The Investigation of LRP5-Loaded Composite with Sustained Release Behavior and Its Application in Bone Repair

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Low-density lipoprotein receptor-related protein 5 (LRP5) plays a vital role in bone formation and regeneration. In this study, we developed an injectable and sustained-release composite loading LRP5 which could gelatinize in situ. The sustained release of the composite and its efficacy in bone regeneration were evaluated. Sodium alginate, collagen, hydroxyapatite, and LRP5 formed the composite LRP5-Alg/Col/HA. It was found that the initial setting time and final setting time of LRP5-Alg/Col/HA containing 4% alginate were suitable for surgical operation. When the composite was loaded with 40 μg/mL LRP5, LRP5-Alg/Col/HA did not exhibit a burst-release behavior and could sustainably release LRP5 up to 21 days. Up to 18 days, LRP5 released from LRP5-Alg/Col/HA still present the binding activity with DKK1 (Wnt signaling pathway antagonist) and could increase the downstream β-catenin mRNA in bone marrow mesenchymal stem cells. Moreover, LRP5-Alg/Col/HA was found to significantly increase bone mineral density in the defect area after 6 weeks’ implantation of LRP5-Alg/Col/HA into the rats’ calvarial defect area. H&E staining detection demonstrated that LRP5-Alg/Col/HA could mediate the formation of a new bone tissue. Therefore, we concluded that Alg/Col/HA was a suitable sustained-release carrier for LRP5 and LRP5-Alg/Col/HA had a significant effect on repairing bone defects and could be a good bone regeneration material.

1. Introduction

With the development of society and the consequent industrial accidents, traffic accidents, and natural disasters, the number of patients with orthopedic trauma has also increased. Moreover, bone tumors [1] and orthopedic diseases such as skeletal tuberculosis and avascular necrosis have caused numerous patients with bone defects. Therefore, bone defects are not only a severe disease that potentially shorten the life span of individual patients but also an important public health issue that concerns the society. With the aging of the population, the improvement of people’s health awareness and consumption capacity, and the improvement of the national medical security system, the demand for bone repair materials has increased dramatically [2]. In recent years, a variety of synthetic bone repair biomaterials have been widely used in clinics, and the injectable bone repair material is the most important one [3]. Firstly, the injectable bone repair material can be implanted into the body by injection with insignificant trauma, therefore eliminating many complications associated with traditional bone transplantation surgery. Secondly, it has good plasticity, which can fill bone defects of any shape or size, and can be gelatinized by physical or chemical action-mediated sol-gel phase transformation, forming a scaffold material with porous microstructure and exerting bone conduction [4, 5]. Finally, the implanted materials can degrade slowly, resulting in a hydrated network structure that can better simulate the physical and chemical microenvironment of the extracellular matrix and promote cell proliferation, differentiation and secretion of new extracellular matrix with efficient mass transfer performance [4, 6], good regeneration activity to damaged tissue [7, 8], and growth activity for a new bone in the host. Therefore, injectable materials have various
advantages and have attracted increasing attention as carriers capable of carrying compounds, macromolecular drugs, proteins, and cells.

Sodium alginate (NaAlg or Alg) is a cell wall component of brown algae with a chemical composition of 1,4-glycosidically linked β-D-mannuronate (M) and α-L-guluronate (G) combined linear chain anionic polymer, which has a molecular weight of about 50,000 to 250,000 Daltons [9]. The molecular formula is \( (C_6H_7O_5Na) \). Figure 1 shows the monomers constituting the alginate polymer. Alginate is easily bonded to most divalent cations, such as Ca\(^{2+}\), Ba\(^{2+}\), and Cu\(^{2+}\), to form an ion cross-linked alginate hydrogel [10], which is metabolized into monomers of mannose and glucuronic acid by enzymatic hydrolysis in vivo that are nontoxic to humans [11]. In addition, alginate has the advantages of no immunogenicity and a certain biological sustained release [12]. However, alginate hydrogel shows a significant burst-release effect after swelling [13–15]. According to Tan’s report [16], a gel-like composite containing alginate, collagen, and hydroxyapatite (HA) is prepared with sustained-release and injectable properties. On the one hand, collagen and hydroxyapatite act as a “stable stent” to effectively reduce the swelling of the alginate and improve the sustained-release effect of alginate hydrogel. On the other hand, the injectability property of alginate hydrogel can offset the disadvantages of collagen and hydroxyapatite and solid property and poor plasticity, although collagen and hydroxyapatite have the advantages of biodegradability, good biocompatibility, and biological activity [17, 18]. Additionally, local injection is a simple and effective minimally invasive surgery with low complications. Therefore, alginate hydrogel can be combined with collagen and hydroxyapatite to prepare a gel composite suitable for bone tissue repair.

Low-density lipoprotein receptor-related protein 5 (LRP5) is a member of the low-density lipoprotein receptor-related protein (LRP) family and is widely expressed in a variety of tissues, including the fibroblasts, macrophages, central nervous system, digestive tract epithelial cells, liver, and kidney [19]. In the bone tissue, LRP5 is mainly expressed in the osteoblasts on the endosteal and trabecular bone surfaces, but not in the osteoclasts that are not conducive to bone repair. The role of LRP5 is mainly to promote the accumulation of bone mass, and its loss-of-function mutation can lead to a decrease in bone mass [20], while a functionally acquired mutation can increase bone mass [21–23]. A peptide derived from an LRP5 gene also enhances stem cell aggregation and chondrogenic differentiation [24]. This effect is mainly through the Wnt signaling pathway. The Wnt and LRP5/6 complex regulate the classical β-catenin signaling pathway, which plays a vital role in the bone differentiation of the bone marrow mesenchymal stem cells, osteoblast proliferation, or apoptosis, and maintain normal bone. LRP5/6 activation is inhibited by secreted proteins belonging to the Dickkopf (DKK) family [25, 26]. Fleury et al. [27] showed DKK1 and LRP5 interaction in vitro. It suggests that the interference with LRP5/DKK1 interaction can be a viable approach for maintenance of normal Wnt signaling pathway and therapeutic intervention to increase bone mass. Clinically, bone defects with varying degrees of bone loss are extremely common and frequent, and the bone tissue has a limited self-repair ability. Therefore, various studies have tried to apply a variety of exogenous beneficial proteins to local bone defect areas to promote osteogenesis. However, the efficacy of LRP5 still lacks research reports on bone defect repair.

In this study, a composite containing alginate, hydroxyapatite, and collagen was used as a carrier. After loading with LRP5, the sustained-release capability, biocompatibility, and repair of bone defects in vivo were studied.

2. Method

2.1. LRP5-Alg/Col/HA Preparation. The preparation of calcium sulfate slurry consists of weighing calcium sulfate (Sigma) and deionized water, electromagnetic stirring until no obvious particles are seen, leaving for more than 24 hours, removing the static electricity, and standing for further use.

At less than 10°C, the bovine type I collagen (Sigma) was dissolved in HCl of pH = 2 and prepared an acidic collagen solution with a concentration of 5 mg/mL. Thereafter, Na\(_4\)PO\(_4\)·12H\(_2\)O solution and a certain proportion of sodium alginate (Sigma) solution were sequentially added with stirring. After that, NaOH solution was added to adjust the pH to 7.4, and different concentrations of LRP5 (Novus Biologicals) or BSA were added as needed. Afterward, hydroxyapatite slurry was added in equal volume and mixed evenly.

Finally, the above mixture was uniformly mixed with the calcium sulfate slurry and allowed to stand for 15 min. The inside of the mixture was cross-linked in situ to obtain a solid composite suitable for bone tissue repair.
bone repair material, which was named LRP5-Alg/Col/HA. The LRP5-Alg without collagen and hydroxyapatite was prepared in the same manner as above. BSA-Alg/Col/HA was prepared in the same manner as above with a different concentration of BSA. The final concentrations of each substance were calcium sulfate (5 mM), collagen (2.5 mg/mL), Na3PO4 (1.3 mM), alginate (2%, 3%, 4%, and 5%), and hydroxyapatite (10 mg/mL).

2.2. The Injectability Study. At 37°C, the gelation time of the liquid after mixing with the calcium sulphate slurry was recorded including the initial setting time and final setting time, using a tube rotation method.

2.3. Sustained-Release Behavior Investigation. BSA was used as a model protein to be loaded into Alg/Col/HA, and the release of protein was studied. BSA-Alg/Col/HA with a final concentration of 10, 20, 40, 80, 150, and 300 μg/mL BSA was prepared. 5 mL of saline was added as a drug-release medium in a test tube. 1 mL BSA-Alg/Col/HA gel was injected and placed in a 37°C water bath shaker (60 r/min) for 2 weeks. At 0.25, 1, 3, 6, 9, 12, and 15 days, 0.5 mL of the release medium was taken out, and an equal amount of the fresh saline solution was added. The BCA colorimetric method was used to detect the BSA concentration, and the OD value of the solution was measured at a wavelength of 570 nm. The BSA concentration in the solution was converted according to the standard curve of the concentration of the BSA solution.

The method for the sustained-release manner of LRP5-Alg/Col/HA or LRP5-Alg (no collagen and hydroxyapatite) was the same as above. The release time was recorded until 3 weeks, and the concentration of LRP5 in the release solution was detected at 0.25, 1, 3, 6, 9, 12, 15, 18, and 21 days. The method was performed by the enzyme-linked immunosorbent assay kit (PeproTech, USA) according to the manufacturer's instructions. The concentration of LRP5 in the released solution was determined by comparing with the standard curve; calculate the percentage of total BSA or LRP5 released at each time point and plot the cumulative release profile.

2.4. DKK1 Binding Activity Detection of LRP5 Released from LRP5-Alg/Col/HA. At day 1, day 6, day 12, and day 18, saline was replaced with DMEM. After 24 hours, DMEM was collected and filtered as the conditioned medium. The binding activity of LRP5 to DKK1 was detected using a competitive assay. After the overnight incubation of 10 yg/mL DKK1 solution in a 96-well plate, the plate was washed 3 times and then blocked using 5% BSA for 4 hours. Then, different conditioned mediums and 50 ng/mL LRP5-FITC were added and incubated for 2 hours at room temperature. After washing, FITC fluorescence was read immediately using an Envision plate reader.

2.5. Biological Activity of LRP5 Released from LRP5-Alg/Col/HA. The biological activity of LRP5 in the conditioned mediums collected at different times was indirectly evaluated by the expression level of the downstream gene β-catenin of the rat bone marrow-derived stem cells (MSCs).

Rat MSCs (Fuyang Biotech, Shanghai, China) were seeded into 6-well plates at a concentration of 1 × 10^5 cells/well, incubated in a DMEM medium containing 10% fetal bovine serum and penicillin–streptomycin at 37°C in a 5% CO2 incubator. After 24 hours of culture, the medium was removed, and the conditioned mediums of the Alg/Col/HA sample without LRP5 at different times were added as the negative control, and the conditioned mediums of the LRP5-Alg/Col/HA at different times were used as the experimental. Then, Wnt3a and DKK1 were added into every well of the final concentration of 100 ng/mL. After 2 days culture, the medium was removed and the cells were collected for detection. Then, RT-qPCR assay was preformed to detect the gene level of β-catenin.

2.6. Real-Time Quantitative PCR (RT-qPCR). Total RNA from rat MSCs were extracted using TRIzol (Invitrogen). First-strand cDNA was made using SuperScript III (Invitrogen). qPCR was run on the ViiA Real-Time PCR (Applied Biosystems) using the SYBR Green method. The β-catenin relative expression level was calculated by comparing the cycle times to those of β-actin. PCR primers were listed as follows:

forward5′-ACCTCCCAAGTCTTTATG-3′ and reverse5′-TACAACGGGCTGTTTCTAC-3′, for β-catenin

and forward5′-CCAGAGCAAGAGAGGCATC-3′ and reverse5′-CTCAGGAGGCAATGATCT-3′, for β-actin.

2.7. Bone Defect Model Preparation and Drug Administration. Twenty male Sprague-Dawley rats were randomly divided into 4 groups, with 5 rats in each group. Except for 5 healthy controls, in the other 3 groups, the rat parietal and frontal bones were exposed after the median incision of the skull. The parietal bone was made at 5 mm of full-thickness bone defect, and the dura mater should be kept intact. Three different experimental materials were randomly implanted; then, the periosteum, soft tissue, and skin were sutured layer-by-layer. Rats in the vehicle group were not treated; rats in the Alg/Col/HA group were implanted with Alg/Col/HA without LRP5; and rats in the LRP5-Alg/Col/HA group were implanted with LRP5-Alg/Col/HA. Rat activities, diet, mental state, and incision status were observed daily for 6 weeks.

2.8. Bone Mineral Density (BMD) Testing. The rats were euthanized at 6 weeks, and cranial bone samples were taken for microCT scan to analyze BMD of the rat calvarial defect area. The scanning conditions were 55 kVp, 109 μA, 10.5 μm resolution, and 200 ms exposure time.

2.9. Hematoxylin-Eosin (H&E) Staining. The formalin-fixed rat cranial bones were thoroughly washed with PBS and decalcified in a solution of 10% EDTA (pH 8.0) at 4°C for 20 days. The samples were treated with ethanol gradient dehydration, xylene transparent, and paraffin-embedding, continuous longitudinal tissue section (5 μm thickness), and then H&E staining was performed. The bone tissue structure of each group was observed under a microscope.
2.10. Statistical Analysis. SPSS19 statistical analysis software was used. Data was analyzed by the 2-tailed t-test or one-way analysis of variance (ANOVA), followed by the Tukey post hoc comparisons. Data was presented as mean ± SD, and P values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effect of Alginate Concentration on Gelation Performance. After the cations are added to the aqueous sodium alginate solution, Na+ on the α-L-guluronate (G) unit undergoes an ion-exchange reaction with the divalent ion, and the α-L-guluronate residues accumulate to form a cross-linked network, thus transforming into a hydrogel. In this process, Ca2+ is captured to form calcium alginate gel, which can inhibit the water flow. In this study, we examined the effect of different alginate concentrations on the gelation time of the composite according to the optimal criteria of solidification time including (1) “3 min initial setting time < 8 min” and (2) “final setting time ≤ 15 min” [28]. As shown in Figure 2(a), the Alg/Col/HA gelation rate was positively related to the alginate concentration. The higher alginate concentration induced the faster gelatinization. When the alginate concentration was 2%, 3%, 4%, and 5%, the initial setting times of the composite were 578 ± 14.3 sec, 298.5 ± 12.3 sec, 254.3 ± 10.5 sec, and 204.7 ± 12.7 sec, respectively, and the final setting times were 1,223.1 ± 54.9 sec, 682.4 ± 23.1 sec, 603.7 ± 12.3 sec, and 432.8 ± 14.6 sec, respectively. It indicated that 3%~5% of alginate concentration could provide relatively controllable initial and final setting times, which were suitable intervals for the surgical operation [28]. As the alginate concentration increased, the injectable ability of Alg/Col/HA decreased. The main reason is that the viscosity increases along with the increase of alginate [29]. However, when alginate concentration is too low, the formation of the network structure and the release efficiency are also affected. Therefore, the composite material
with 4% alginate concentration was selected as the material for subsequent research. Because the isoelectric point (pI) of LRP5 (5.11) is similar to BSA pI (4.70) and BSA is easy to obtain, we use BSA as the model protein to verify whether the loaded protein can affect the gelation time of the composite with 4% alginate concentration. As shown in Figure 2(b), when BSA concentration was in the range of 10~300 μg/mL, it was found that there were no obvious change trends in the initial and final setting times. It indicated that loaded protein had no influence on the gelation time.

3.2. Sustained-Release Performance Study of LRP5-Alg/Col/HA. As a protein, LRP5 is prone to be degraded. In order to overcome this, a few sustained-release carrier loaded LRP5 have been reported [24]. In this study, we investigated the in vitro sustained LRP5 release behavior of LRP5-Alg/Col/HA. Based on feasibility and cost considerations, BSA was first used as a model protein to study the feasibility of Alg/Col/HA as a protein sustained-release carrier. As shown in Figure 3, as the amount of loaded BSA increased, the release rate of BSA increased. It took 15 days to observe BSA release from BSA-Alg/Col/HA loaded with different amounts of BSA. When BSA concentrations were set at 20 μg/mL and 10 μg/mL, the cumulative release of BSA could exceed 60% and 40% within 2 weeks, respectively. When BSA concentration was 40 μg/mL, the cumulative release of BSA could reach more than 80% within 2 weeks. However, samples with BSA concentration of 80 μg/mL and 150 μg/mL, the cumulative release reached 80% within 1~1.5 weeks. Therefore, in the range of BSA concentration of 10~40 μg/mL, BSA-Alg/Col/HA had considerable controlled-release properties, and this concentration range might be a feasible condition to prepare LRP5-Alg/Col/HA.

Considering the largest loading and considerable sustained release, 40 μg/mL LRP5 was loaded into LRP5-Alg/Col/HA. In Figure 4, LRP5-Alg/Col/HA showed a good sustained-release property at a relatively stable release rate for 21 days. No burst release of LRP5 was found on day 1 when compared to the same amount of loaded LRP5 in LRP5-Alg. However, the burst-release effect of LRP5-Alg without collagen and hydroxyapatite was obvious, and the cumulative release of LRP5 was nearly 80% in the first 6 hours and 96% in 24 hours. Previous reports have studied that alginate hydrogels have controlled-release properties, but their burst-release effects are apparent, due to the excessive swelling of the hydrogel [13]. In this study, the alginate hydrogel was modified by the addition of collagen and hydroxyapatite. Collagen and the alginate form an interpenetrating polymer network (IPN) [30, 31]. The two polymers are intertwined and entangled through the network, which retain not only the secondary structure.
of collagen but also the network structure of calcium alginate, ensuring the normal biofunction of both polymers. Furthermore, collagen and hydroxyapatite also act as a “stable stent” for calcium alginate gels due to their significant ability of water absorption. Collagen absorbs water and greatly reduces the excessive swelling of the alginate gel, thereby increasing the sustained release of the composite. On the other hand, collagen and hydroxyapatite also adsorb protein drugs and improve the sustained-release performance.

3.3. Bioactivity of LRP5-Alg/Col/HA. The biological activity of LRP5 is mainly achieved by the Wnt signaling pathway. During the osteogenesis process, the Wnt/LRP5/β-catenin pathway plays an important role. The normal function of the Wnt/LRP5/β-catenin pathway mediates intracellular signals through LRP5 and stabilizes intracellular β-catenin, which enters the nucleus to bind to transcription factors, thereby regulating the expression of genes involved in osteoblast proliferation and functionality and promoting skeletal development. Loss of function of LRP5 leads to osteoblast dysfunction, which affects the accumulation of bone mass [32–34]. DKK1, a Wnt antagonist, has been reported to specifically inhibit the signaling pathway by binding to LRP5, whereas the exogenous LRP5 prevents the interaction of endogenous LRP5 with DKK1. In order to study whether released LRP5 from LRP5-Alg/Col/HA still remained biologically active, we tested both the binding and biological activities of released LRP5 by competitive binding assay and cellular assay, respectively. Through the binding assay, FITC fluorescence increased on days 1, 6, 12, and 18, indicating that the LRP5 binding activity to DKK1 decreased over time in the conditioned mediums (Figure 5(a)). In cellular assay, the conditioned mediums containing released LRP5 were cultured with MSCs, and the downstream mRNA expression level was detected to indirectly identify LRP5 bioactivity. On days 1, 6, 12, and 18, compared with the Alg/Col/HA control group (Figure 5(b)), the LRP5-conditioned mediums in the LRP5-Alg/Col/HA group significantly increased the mRNA level of β-catenin in MSCs, indicating that LRP5-Alg/Col/HA could maintain the biological activity of LRP5 up to 18 days. Further, it was found that LRP5 release showed a uniform release rate during 1, 6, 12, and 18 days according to Figure 4. However, in Figure 5, the downstream β-catenin mRNA expression level decreased along with the release time, which was not consistent with the in vitro release. It was demonstrated that the released LRP5 has certain attenuation on biological activity over time. Taken together, it still had acceptable retention of binding activity and bioactivity, which was confirmed by the in vitro and cellular experiments in Figure 5. Overall, Alg/Col/HA was a good sustained-release carrier of LRP5, which not only had good controlled-release properties but also maintained the biological activity of LRP5.

3.4. Effect on the BMD of LRP5-Alg/Col/HA in Bone Repair. Compared with other bone defect models, the rat skull critical-size defect model is a reliable model for evaluating the repairing ability of bone biomaterials. This model does not have the ability to repair itself and requires certain material filling support to achieve healing [35–39]. Moreover,
experimental operation is relatively easy and repeatable. Therefore, we used this model to evaluate the ability to induce the osteogenesis of the LRP5-Alg/Col/HA composite. During the surgery, the composite could form into gel state in the defect area after injection. It implied the applicable possibility of LRP5-Alg/Col/HA in minimally invasive surgery to treat other types of bone defects according to the injectability of the LRP5-Alg/Col/HA composite. At 6 weeks, as shown by BMD measurement in the defect area in Figure 6, the density of the defect area in the LRP5-Alg/Col/HA group was significantly improved (P < 0.05) compared with the vehicle group, which was still a large gap in density compared to normal cranial bone density. Additionally, the improvement effect of LRP5-Alg/Col/HA was extremely obvious, and the bone density was significantly increased (P < 0.05) when compared with the Alg/Col/HA group. The histological evaluation results (Figure 7) revealed that the Alg/Col/HA group had an obvious new trabecular bone formation at the defect site, and no cartilage-like tissue was observed. Also, no cartilage-like tissue was observed in the LRP5-Alg/Col/HA group, but the trabecular bone distribution direction was more consistent, and the trabecular bone width was more uniform. In the vehicle group, the defect site was filled with fibrous tissue (black arrow in Figure 7), which affected cellular infiltration and growth. The results of the histological evaluation (Figure 7) and BMD (Figure 6) confirmed that LRP5-Alg/Col/HA had a potent ability to induce osteogenesis and had a great healing effect on the repair of bone defects. On the one hand, LRP5-Alg/Col/HA materials could facilitate the formation of a new bone in the defect site. Moreover, LRP5-Alg/Col/HA had good biocompatibility and osteogenic capability. In addition, the sustained release of LRP5 accelerated osteogenesis in vivo, which was consistent with previous reports. In the femoral fracture report [40], the healing effect of the whole-knockout LRP5 mice was significantly weaker than that of the wild-type mice, the healing area was smaller, and BMD was lower in the region.

4. Conclusion

LRP5-Alg/Col/HA has good injectability and is capable of the sustained release of LRP5 for up to 3 weeks and maintains the biological activity of LRP5 for more than 2 weeks. Furthermore, LRP5-Alg/Col/HA can promote the formation of a new bone in the rat calvarial defects and promote bone mineral density increase.

Data Availability

All the data is available with the handwritten notebook documented in our lab and could be provided from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Yanhai Xi and Tingwang Jiang are equal contributors to this work.

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