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Synthesis of strontium chondroitin sulfate and the evaluation of its capability to attenuate osteoarthritis

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Highlights

•A new chemical, strontium chondroitin sulfate (SrCS) was fabricated and characterized.

•The chemical and structural analysis suggested that SrCS is a polysaccharide-metal ion complex.

•The cell proliferation results indicated that the SrCS is safe to both chondrocyte and osteoblast.

•Biochemical results indicated that SrCS can increase collagen formation and has significant anti-inflammatory effects.

•Animal study results suggested that SrCS should have a positive impact on OA cartilage remodeling.

Abstract
Osteoarthritis (OA) is the most prevalent musculoskeletal disorder and the leading cause of joint disability in elderly patients. In this study, we fabricated strontium chondroitin sulfate (SrCS), a new polysaccharide-metal ion complex that is the combination of chondroitin sulfate and strontium, which are two widely adopted chemicals in OA clinical management. The structural, chemical compositions and morphology of as-fabricated SrCS were systematically investigated. Cell proliferation test, RT-PCR and preliminary animal studies were conducted to evaluate the clinical potential of SrCS on OA treatment. The materials characterization results verified that the Sr was successfully integrated into CS by replacing sodium in the original structure and formed a new polysaccharide-metal ion complex. The cell proliferation results indicated that the SrCS has excellent biocompatibility for both chondrocyte and osteoblast. The RT-PCR results showed that the SrCS can significantly increase the expression of COLII and ACAN, decrease MMP1 and MMP13 in chondrocyte and decrease the IL-6 and IL-1β in both chondrocyte and osteoblast. Preliminary animal studies demonstrated that SrCS can effectively simulate the articular cartilage formation in SD-rats after modified Hulth's OA modeling surgery. We therefore believed that the SrCS should be a rather effective chemical for OA clinical management as well as a beneficial component for various biomaterials in cartilage tissue engineering.

Keywords
Chondroitin sulfateStrontiumCartilageOsteoarthritis

1. Introduction

Osteoarthritis (OA) is the joint disorders that generally characterized by articular cartilage (AC) degradation, subchondral bone sclerosis and osteophyte formation. It is prevalent worldwide and now is the leading cause of physical disability among aging adults (Clegg et al., 2006, Gutierrez et al., 2015, Wandel et al., 2010). There is no satisfactory clinical treatment for OA so far: pharmacological treatment of OA is limited to only relieve the symptom but fail to slow down the OA's progression, and total joint arthroplasty that might yield various complications is the final solution (Henrotin et al., 2001, Sharma, 2016). Although the etiology of OA is still unknown, it is generally accepted that the entire joint, including AC, subchondral plate and trabecular bone, are altered by OA and the cross-talk between these joint tissues should be rather important in OA's pathology (Grover and Samson, 2016, Provenza et al., 2015; Xiao et al., 2016). Recent studies reported that the disequilibrium of bone metabolism will result in abnormal osteoid islets on subchondral bone, which is one of the critical steps in OA progression (Xiao et al., 2016, Zhen et al., 2013). Hence, both the AC and subchondral bone should be well considered for effective clinical treatment of OA.

Chondroitin sulfate (CS) is a complex, heterogeneous polysaccharide. It is the basic component of all connective tissues (Barnhill et al., 2006; du Souich, 2014, Ronca et al., 1998). Commercially available chondroitin sulfate extracted from cartilage generally exists in the form of sodium salt. The degree of sulfation and the chain length of extracted CS vary from different species and tissues, with the molecular weight range from 10 to 100 kDa (Tat, Pelletier, Mineau, Duval, & Martel-Pelletier, 2010). Chondroitin sulfate is anti-inflammatory, beneficial in collagen production and the anabolic/catabolic balance of chondrocytes (Ronca et al., 1998). Therefore, chondroitin sulfate currently is widely adopted on pharmacological treatment for OA, which was reported to be able to effectively relieve the pain, improve the AC function and slow down the radiological progression of OA (Grover and Samson, 2016, Provenza et al., 2015; Roubille et al., 2015, Wandel et al., 2010, Zhang et al., 2008).

Strontium (Sr) is a trace element of human body that mainly exists in skeleton (Li et al., 2007). Sr is known as anti-inflammatory and antioxidant, and has been employed as antirheumatic drug for decades (Morandi, 1956). Strontium has beneficial effects on both bone and cartilage metabolism (Henrotin et al., 2001, Marie, 2006, Reinholt et al., 1985). Recent clinical studies suggested that Sr can reduce bone resorption, increase bone formation and simulate the formation of collagen matrix. Therefore, Sr has been gradually adopted in the OA treatment (Sharma, 2016, Wang et al., 2015; Wyland, 2015, Yu et al., 2013).

In this study, strontium chondroitin sulfate (SrCS), a new chemical combining chondroitin sulfate and strontium was first fabricated through ion exchange method. We systematically investigated the chemical composition, structure and morphology of as-prepared SrCS and evaluated its potential capability on OA treatment. The results suggested that the proposed fabrication protocol can successfully integrated Sr into CS to form SrCS with high purity. Moreover, the SrCS is anti-inflammatory, rather effective in simulation collagen formation and helping the growth of cartilage. We therefore believed that SrCS fabricated in this work should be a promising chemical for various biomedical applications, especially those related to OA.

2. Materials and methods

2.1. Materials

The chondroitin sulfate used in this study was supplied by Henan Xingyuan Chemical products Co. Ltd., which is chondroitin sulfate A extracted from bovine trachea, with molecular weight 18 kDa (Supplementary Fig. 1). The cation exchange resin and strontium chloride used were purchased from Sinopharm Chemical Reagent Co., Ltd. Strontium hydroxide used in this study was purchased from Shanghai Chemical Reagent Co., China.

2.2. Synthesis of SrCS

Fig. 1 illustrates the synthesis pathway of SrCS. The fabrication process includes ion exchange, pH adjustment, CS-strontium chloride reaction and the SrCS precipitation. In the ion exchange procedure, a chromatographic column filled with pre-processed ion exchange resin (732 sodium cation resin) was employed to absorb sodium ions in CS. The CS (10 wt.%, 10 mL) solution was gradually added into the column with carefully controlled flow rate (1 mL/min) to ensure the maximum absorption. The output liquid that mainly contains chondroitin sulfate acid was collected using a clean beaker. With the purpose of improving the reaction conversion rate, pH adjustment was performed by adding suitable amount of strontium hydroxide to ensure the pH value of the collected liquid to be around 6.5. After that, strontium chloride solution was added into the pH-adjusted liquid to exchange residual sodium and hydrogen ions, and formed SrCS. Finally, 95% ethanol was added into the mixture to precipitate SrCS. The collected precipitation was washed with 95% alcohol for at least three times to remove the possibly existed impurities. Before further experiments, the cleaned product was dried in a vacuum oven at 50 °C for 24 h.

Fig. 1

Fig. 1. Synthesis pathway of SrCS (95% ethanol was applied to precipitate SrCS powder from SrCS liquid). The Sr^{2+} ions will substitute Na^{+} ions and bond either $–SO_4–$ or –COO– site (Cael, Winter, & Arnott, 1978).

2.3. Materials characterization

The morphology of the fabricated product was examined using a TESCAN VEGA 3 LMH SEM (Zeiss, Germany). Gold sputtering was applied to enhance conductivity before SEM observation. The quantitative analysis of the strontium composition was conducted using the EDS integrated in the SEM. The crystal structure of the raw materials used for the fabrication and the final products were characterized using a D8 Advance ECO XRD (Bruker, Germany). The Fourier transform infrared (FTIR) spectra of CS and SrCS from 4000 cm^{-1} and 400 cm^{-1} were recorded in KBr at room temperature on a FTIR spectrophotometer Bruker vertex 70 (Bruker, Germany). The powder samples were mechanically pressed to be pellets before FTIR test. The in vitro stability of SrCS was investigated using the previous established protocol (Supplementary Fig. 2) (Hui, Zhang, Niu, Wang, & Sun, 2011).

1H NMR was used to further explore the structure of the CS and the prepared SrCS. The 1H NMR spectra were performed on a Bruker Avance III 400 with D_2O as the solvent. Mnova Software 9.0 (Mestrelab Research) integrated in the NMR was employed for spectral signal processing.

2.4. Cell proliferation test

The primary chondrocytes and osteoblasts were extracted from the articular cartilage disposal of an OA patient after total joint arthroplasty. The culture medium used for chondrocytes was HyClone medium containing DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum, Gemini). Human primary osteoblasts were maintained in DMEM/F12(Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, GIBCO) with 10% FBS. Cells were incubated in an incubator at 37 °C in 5% CO2.

The proliferation tests of OA chondrocytes and osteoblasts treated with various chemicals were performed with the Cell Counting kit-8 (DOJINDO) following the manufacturer's instruction. First, cells were seeded onto the 96well plate with identical density 4 × 104 cells/mL and incubated for 24 h before chemical treatment. After that, cells were treated with different concentrations of CS or SrCS and incubation for another 24 h. For proliferation assay, at each of the designated time points (1, 3 and 5 days), 10 μL of CCK-8 solution was added to 100 μL of culture medium and incubated for 2 h at 37 °C. The absorbance of solution was measured using a microplate reader (Thermo, Multiskn Go) at 450 nm.

2.5. RT-PCR assay

Total RNA from cell and tissue samples were extracted with RNAiso plus following the instructions provided by the supplier. Total RNA samples were used to synthesize cDNA though reverse transcription kit (TAKARA). Briefly, a 10 μL reaction including 0.5 μg total RNA, reverse transcription was performed at 37 °C for 15 min, and then terminated by heating at 85 °C for 5 s.

RT products were performed in triplicate for microRNA or genes to be tested, using SYBR Green I. The PCR was processed at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. In addition, relative quantities (RQs) were measured with the comparative 2△CT method. The primers used for gene detection were as follows:

F: 5′-AGATGAAGTGCTCCTTCCAGG-3′ IL-1β
R: 5′-TGGTCGGAGATTCGTAGCTG-3′ IL-1β
F: 5′-CTCAGCCCTGAGAAAGGAGA-3′ IL-6
R: 5′-TGATTTTCACCAGGCAAGTCT-3′ IL-6
F: 5′-TAGAGGATGTGAGTGGTCTT-3′ ACAN
R: 5′-TCCACTAAGGTACTGTCCAC-3′ ACAN
F: 5′-CCTCTGCGACGACATAATCT-3′ COLII
R: 5′-CTCCTTTCTGTCCCTTTGGT-3′ COLII
F: 5′-AGAAGGCTGGGGCTCATTT-3′ GAPDH
R: 5′-GGTGCTAAGCAGTTGGTGGT-3′ GAPDH

2.6. Animals and hematoxylineosin staining

The SD rats (male, 21 days old) were supplied by Guangdong Medical Laboratory Animal Center (Guangdong, China). The knee osteoarthritis was induced through modified Hulth's OA modeling method. In brief, rat's right knee was skin perpetrated, sterilized and made an inside-incision. After joint-space opening, anterior cruciate ligament transection (ACLT) was

performed to cut off two-thirds of the medial meniscus. The control group was sham-operated. The postoperative rats were started to gavage with CS (1445 mg/kg), SrCS (1700 mg/kg) or vehicle for one month. The designed dose ensures that the amount of given chemical is the same for each group. All animal experiments were with the approval of the Animal Study Committee of Guangdong Medical Laboratory Animal Center.

In the hematoxylineosin staining experiment, knee joint tissues were firstly fixed in 4% paraformaldehyde for 24 h and decalcified in 15% EDTA solution at 4 °C for 3 weeks for HE staining. The tissues were then processed for the standard dehydration, transparency and paraffin-embedding schedule. The paraffin blocks containing the cartilage tissues were cut at 4 μm sections and stained with hematoxylin and eosin.

3. Results and discussion

3.1. Energy dispersive spectrometer (EDS)

We varied the weight percentage of SrCl2 during the fabrication (0, 10, 20, 30, 40, 50, and 60% respectively) to adjust the amount of Sr in the final product. Fig. 2(a) shows the EDS results of the products yielded with various amount of SrCl2. Significant peak of Na was detected in the final product without SrCl2 (0% group), but for the final products fabricated with SrCl2, the Na peaks disappeared. This suggested that the introduction of SrCl2 enables the Na ions be exchanged completely. The atomic weight percentage of both Sr and Cl in the final product increased with the increasing amount of SrCl2, which confirmed that the Sr content in the final product could be adjusted easily by controlling the addition of SrCl2 during the fabrication. The small amount of Cl detected here should mainly be HCl residuals, which cannot be removed during precipitation process in current fabrication protocol. The theoretical value of Sr in SrCS is around 16%, if assume all the –SO4– or –COO– sites are bonded with Sr. Thus the Cl detected in 50% and 60% group should be due to the existence of both HCl and SrCl2. Therefore, the SrCS for 40% group, which should have the highest amount of Sr but without SrCl2 impurity, was used in materials characterization and biological experiments hereafter. It also can be further confirmed by the EDS results of weight ratio of strontium/sulfur in Fig. 2(b).

Fig. 2

Fig. 2. (a) EDS spectra and the corresponding amount of elements (atomic weight percentage) in the final products fabricated with various amount of SrCl2. (b) weight ratio of sodium/sulfur in raw material CS and strontium/sulfur in final product SrCS.

3.2. SEM and XRD

Fig. 3 is the typical SEM images of CS and SrCS. The morphology of SrCS and CS was found to be different: the CS was large particles with irregular shape and rough surface while the SrCS particles were rather small with round shape and smooth surface. Fig. 3(c) shows the XRD diffraction patterns of the raw materials: CS, SrCl2, the physical mixture of SrCl2/CS and the as-prepared SrCS. There are no significant peaks on the XRD pattern of CS, while SrCl2 diffraction patterns have certain significant peaks, which responses to its crystal orientations. The physically mixed SrCl2/CS exhibits similar peaks as those of SrCl2. This confirmed that simply mixing the two stuffs will not change their crystal structures. The XRD result of SrCS exhibits no significant peaks, indicating its amorphous nature. The XRD

characterization together with the EDS results confirmed that strontium ions were successfully introduced to the CS and formed a new complex.

Fig. 3
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Fig. 3. Typical SEM image (a) CS, (b) SrCS and (c) XRD diffraction spectra of CS, SrCl2, SrCS and CS&SrCl2 mixture.

3.3. The structure characterization of SrCS particles
Fig. 4(a) shows the FTIR spectra of the raw material CS and the final product SrCS. FTIR spectra significant absorption peak of CS and SrCS appeared at 3433 cm−1 (OH stretching), 2930 cm−1 (CH2 stretching), 1642 cm−1 (amide I band), 1571 cm−1 (NH deformation), 1066 cm−1 (Csingle bondO stretching). Absorption peak 1257 cm−1 is asymmetric SO2 stretching and 1127 cm−1 should be due to the symmetric SO2 stretching. There are different types of CS, including chondroitin sulfate A, C–E. The characteristic adsorption peaks 928 cm−1 and 852 cm−1 found here belong to chondroitin sulfate A (Mathews, 1958). Blue shifts were found for SrCS FTIR spectra in absorption bands. For instance, peak 1414 cm−1 shifted to 1420 cm−1 and peak 1036 cm−1 shift to 1039 cm−1. Na's electronegativity is slightly lower than that of Sr, and so the blue shifts found in the FITR spectra should be the consequence of Sr-Na substitution. Comparing with the FTIR spectra for CS, peak intensity for SrCS spectrum increased remarkably, which indicates the formation of strong hydrogen bonding. FTIR spectra results therefore suggested that strontium has successfully bonded with CS molecule.

Fig. 4
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Fig. 4. FTIR spectra and 1H NMR spectra (400 MHz, 298 K, D2O) of strontium chondroitin sulfate (SrCS) and chondroitin sulfate (CS).

Representative 1H NMR spectra of SrCS and CS samples are illustrated in Fig. 4(b) for the range 0–6 ppm. Certain characteristic signals in the 1H NMR spectra related to SrCS and CS were observed. D2O solvent peak can be seen in 4.5-4.80 ppm region. Result showed the presence of characteristic peaks CH (3.6 ppm) in SrCS and CS spectrum. Meanwhile, the signal 1.9 ppm can be readily assigned to the methyl protons (CH3CO) in SrCS and CS. The 1H NMR data of CS and SrCS shows resonances at 3.29 ppm and 3.48 ppm, characteristic H-2 and H-3 protons of the glucuronic acid (GlcA). Each spectrum has distinctive features but their respective patterns are identical. This phenomenon indicated their similar position of protons in CS and SrCS. It is therefore believed that chondroitin sulfate and strontium ions were combined to form a polysaccharide-metal ion complex.

3.4. Cell proliferation test
Fig. 5 shows the cell viability of osteoblast and chondrocyte cultured with various concentrations of SrCl2, CS and SrCS respectively. The SrCl2 significantly inhibited the proliferation of both osteoblast and chondrocyte with the increase of concentration. Although the cell viability of both osteoblast and chondrocyte treated by CS or SrCS at various concentrations does not have statistically significant variations, slight improvements on cell proliferation can be observed at certain concentrations. Therefore, we believed that

the existence of CS and SrCS in the culture medium did not inhibit the cell proliferation, and the SrCS should be a safe chemical for clinical trial.

Fig. 5
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Fig. 5. Cell viability of osteoblast (a–c) and chondrocyte (d-f) treated with SrCl2, CS and SrCS at different concentrations. Data are presented as mean ± S.D of the samples from three biological replicates at various treatment concentrations. *represents p value < 0.05, and *** represents p value < 0.001.

3.5. mRNA expression of cell examined using RT-PCR
Fig. 6 is the mRNA expression related to the cartilage formation (COLII, ACAN) and inflammation (IL1β and IL6) of cells after different chemical treatments. We investigated the mRNA expression of collagen type II and aggrecan in human primary chondrocytes, which are the major components of cartilage and extracellular matrix. It is obvious that both COLII and ACAN mRNA expression levels see a remarkable increase after dealing with SrCS, while no significant enhancement of COLII and ACAN mRNA level can be observed in the chondrocytes after CS treatment (Fig. 6(a)–(b)). Conversely, the addition of SrCS or CS lead to a reduction of MMP1 and MMP13 (Fig. 6(e)–(f)), which are essential for cartilage degradation in OA.

Fig. 6
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Fig. 6. Gene expression analysis of OA chondrocytes and osteoblasts after CS or SrCS treatment. Data are present as mean ± S.D for three biological replicates. *represents p value < 0.05, ** represents p value < 0.01, and *** represents p value < 0.001.

SrCS was found to have significant anti-inflammatory effect. Fig. 6(c)–(d) shows the expression levels of IL1β and IL6 of the chondrocytes, which are markedly reduced after SrCS treatment while increased by CS treatment. Similar results were found in osteoblasts treated with SrCS or CS (Fig. 6(g)–(h)). The strong anti-inflammatory effect of SrCS should be attributed to the existence of Sr, which is well-known anti-inflammatory and anti-oxidant (Bruyere et al., 2008, Henrotin et al., 2001). Based on the Cell proliferation and RT-PCR results, it is believed that the SrCS should be a safe and rather effective chemical for OA clinical treatment.

3.6. SrCS or CS attenuates progression of OA in rats osteoarthritis model
To investigate whether SrCS can inhibit progression of osteoarthritis, we employed the rat surgery osteoarthritis model for preliminary in vivo study, and the histological results are shown in Fig. 7. It is clear that there were large extent of AC destruction for each group after surgery-induced osteoarthritis, but the vehicle treated rat cartilage thickness decreased the most, only 308 μm. Compared with vehicle-treated group, CS and SrCS treated rats cartilage increased to 397 μm and 480 μm respectively, indicating that both CS and SrCS may have beneficial effects for the recovery of AC suffering surgery-induced OA. Moreover, SrCS-treated rat cartilage significantly increased in non-surgery rat group compared to CS-treated rat, suggesting that SrCS may have a better impact on OA cartilage remodeling. The in vivo

results thus indicate that the SrCS might be an effective drug for OA pathogenesis, and this is consistent with the previous in vitro results (Fig. 8).

Fig. 7
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Fig. 7. AC destruction in surgery-induced OA rat model after different treatment by CS or SrCS.

Fig. 8
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Fig. 8. Model of the effects of SrCS in attenuating the degradation of OA cartilage. The Sr in SrCS is anti-inflammation and can help in maintaining the bone metabolism and simulate collagen formation; the CS is also helpful in the collagen formation. Therefore, SrCS should be an effective chemical for OA treatment.

4. Conclusions

In this study, SrCS, a new complex that is the combination of two effective chemicals (CS and Sr) in OA treatment was fabricated using ion exchange method. The material characterization of SrCS were performed using SEM, XRD, EDS, etc.; it was found that the CS was successfully integrated with Sr, and the amount of Sr in the final product can be well controlled by the amount of $SrCl_2$ adopted during the fabrication. The results of cell experiments showed that the fabricated SrCS is biological safe and effective in anti-inflammation. Moreover, it can effectively simulate the collagen formation and attenuate the degradation of OA AC. Preliminary animal study results agree with those found in vitro. Therefore, it is believed SrCS fabricated in this study should be a promising chemical in OA related biological applications.

Competing interests

The authors declare no competing financial interests.

Contributions

Tang B. and Wen C.Y. designed the work; Ma F.B. performed the materials related experiments, Liu N. and Hu N. performed the biological related experiments. All authors contributed to the data analysis. Ma F.B., Liu N., and Tang B. wrote the manuscript.

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