutic option for OA, which can not only prolong the residence time of drugs in the joint cavity, but also deliver drugs to the target site by linking targeting molecules[8].
Polydopamine (PDA) has been widely used in drug delivery due to its inherent advantages[9]. Dopamine molecules can easily self-polymerize in a weak alkaline environment, resulting in the deposition of PDA coatings on various surfaces[10]. PDA with enriched phenol groups can scavenge reactive oxygen species (ROS) and reduce acute inflammation[11], but its role in chronic inflammation has not been reported. Oxidative stress refers to an imbalance caused by excess production of ROS or oxidants over the ability of the antioxidant defense system, which is elevated in OA cartilage and contributes to chronic inflammation[12]. At the same time, inflammatory mediators increase the production of ROS and the expression of matrix metalloproteinases (MMPs), leading to the degradation of the cartilage matrix. In fact, ROS and inflammation are interdependent and mutually reinforcing[13, 14], which can be an ideal target for OA treatment[12]. Our study wished to investigate the role of PDA as a ROS scavenger in the chronic inflammatory environment of OA.

Synovial fluid contains different kinds of mesenchymal stem cells, which are increased in the early stage of OA[15] and can be used as a source of chondrocyte regeneration. Kartogenin (KGN) was found to promote the selective differentiation of bone marrow mesenchymal stem cells (BMSCs) into chondrocytes [16–19]. This study designed a nanoparticle delivery system KGN@PLGA/PDA-PEG-E7 nanoparticles that can target BMSCs and sustainably release KGN. Firstly, polylactic-co-glycolic acid (PLGA) was used as the basic carrier, which is biodegradable and biocompatible and has been approved by the FDA for clinical use. Then, KGN@PLGA was surface-modified with PDA to improve hydrophilicity and impart antioxidant capacity. Finally, E7 (EPLQLKM) was linked by PEGylation to form an affinity for BMSCs[20]. The Synthesized KGN@PLGA/PDA-PEG-E7 nanoparticles were used to induce chondrocyte regeneration under oxidative stress conditions in vitro and to treat OA in vivo in an ACLT rat model. Herein, we aimed to investigate the antioxidant capacity of PDA and the efficacy of targeted delivery in the treatment of OA.

2. Materials And Methods

2.1 Materials and Reagents

KGN was purchased from MedChemExpress, USA. E7 (EPLQLKM, 99.51% purity) was obtained from SciLight Biotechnology, China. Poly(lactic-co-glycolic acid) (PLGA, 50/50, 20,000 Da), polyvinyl alcohol (PVA), and dopamine hydrochloride were purchased from Sigma-Aldrich, USA. NH₂-mPEG-SH(2000), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), n-hydroxysuccinimide (NHS), Tris(hydroxymethyl)aminomethane (Tris-HCl) were purchased from Aladdin Bio-Chem Technology, China. All primary antibodies were purchased from Abcam, UK, and secondary antibodies were purchased from Beyotime Biotechnology, China.

2.2 Synthesis of KGN@PLGA

The KGN@PLGA nanoparticle was synthesized by the emulsification-solvent evaporation method[21]. Firstly, 3 mg KGN was first dissolved in 100µL DMSO and mixed with 4 mL CH₂Cl₂ containing 100 mg
PLGA. The mixture solution was added to 25 mL of 5% PVA solution and then sonicated on a Misonix sonicator 3000 at 80W for 15 minutes. After sonication, the emulsion solution was mixed with 30 mL ddH$_2$O and stirred overnight to evaporate the organic solvent. On the second day, a milky KGN@PLGA mixture was obtained by centrifugation (10,000 rpm, 10 min) and washed with ddH$_2$O three times.

2.3 Synthesis of KGN@PLGA/PDA

The dopamine coating on the surface of KGN@PLGA nanoparticles was based on a previously reported method [22]. Briefly, 30 mg KGN@PLGA nanoparticles were dispersed in 15 mL Tris-HCl buffer (10 mM, pH 8.5), then 5 mL of dopamine solution (2 mg/mL) was added dropwise, followed by stirring overnight. The KGN@PLGA/PDA nanoparticles were centrifuged at 10,000 rpm for 10 min and washed with ddH$_2$O three times.

2.4 Synthesis of KGN@PLGA/PDA-PEG-E7

30 mg KGN@PLGA/PDA NPs were dispersed in 15 mL deionized water with 4 mg NH$_2$-mPEG-SH, stirred for 1 hour, centrifuged at 10,000 rpm for 10 min, and washed three times to obtain KGN@PLGA/PDA-PEG nanoparticles. To modify the BMSCs specific affinity peptide EPLQLKM (E7) in KGN@PLGA/PDA-PEG NPs, 1 mg E7 peptide, 0.5 mg EDCI and 0.4 mg NHS were added into 30 mL KGN@PLGA/PDA-PEG solution (2 mg/mL) with magnetic stirring. Six hours later, the KGN@PLGA/PDA-PEG-E7 NPs were obtained after the removal of the excess reactant by centrifugation (10,000 rpm, 10 min).

2.5 Characterization of nanoparticles

The three kinds of KGN-loaded nanoparticles were dispersed in PBS buffer (0.2 mg/mL), then the hydrodynamic size and zeta potential were measured by dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Instruments, UK). And particle size stability of these nanoparticles was monitored by DLS for 7 days. The UV-Vis spectrum (UV-3600Plus, Shimadzu, Japan) of KGN@PLGA nanoparticles before and after dopamine coating was recorded. The morphology of nanospheres was observed by transmission electron microscope (TEM, Talos F200S, Thermo Fisher Scientific, USA). The KGN concentration in the supernatant of these nanoparticles was measured by high-performance liquid chromatography (HPLC, Agilent 1200, Agilent Technologies, USA) using a C-18 column (150mm×4.6 mm, 5µm). The analysis was carried out with a flow rate of 1.0 mL/min and recorded at 274 nm for 20 min. The calibration curve for KGN was established in the range of 2–200 mg/L. And the encapsulation efficiency (EE%) and loading capacity (LC%) of KGN was calculated according to the following formula:

\[
\text{encapsulation efficiency} = \left( \frac{\text{KGN}_{\text{total}} - \text{KGN}_{\text{free}}}{\text{KGN}_{\text{total}}} \right) \times 100\%;
\]

\[
\text{loading capacity} = \left( \frac{\text{KGN}_{\text{total}} - \text{KGN}_{\text{free}}}{\text{total nanoparticle weight}} \right) \times 100\%.
\]

2.6 In vitro release profile

The release profile of KGN from KGN@PLGA/PDA-PEG-E7 NPs was measured at pH 5.0 and pH 7.4. Briefly, 10 mg of KGN@PLGA/PDA-PEG-E7 nanoparticles were dissolved in 2 mL of PBS buffer, then divided into 1 mL and adjusted to pH 5.4 and 7.4 respectively, and placed in a shaking incubator (100
rpm). The supernatants were collected after centrifugation and replaced with fresh PBS buffer at each sampling time. The concentration of released KGN in the supernatant was evaluated by HPLC.

2.7 Extraction and Culture of primary rat bone marrow mesenchymal stem cells (BMSCs)

Primary BMSCs were obtained from Sprague Dawley (SD) rats. The femoral medullary cavity was flushed with α-MEM medium, and the released cells were collected into a 75 cm² culture flask with 10 ml of α-MEM medium containing 10% FBS. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ with medium changes every three days. Cells at passage 3 were used for subsequent experiments.

2.8 Assessment of BMSCs affinity In vitro

The E7 peptide was used as a competitive agent to compete with KGN@PLGA/PDA-PEG-E7 NPs for BMSCs binding. First of all, KGN@PLGA/PDA-PEG-E7 was labeled with cyanine 7(cy7, cy7-KGN@PLGA/PDA-PEG-E7) to evaluate the uptake of nanoparticles by BMSCs. The pre-blocking group was pretreated with E7 peptide for 12 hours, followed by co-culture with cy7 -KGN@PLGA/PDA-PEG-E7 NPs for 4 hours. Cells were then rinsed with PBS to remove residual NPs in the medium. 4,6-Diamidino-2-phenylindole (DAPI) and phalloidin were used to indicate the nucleus and skeleton of BMSCs, respectively, and the uptake of nanoparticles by BMSCs was observed under a fluorescence microscope.

2.9 In vitro Cytotoxicity

The cytotoxicity of the above nanoparticles on BMSCs was evaluated by Calcein/PI live-dead staining and the CCK-8 method. The concentration of KGN suitable for in vitro intervention of BMSCs is approximately 100 nM[16–18]. Specifically, BMSCs were intervened with KGN@PLGA, KGN@PLGA/PDA, and KGN@PLGA/PDA-PEG-E7 NPs that can release KGN to 100 nM according to release profile, respectively, and live-dead staining was performed 7 days later. On the other hand, KGN@PLGA/PDA-PEG-E7 nanoparticles that can release KGN from 0 to 1000 nM were used to intervene BMSCs, and the activity of BMSCs was detected by CCK8 at 1 day, 4 days, and 7 days, respectively.

2.10 In vitro chondrogenic differentiation

Chondrocytes were induced by the micromass method, using a conditioned medium (DMEM high glucose medium supplemented with 10 ng/mL TGF-β3, 100 nM dexamethasone, 50 mg/mL vitamin C and 1% ITS). 100 nM KGN and 4.07mg/L KGN@PLGA/PDA-PEG-E7 NPs were added to the conditioned medium to evaluate their ability to induce chondroblasts, respectively. The medium was changed every 3 days for a total of 14 days of induction. The results were evaluated in the following ways. ( ) Safranin O and Alcian blue staining: The BMSCs cultured for 2 weeks were fixed with 4% paraformaldehyde, stained with alcian blue and safranin 0 to detect proteoglycans, and then washed three times with PBS. ( ) Immunocytochemical staining: After 14 days, the cells were fixed with 4% paraformaldehyde and then incubated with anti-collagen II primary antibody (1:500) for 16 hours at 4°C, and washed three times with PBS. Then the cells were incubated with FITC-conjugated goat anti-mouse IgG (1:1000) for 1 h and
washed three times with PBS. Finally, the nucleus and cytoskeleton were stained sequentially with DAPI (0.5µg/mL) and phalloidin (1:1000). ( )Western bolt: After cells were collected by centrifugation, RIPA lysis buffer (Beyotime Biotechnology, China) was added to extract proteins, then the total protein content of the lysates was measured using the BCA protein assay kit (Beyotime Biotechnology, China). Proteins were separated and identified by 10% SDS-PAGE and transferred onto nitrocellulose membranes at 300 mA for 1h. Then the membranes were blocked with 5% milk in TBST for 1 hour and incubated with primary antibody overnight. After three washes with PBS, the membranes were incubated with secondary antibody for 2 hours. The results were visualized on a chemiluminometer reader (Bio-Rad, USA).

2.11 In vitro oxidative stress model

To study the anti-oxidative stress effect of PDA, an in vitro oxidative stress model was established by intervening BMSCs with H2O2. Firstly, BMSCs were intervened with H2O2 gradients of 0 µM, 25 µM, 50 µM, 100 µM, 200 µM, and 400 µM for 7 days, and the non-toxic concentration of H2O2 was 100 µM according to the CCK8 method. Therefore, a model of oxidative stress in vitro was established using 100 µM H2O2. KGN@PLGA/PDA(4.07mg/L) and KGN@PLGA/PDA-PEG-E7(4.07mg/L) were added to the medium containing 100 µM H2O2 for 7 days of intervention, respectively, and the ROS was assessed with a ROS detection assay kit (Beyotime Biotechnology, China). Next, BMSCs were induced with a chondrogenic conditioned medium containing 100 µM H2O2 to explore the chondrogenic inducibility of KGN@PLGA/PDA and KGN@PLGA/PDA-PEG-E7 under oxidative stress. The results were evaluated using the methods stated above.

2.12 Anterior cruciate ligament transection (ACLT) rat OA model

3-month-old male Sprague Dawley rats (300-350g) in the animal experiments were provided by the Laboratory Animal Center of Soochow University, and OA was induced by anterior cruciate ligament transection (ACLT) surgery. All procedures were performed under the Guide for the Care and Use of Laboratory Animals. Briefly, after anesthesia, a medial parapatellar incision was made through the retinaculum, then the anterior cruciate ligament was found with the keen in a flexed position, which was cut by microsurgery scissors. Before closing the surgical incision, an anterior drawer test was performed to ensure a successful OA model. The rats were randomly divided into 6 groups (n = 5): PBS group, KGN group, KGN@PLGA group, KGN@PLGA/PDA group, KGN@PLGA/PDA-PEG-E7 group, and Sham group. Intra-articular drug injection was started 1 month after surgery, once every 3 weeks. Specifically, the PBS group and the Sham Group were intra-articular injected with 100 µl of PBS, the KGN group was given 100 µl of KGN (100 µM), and the other groups were given 100 µl of nanoparticles(4.07 mg/mL) that can release 100 µM KGN. All rats were sacrificed 3 months after OA induction (Fig. 1B).

2.13 Retention time in the OA joint

The cy7-labeled KGN@PLGA/PDA-PEG-E7 NPs were injected 1 month after ACLT surgery to evaluate the retention time in the OA joint(n = 4). After injection of free cy7(100µl, left knee) and cy7-labeled
KGN@PLGA/PDA-PEG-E7 NPs (100µl, right knee) into the same rat, the fluorescence was detected using an in vivo imaging system (IVIS Lumina XR III, PerkinElmer, USA) within the next 4 weeks. Images were taken on days 0, 1, 7, 14, 21, and 28 post-injection, and the fluorescence intensities of the two groups were adjusted to the same level on day 0.

2.14 Micro-CT analysis

Rat knee joint specimens were scanned with micro-CT (SkyScan 1176, Bruker, Belgium) at 80 kV, 500 µA and reconstructed at 18 µm resolution. Traumatic osteoarthritis in patients with anterior cruciate ligament injury primarily affects the medial knee joint[23, 24]. Therefore, the subchondral bone of the tibial plateau is divided into medial tibial plateau (MTP) and lateral tibial plateau (LTP). The region of interest (ROI) is the area below the calcified cartilage and above the epiphyseal line of the tibial, and bone surface osteophytes, subchondral bone thickness(SBT) of the medial 1/3 of the tibial plateau, bone volume fraction (BT/TV), and bone mineral density (BMD) were measured according to previously reported methods[25].

2.15 Histological and immunohistochemical evaluation

( ) Histology: The knee joints were fixed with 10% formalin solution for 24 hours and decalcified with 10% EDTA for 2 months. The paraffin-embedded joints were then cut into 5 µm sections for H&E staining and Safranin O-fast green staining. The depth and extent of the cartilage lesion were accessed by two independent investigators according to the recognized OARSI score[26]. ( ) Immunohistochemistry (IHC): After deparaffinization and hydration, the sections adjacent to those assessed by histology were incubated with hyaluronidase and pepsin at 37°C for 1 hour. The sections were then blocked with 5% BSA for 30 minutes, incubated with primary antibody overnight at 4°C and secondary antibody for 1 hour at room temperature. DAB substrate was used for color development and Image J software was used to quantify stained images.

2.16 Statistics analysis

All data were expressed as mean ± SD. Multiple treatments were analyzed by one-way ANOVA with Tukey’s test, and unpaired t-test was used for comparing two groups. Statistical analyses were performed using the SPSS software (version 21.0; IBM, USA). P < 0.05 was considered statistically significant.

3. Results

3.1 Synthesis and characterizations of NPs

In this work, the targeted drug-loaded nanoparticles KGN@PLGA/PDA-PEG-E7 NPs have a "core-shell" structure (Fig. 1A). Firstly, PLGA was used as the basic carrier of KGN, and the feeding ratio for KGN/PLGA was 3% (3mg KGN/100 mg PLGA). The color of the KGN@PLGA/PDA changed from milky white to dark black (Fig. S1), indicating that polydopamine was coated on the KGN@PLGA NPs. Due to the lack of thiol and amine groups on the surface of PLGA NPs, the coating of PDA on PLGA may be
through hydrophobic interaction[27]. To enhance the stability and biocompatibility of the KGN@PLGA/PDA NPs, NH$_2$-mPEG-SH was modified through the Michael addition reaction between sulfhydryl groups and catechol of the PDA layer[28]. Finally, the BMSCs affinity peptide E7 was conjugated to the amino group of NH$_2$-mPEG-SH for targeting BMSCs in vitro and in vivo.

The hydrodynamic size of KGN@PLGA was about 200.04 nm measured by dynamic light scattering (DLS), the coating of the PDA layer increased the size of KGN@PLGA/PDA to 233.58 nm. Modification of the PEG protection layer and E7 peptide slightly increased the size of KGN@PLGA/PDA-PEG-E7 NPs to 245.26 nm (Fig. 2A&B), which is consistent with the TEM observations (Fig. 2H). The zeta potential of the synthesized NPs changed at different steps from KGN@PLGA to KGN@PLGA/PDA-PEG-E7, indicating successful modification throughout various steps of the synthesis process (Fig. 2D). Due to a PEG protective layer and negative surface charge (-19.52 mV), KGN@PLGA/PDA-PEG-E7 NPs remained stable in PBS buffer without significant size change and aggregation for 7 days (Fig. 2C). The absorption of KGN@PLGA/PDA NPs increased after coating with polydopamine (Fig. 2E). Centrifugation was used to collect nanoparticles, which is not an efficient method. The final loading capacity (LC%) and encapsulation efficiency (EE%) of KGN@PLGA/PDA-PEG-E7 were 2.42% and 31.53%, respectively (Table 1). The release profiles of PDA-modified nanoparticles are pH-responsive[9, 21]. As is shown in Fig. 2F, 66.47% of KGN was released at pH 5.4 within three days, while only 61.76% of KGN was released at pH 7.4 after 4 weeks.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Loading capacity (LC%)</th>
<th>Encapsulation efficiency (EE%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGN@PLGA</td>
<td>2.59 ± 0.23</td>
<td>32.67 ± 2.87</td>
</tr>
<tr>
<td>KGN@PLGA/PDA</td>
<td>2.47 ± 0.34</td>
<td>32.48 ± 3.10</td>
</tr>
<tr>
<td>KGN@PLGA/PDA-PEG-E7</td>
<td>2.42 ± 0.19</td>
<td>31.53 ± 2.18</td>
</tr>
</tbody>
</table>

### 3.2. Uptake and toxicity of nanoparticles by BMSCs

The targeting affinity of KGN@PLGA/PDA-PEG-E7 to BMSCs was observed in vitro. KGN@PLGA/PDA-PEG-E7 NPs were labeled with red fluorescent dye cy7. As is shown in Fig. 3A&B, after 4 h of co-culture, Cy7 fluorescence was significantly increased in BMSCs, indicating that KGN@PLGA/PDA-PEG-E7 NPs were easily taken up by BMSCs. Conversely, the pretreatment of BMSCs with E7 peptide for 12 h resulted in competition with the subsequent binding of KGN@PLGA/PDA-PEG-E7 NPs and thus significantly decreased cy7 intensity in the pre-blocked group. The results suggest that the BMSCs-targeting peptide E7 conferred effective BMSCs affinity to KGN@PLGA/PDA-PEG-E7 in vitro. Consistent with previous reports, KGN at a concentration of 100 nM was not toxic to BMSCs cells measured by CCK8(Fig. 3.D), and the corresponding concentration of KGN@PLGA/PDA-PEG-E7 NPs was also non-cytotoxic, which was used as the intervention concentration for subsequent in vitro experiments. After co-culture of the three
nanoparticles with BMSCs for one week, the Calcein/PI live and dead staining showed abundant green fluorescence and slight red fluorescence (Fig. 3C), which was similar to the blank group, proving that the nanoparticles have good biocompatibility for BMSCs.

### 3.3. In vitro chondrogenic differentiation

The role of KGN in promoting chondrocyte differentiation is dose-dependent[16], and maintaining long-term sufficient KGN is beneficial to the differentiation of BMSCs into chondrocytes. As is shown in Fig. 4A&B, the intensity of safranin O staining (red) and alcian blue staining (blue) in the KGN@PLGA/PDA-PEG-E7 NPs group was the strongest, followed by the KGN group and the weakest in the blank group, reflecting the accumulation of proteoglycans: KGN@PLGA/PDA-PEG-E7 group > KGN group > Blank group. Type II collagen (Col-2) is a specific protein secreted by chondrocytes and is an important component of the cartilage matrix. Immunofluorescence showed that little Col-2 was expressed in the blank group, while the KGN@PLGA/PDA-PEG-E7 group expressed more Col-2 than the KGN group (Fig. 4C&E). SOX9 protein is a key factor regulating the differentiation of BMSCs into chondrocytes and can inhibit the progression of OA[29]. Western Blot showed that the expression of SOX9 and Col-2 in the KGN group was slightly higher than that in the blank group but lower than that in the KGN@PLGA/PDA-PEG-E7 group (Fig. 4D&F). Overall, these results suggest that KGN@PLGA/PDA-PEG-E7 can enhance the intracellular delivery efficiency of KGN and thus promote chondrogenesis of BMSCs more efficiently than free KGN, suggesting a potential strategy for repairing OA cartilage.

### 3.4 Chondrogenic differentiation under oxidative stress conditions

Oxidative stress plays an important role in the development of OA and is mutually causal with the chronic inflammation environment. H$_2$O$_2$ was used to establish an oxidative stress model of BMSCs. CCK8 method showed no apparent BMSCS toxicity with 100 µM H$_2$O$_2$ that was taken as the intervention concentration of the subsequent experiment (Fig. S2). The polyphenolic structure of PDA can scavenge free radicals and reduce ROS production. The ROS level was detected by the DCFH-DA probe, as is shown in Fig. 5A, after modification with PDA, both KGN@PLGA/PDA and KGN@PLGA/PDA-PEG-E7 nanoparticles exhibited great ROS scavenging ability, and functional modification did not reduce the antioxidant capacity of KGN@PLGA/PDA NPs.

To investigate the ability of PDA-modified nanoparticles to promote chondrogenic differentiation under oxidative stress, KGN@PLGA/PDA and KGN@PLGA/PDA-PEG-E7 NPs were added into the medium containing 100 µM H$_2$O$_2$ for 14 days of induction. Safranin O and Alcian blue staining showed that oxidative stress had a significant inhibitory effect on chondrogenic differentiation of BMSCs (Fig. 5B); the expression of proteoglycan in the KGN@PLGA/PDA-PEG-E7 group was higher than that in KGN@PLGA/PDA group, and both were higher than that in the blank group, suggesting that PDA modification has a strong antioxidant effect, and targeted delivery of KGN can enhance the effect of
chondrogenic differentiation in vitro. Similar results were also obtained for Col-2 expression in immunofluorescence and western blot (Fig. 5C&D). COX-2 is a common mediator in inflamed tissues, and the expression of COX-2 was induced by ROS in OA joints, which could be inhibited by antioxidants in vitro and in vivo[30]. Western blot also showed that there was no significant difference in the expression of COX-2 between the blank group and the KGN@PLGA/PDA group or the KGN@PLGA/PDA-PEG-E7 group. In addition, H$_2$O$_2$ significantly increased the level of inflammatory factor IL-6, while antioxidant treatment with PDA-modified nanoparticles could significantly inhibit the expression of IL6, which also verifies the good biocompatibility of the nanoparticles without any apparent pro-inflammatory effect after cellular uptake.

### 3.5 Residence time in the joint cavity

The residence time of the drug in the joint cavity is critical to the effect of the drug acting in the joint. As a hydrophobic small-molecule drug, KGN can be rapidly absorbed by synovial capillaries and cleared from joints. Drug-loaded nanoparticles can significantly prolong drug residence time. In addition, both surface modification and PEGylation of PDA can improve the hydrophilicity of nanoparticles, thereby reducing the phagocytosis of reticulated macrophages and further prolonging the residence time. In this research, cy7 was used to label KGN@PLGA/PDA-PEG-E7 NPs to study their retention in the joint cavity, while free cy7 fluorochromes were used as a control. The IVIS showed that the fluorescence intensity of free cy7 fluorochromes decreased rapidly within 7 days and almost no fluorescence signals were detected on the 14th day, while the KGN@PLGA/PDA-PEG-E7 nanomedicine still retained about 18% of the fluorescence intensity on the 28th day. The results indicated that the nanomedicine could stay in the joint cavity for 1 month, which can ensure the long-term sustained release of KGN after a single intra-articular injection.

### 3.6 Therapeutic effect in the rat ACLT model

All rats were confirmed by the anterior drawer test to establish the OA model successfully, and no death occurred within three months. Pain symptoms and imaging findings are often inconsistent in patients with OA[1]. Articular cartilage is not innervated, so it is subchondral bone sclerosis rather than cartilage damage that causes joint pain in the late stage of OA[31, 32]. In this work, Micro-CT was used to evaluate osteophyte formation and subchondral bone changes in the OA knee 12 weeks after ACLT surgery. As is shown in Fig. 7A&B, osteophyte formation was significantly reduced by nanomedicine treatment, with the least osteophyte in the KGN@PLGA/PDA-PEG-E7 group, which was lower than that in the KGN@PLGA/PDA group. In addition, the thickness of subchondral bone and cancellous bone was significantly reduced in the ACLT model (PBS group), and treatment with KGN@PLGA/PDA-PEG-E7 could significantly inhibit bone resorption, which was similar to the Sham group (Fig. 7C&D). The medial knee joint is mainly affected in patients with ACL injury [23, 24], which may be due to partial knee varus. In the subchondral bone of the medial tibial plateau (MTP), the bone volume fraction (BV/TV) and bone mineral density (BMD) of the KGN@PLGA/PDA group and the KGN@PLGA/PDA-PEG-E7 group were significantly higher than those of PBS. While in the lateral tibial plateau (LTP), only the Sham group showed significantly higher BV/TV and BMD than the PBS group.
Rats were sacrificed 3 months after the induction of OA. Compared with the Sham group, intra-articular injection of nanoparticles did not cause joint swelling. Specimens were collected for histological evaluation. Firstly, the autopsy results of the heart, liver, kidney, spleen, and lung showed no significant histological differences between the 6 groups (Fig. S3), suggesting that these nanoparticles have a good biosafety profile. Cartilage degeneration was assessed with H&E staining and Safranin O-fast green staining (Fig. 8A&B). The cartilage structure was complete and the chondrocytes were neatly arranged in the Sham group. In the PBS group, however, the damaged cartilage structure was lightly stained, with fibrotic areas in the superficial cartilage and cracks in the deep layer, in which large areas of chondrocytes were lost and the remaining chondrocytes were swollen. Among the treatment groups, the KGN@PLGA/PDA-PEG-E7 group had the best cartilage integrity, the least surface erosion, and the highest chondrocyte density, similar to the Sham group. Compared with the PBS group, the OARSI scores of the KGN group, KGN@PLGA group, KGN@PLGA/PDA group, and KGN@PLGA/PDA-PEG-E7 group decreased by 46.31%, 56.31%, 68.77%, and 80.52%, respectively. The OARSI score of the KGN@PLGA/PDA-PEG-E7 group (2.22 ± 0.76) was similar to that of the Sham group (1.68 ± 0.51), and lower than that of the KGN@PLGA/PDA group (Fig. 8D). In addition, immunohistochemistry found that Col-2-positive cells were significantly increased after KGN treatment, and the proportion of KGN@PLGA/PDA-PEG-E7 group was higher than that of KGN@PLGA/PDA group, and both were higher than that of KGN group (Fig. 8C&E). Compared with the PBS group, the expression of the inflammatory factor IL-6 was significantly decreased in each nanoparticle group, indicating that the nanoparticles had no obvious pro-inflammatory effect. There was no significant difference in the IL-6-positive cells between the KGN@PLGA/PDA group and the KGN@PLGA/PDA-PEG-E7 group, and they were all significantly lower than those in the KGN group, possibly due to the antioxidant effect of PDA to reduce inflammation.

4. Discussion

Cartilage damage is a key pathological feature of OA, which occurs in the early stage and throughout the disease. Due to the lack of blood vessels and nerves, the regeneration capacity of damaged cartilage is minimal, so the progression of OA is difficult to reverse. KGN was found to treat cartilage damage in early OA [4–7] and several drug delivery systems have been reported to prolong the intra-articular residence of KGN and improve its efficacy [17, 18, 33–35]. However, these strategies using the entire joint cavity as a drug repository is more suitable for drugs acting on multiple targets. Few studies have reported targeted delivery of KGN to MSCs, and few literatures have also focused on the chronic inflammatory environment of OA. In this work, we used PDA for intra-articular drug delivery to verify the effect of its antioxidant capacity on chronic inflammation, and also achieved targeted delivery of KGN to MSCs by linking stem cell affinity peptide E7.

The size of nanoparticles is significant for the efficacy of intra-articular delivery, which affects the clearance rate, tissue distribution, and cellular uptake of nanoparticles in the joint cavity. The optimal size for intra-articular drug delivery remains controversial. On the one hand, nanoparticles of larger size are removed more slowly, and substances larger than 250 nm can stay in the joint cavity for a longer time [36]. On the other hand, the unique dense extracellular matrix (ECM) of cartilage tissue makes it difficult for large nanoparticles to penetrate, with a threshold size around 55–140 nm [8]. Notably, pathological
changes caused by OA can also affect the clearance rate of nanoparticles. It was reported to deliver KGN with nanoparticles and microparticles, respectively, but no significant difference was found in OARSI scores. The prepared KGN@PLGA/PDA-PEG-E7 NPs were about 245 nm in size, which could stay in the joint cavity for more than 4 weeks to continuously release KGN and repair the damaged cartilage in early OA.

Polydopamine (PDA) has been widely used in cancer therapy, antibacterial, anti-inflammatory, and tissue regeneration due to its excellent biocompatibility and degradability, and good drug loading capacity. PDA can be modified under simple conditions, and easily performed secondary modification, the abundant amino and hydroxyl functional groups in PDA polymers can be functionalized and modified by covalent bonding (e.g., PEGylation), hydrogen bonding, metal coordination, π-π stacking, and electrostatic interaction. In addition, the PDA-modified nanocarriers exhibited pH-responsive properties, in this study, KGN was released from nanoparticles faster at pH 5.4 than at pH 7.4. Such acid-triggered drug release is owed to the protonation of amino groups on PDA NPs under acidic conditions. The accepted pH of body fluids is 7.4, while intracellular nanoparticles are mainly located in lysosomes (pH 5–6). Therefore, KGN was released at a slower rate in the joint cavity with reduced toxicity, and was rapidly released from KGN@PLGA/PDA-PEG-E7 NPs after being taken up by BMSCs. This acid-triggered drug release can achieve low toxicity to normal tissues, enabling precise treatment of OA.

We also found that the ROS-scavenging effect of PDA can attenuate chronic inflammation in OA. Oxidative stress elicited by ROS accumulation is one of the drivers of cartilage catabolism. Excessive ROS not only reduces the chondrogenic differentiation ability of stem cells, but also inhibits the synthesis of proteoglycans and type II collagen by chondrocytes. Chondrocytes could reprogram their metabolism towards glycolysis and increase ROS production under inflammatory conditions, and scavenging ROS could reduce inflammation and cartilage catabolism. Antioxidant drugs or free radical scavengers have been used to treat OA, mainly limited by poor drug stability or short residence time. As a ROS scavenger, PEG-MnO2 nanoparticles-treated chondrocytes had similar expression of type II collagen and proteoglycans to healthy chondrocytes. The abundant phenolic groups of PDA could scavenge ROS, which had a significant anti-inflammatory effect on acute inflammation by reducing neutrophil infiltration. Notably, PDA NPs can also reduce ROS production by increasing the efficiency of mitochondrial oxidative phosphorylation. In vitro, PDA modification could significantly reduce ROS levels and the expression of COX-2 in BMSCs, and functional modification did not reduce this antioxidant capacity. Interestingly, KGN@PLGA/PDA-PEG-E7 nanoparticles could promote the differentiation of BMSCs into chondrocytes under 100 µM H₂O₂, even stronger than the negative control group. In vitro and in vivo, the expression of IL-6 in the KGN@PLGA/PDA group and KGN@PLGA/PDA-PEG-E7 group was significantly lower than that in the KGN group, suggesting that PDA, as an antioxidant, can reduce inflammation in early OA.

In addition, targeted drug delivery can enhance the efficacy of OA treatment. The off-target effects of drugs that act on a single target cannot be ignored. For example, KGN may stimulate the overgrowth
of normal tissue[49]. In this work, stem cell affinity peptide E7 was modified by PEGylation, and the cellular uptake results indicated that the E7 peptide conferred effective BMSCs affinity to KGN@PLGA/PDA-PEG-E7 in vitro. As a result, the expression of proteoglycan and Col-2 in the KGN@PLGA/PDA-PEG-E7 group was higher than that in the KGN@PLGA/PDA group. In vivo, it was reported that the E7-modified carrier could facilitate the migration of exogenous MSCs into the cartilage layer[48]. In a rat ACLT model, compared with the non-targeted KGN@PLGA/PDA group, the KGN@PLGA/PDA-PEG-E7 group had lower OARSI scores and higher Col-2 positive cells, Micro-CT also showed that the KGN@PLGA/PDA-PEG-E7 group had the least osteophyte formation and bone resorption. We found the OARSI score of the KGN@PLGA/PDA-PEG-E7 group decreased by 80.52% compared with the PBS group.

To our knowledge, this is the first study to use PDA for intra-articular drug delivery and to demonstrate the efficacy of its antioxidant capacity in OA, which also enables targeted drug delivery to BMSCs. However, the anti-inflammatory mechanism of PDA has not been explored, and the effect of nanocarriers on advanced OA has not been studied, which will be our future work.

5. Conclusion

In this work, we synthesized a KGN-loaded nanocarrier based on PLGA/polydopamine core/shell structure to treat OA. In vitro, these well-biocompatible KGN@PLGA/PDA-PEG-E7 nanoparticles could target BMSCs and reduce ROS production, which could promote the differentiation of BMSCs into chondrocytes even under oxidative stress conditions. In vivo, KGN@PLGA/PDA-PEG-E7 nanoparticles could stay in the articular cavity for more than four weeks in a rat ACLT model and effectively protect cartilage and subchondral bone. Due to its simple fabrication process and good biocompatibility, this novel nanocarrier holds promise for clinical application. This therapeutic strategy could also be extended to the delivery of other drugs, targeting other tissues to treat joint diseases.

Declarations

Ethics approval and consent to participate

The animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (eighth Edition, 2011).

Consent for publication

All authors agree for publication.

Availability of data and materials

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as
you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Competing interests

The authors have declared that no competing interest exists.

Funding

This review work was supported by the National Natural Science Foundation of China (82272157), the Natural Science Foundation of Jiangsu Province (BK2021650), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Jiangsu Medical Research Project (ZD2022021), and Special Project of Diagnosis; Treatment Technology for Key Clinical Diseases in Suzhou (LCZX202003), the Program of Suzhou Health Commission (GSWS2022002) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX22_3217).

Acknowledgements

Not applicable.

Authors' information

Authors and Affiliations

Department of Orthopaedics, The First Affiliated Hospital of Soochow University, Soochow University, Suzhou, China.

Lujie Zong, Qing Wang, Qian Wu, Yaozeng Xu, Huilin Yang and Dechun Geng

Department of Orthopedics, The First People's Hospital of Changzhou, Soochow University, Changzhou, China.

Lujie Zong

Department of Orthopedics, Qilu Hospital of Shandong University, Jinan, China.

Houyi Sun
Department of Orthopedics, Hai’an People's Hospital, Hai’an, Jiangsu, China.

Shujun Lv

Department of Orthopaedics, Beijing Friendship Hospital, Capital Medical University, Beijing, China.

Liang Zhang

Authors' contributions

DG, LZ and SL contributed to experimental conception and design as the corresponding author. LZ and QW did manuscript writing and revising. LZ, QW, HS and QW made substantial contributions and did most contributions in vivo and vitro including data acquisition and analysis. LZ, HS and YX contributed to micro-CT scanning and analysis, LZ, QW and HY contributed to histological and immunohistochemical staining. All authors reviewed the manuscript.

Corresponding author

Correspondence to Dechun Geng, Liang Zhang and Shujun Lv.

References


38. Z. Du, Y. Mao, P. Zhang, J. Hu, J. Fu, Q. You, J. Yin, TPGS-Galactose-Modified Polydopamine Co-delivery Nanoparticles of Nitric Oxide Donor and Doxorubicin for Targeted Chemo-Photothermal


Figures
Figure 1

Illustration of the procedures to synthesize KGN@PLGA/PDA-PEG-E7 NPs (A) and in vivo experimental procedures in the ACLT-induced OA model (B).
Figure 2

Physical characterization of the nanoparticles. (A-B) The hydrodynamic size and size distribution of KGN@PLGA/PDA-PEG-E7 NPs measured by DLS. (C) Particle size stability of KGN@PLGA, KGN@PLGA/PDA and KGN@PLGA/PDA-PEG-E7 dissolved in PBS at 37 °C for 7 days. (D) Zeta potential of NPs changed from KGN@PLGA to KGN@PLGA/PDA-PEG-E7 NPs. (E) UV-Vis absorption spectra of the nanoparticles. (F) The drug release profiles of KGN in media with different pH values (7.4 and 5.4). (H) TEM images of the nanoparticles.
Figure 3

Uptake and toxicity of nanoparticles by BMSCs. (A-B) Evaluation of the targeting effect of KGN@PLGA/PDA-PEG-E7 NPs on BMSCs by pretreating BMSCs with E7 peptide (n=5). (C) Calcein/PI live and dead staining: BMSCs were intervened with KGN@PLGA, KGN@PLGA/PDA, and KGN@PLGA/PDA-PEG-E7 NPs that can release KGN to 100 nM. (D) CCK8: BMSCs were intervened with different concentration of KGN@PLGA/PDA-PEG-E7 NPs that could release KGN to 10nM, 100nM, and 1000nM, respectively (n=5).
Figure 4

Evaluation of chondrogenic differentiation in vitro. Safranin O (A) and Alcian blue(B) staining reflected the expression of proteoglycans. (C&E) Immunofluorescence staining of Col-2. (D&F) Western bolt (n=3), *compared with Blank group (p<0.05), #compared with KGN group (p<0.05).
Figure 5

Evaluation of chondrogenic differentiation under oxidative stress. (A) The ROS level detected by the DCFH-DA probe. (B)Safranin O and Alcian blue staining. (D&E)Col-2 immunofluorescence staining. (C&F) Western blot (n=3), *compared with Blank group (p<0.05), #compared with H2O2 group (p<0.05).
Figure 6

In vivo fluorescence imaging. (A) IVIS images of free cy7 fluorescent dye (left knee) and cy7-labeled KGN@PLGA/PDA-PEG-E7 (right knee) at different time points after intra-articular injection. (B) Relative fluorescence intensity at each time point.
Figure 7

Micro-CT analysis. (A) Coronal 2-D reconstruction of the knee joint. (B) 3-D reconstruction with osteophytes marked in red shading. (C) Sagittal 3-D reconstruction of the medial tibial plateau. (D) The subchondral bone thickness of the medial 1/3 of the tibial plateau. (E) Bone volume fraction (BT/TV) and bone mineral density (BMD) of the medial tibial plateau (MTP) and lateral tibial plateau (LTP). n=5, *compared with PBS group (p<0.05).

Figure 8

KGN@PLGA/PDA-PEG-E7 NPs reduce cartilage degeneration in rat ACLT models. Representative sections of H&E staining(A), Safranin O-fast green staining(B), and immunohistochemical staining of Col-2 and IL-6(C). (D) OARSI scores. (E) Quantification of Col-2-positive cells and IL-6-positive cells. n=5, *compared with PBS group (p<0.05), #compared with KGN group (p<0.05).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx