Research on a novel chitosan microsphere/scaffold system by double cross-linkers

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RhBMP-2 has shown great promise for the reconstruction of teeth segmental bone defects due to its osteoinductive properties. But the application of rhBMP-2 is limited by its weak drug controlled release. It is usually loaded in a Chitosan Microspheres (CMs) delivery system with excess single cross-linker and then removed before practice. In this study, cross-linkers were replaced with RhBMP-2 which contains vanillin and vitriolic acid, and thus CMs were developed. The materials were studied by SEM, FTIR and drug release experiments. It showed an ideal releasing profile and excellent osteoconductive and osteoinductive performance in the delivery system. Therefore, designing biomaterials with a controllable delivery system composite and releasing profile of rhBMP-2 are critical for applications of bone regeneration and tissue engineering.

Keywords: rhBMP-2, Chitosan microspheres, Double cross-linkers, Delivery

INTRODUCTION
Owing to dramatic development in dental implants placements and alveolar ridge augmentation procedures in the past two decades, there is an increasing demand for adequate bone grafting materials. Large quantities of bone grafting materials that contain hydroxyapatite (HA) as the main natural bone mineral1-3 have been studied. Alzubaydi et al.4-6 reported that HA scaffold can adsorb cells and thus control the release of protein drugs for application in bone tissue engineering. The bone ingrowth from the surrounding bone tissue into the porous scaffold results in biological anchoring.

However, the HA scaffold shows poor osteoinduction in the clinic. Recombined human bone morphology protein 2 (rhBMP-2) is required to be introduced to HA scaffold. RhBMP-2 delivery has been stimulated by the need for more effective treatment in pathologies of the poor osteoinduction7. Recently, an increasing amount of studies have focused on the BMP-2 carrier, especially microspheres. They are small spherical monolithic systems with a particle size range from 0.1 to 1,000 μm8. The main advantages include controllable release of content, good drug protection, long duration of action, high therapeutic efficiency, etc.

There are various methods for preparing microspheres. Chitosan polymers with the smallest low-density microspheres were reported to be synthesized via the emulsion cross-linking method. Common cross-linkers are the glutaraldehyde (GA)9,10, HCl solution11, vitriolic solution12, tripolyphosphate solution (TPP)13,14. The remaining unreacted cross-linkers could thus have potential toxicity or other undesirable effects. However, these excess cross-linker are removed by dialysis or chemical reaction methods prior to practice.

To obtain CMs with biocompatibility, double cross-linkers were used by an emulsion cross-linking method in this study. Previous toxic single cross-linker are replaced by 3-Methoxy-4-hydroxybenzaldehyde (vanillin) and vitriolic acid since vitriolic acid can adjust pH and reduce the potential toxicity of the cross-linker. CMs with loaded rhBMP-2 have been embedded within HA scaffolds for bone repair in segmental defects. The releasing behavior of composite formulation was investigated. Chondrocytes both in vitro and in vivo were characterized.

MATERIALS AND METHODS
Materials
Chitosan (Mw=100 kDa, deacetylation degree: 85%) was obtained from Jinan Haidebei Marine Bioengineering (Jinan, China); rhBMP-2 (Medtronic, USA). All other chemicals have an analytical grade.

Preparation of materials
CMs were prepared using the emulsion cross-linking method. Chitosan and rhBMP-2 were dissolved in 2%
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study were in compliance with approved protocols by the
controlled room. All procedures used in this animal
cage were placed in a ventilated, temperature
Laboratory Animal Technology) were housed in
Three adult male healthy mongrel dogs (Vital River
In vivo experiments

fluorescence microscope.

22, 5-diphenyltetrazolium bromide (MTT) assay. Then
was measured using a 3-(4, 5-dimethylthiazol-2-yl)
contrast microscope (Olympus IX81). Cell viability
Cell morphology was assessed using an inverted phase

scaffolds were evaluated using mouse embryos
In vitro
cell toxicities of HA and CMs/HA/BMP composite
scaffolds were evaluated using mouse embryos
osteoblast precursor cells (MC3T3-E1) as model cells. Cell
cell morphology was assessed using an inverted phase
contrast microscope (Olympus IX81). Cell viability
was measured using a 3-(4, 5-dimethylthiazol-2-yl)
22, 5-diphenyltetrazolium bromide (MTT) assay. Then
F-actin specific cytoskeleton staining was recorded by a
fluorescence microscope.

In vivo experiments

Three adult male healthy mongrel dogs (Vital River
Laboratory Animal Technology) were housed in
cage were placed in a ventilated, temperature
controlled room. All procedures used in this animal
study were in compliance with approved protocols by the
School of Biological Science and Medical Engineering
Committee on the use of Laboratory Animals. The teeth
were assigned into HA, CMs/HA/BMP and a control
group used 876 | 678 tested teeth. At the end of the
eighth week, all animals were killed and their test teeth
socket bone tissue were excised and kept in 10% formalin
for histopathological examination.

Ethics statement
All experiments involving the use of animals
were in compliance with Provisions and General
Recommendation of Chinese Experimental Animals
Administration Legislation and were approved by
Beijing Municipal Science & Technology Commission
(Permit Number: SCXK (Beijing) 2006-0008 and SYXXK
(Beijing) 2006-0025).

Statistical analysis
Data were pooled from at least three independent
experiments and presented as mean±standard deviation
(±s) unless indicated otherwise. Differences between
groups were analyzed using one-way analysis of
variance. All the statistical analyses were performed
with SPSS13.0. *p<0.05 was considered statistically
significant.

RESULTS

SEM of materials
The shape and surface microstructures of all CMs were
shown in Fig. 1. It was notable that the CMs prepared by
vanillin are more round and spherical in shape and also
have smoother surface than those prepared by double
cross-linkers. CMs that were prepared with double cross-
linkers with rough, creased and non-porous structure
were chosen in this study for further research through
comparative study. In Fig. 2, SEM images showed that
the surface of CMs/HA/BMP were denser than that of
pristine HA scaffold substrate, and CMs with smoother
surfaces had tight immobilization onto the CMs/HA/
BMP scaffold surface.

FTIR analysis
FTIR spectra of CS, Vanillin, H2SO4, and CMs which
are prepared with double cross-linkers were studied in
Fig. 3. The peaks at 1,383 cm−1 and 1,210 cm−1 were the
typical band C−N and the bands at 1,154 cm−1 confirmed the
C=N stretching vibration was observed at 1,643 cm−1
in Fig. 3D confirmed the C=N stretching vibration was
the characteristic bond of a Schiff base formed by the
cross-linking reaction. The positively charged amino
group of chitosan as observed with the NH 3+ bending
vibrations (1,550 cm−1) interacted with the negatively
charged SO2−

of H2SO4 acid (1,050 cm−1 and 1,210 cm−1). The chemical reaction route was shown in formula 1.

Also, the schematic diagram of the reaction between CS
and vanillin was presented in Fig. 4A for Schiff base
reaction and hydrogen band formation; B delegates the
cetalization.
Fig. 1 SEM images of (A) CMs prepared with one cross-linker, (B) CMs prepared with double cross-linkers (A1-B1:×10,000; A2-B2:×20,000).

Fig. 2 SEM images of (A) pristine HA scaffold substrate (×400), (B) CMs/HA/BMP surface (×400), (C) the CMs immobilized onto HA scaffold surface (×3,000).

Fig. 3 FTIR spectra of: (A) CS, (B) Vanillin, (C) H2SO4 and (D) CMs (double cross-linkers).

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\text{CS—NH}_2+\text{CH}_3\text{COOH} \rightarrow \text{CS—NH}_3^++\text{CH}_3\text{COO}^-
\]

**The profile of release kinetics of rhBMP-2**

Figure 5 showed rhBMP-2 released from the CMs/HA/BMP scaffolds for over 28 days. The fast-release phase focused on the initial 7 days. It was up to 38.5±2.1% of the total cumulative amount protein. From the 7th day to the 14th day, the accumulative release achieved nearly 47.7±3.4%. The next 7 days, the cumulative release was close to 59.3±2.8%. Overall these data indicated a dual-period release (fast-release and slow-release periods). The release kinetics of rhBMP-2 might be related to the pattern of bone growth16).

**Cell culture**

Compared with other groups, there was obvious cell growth in the CMs/HA/BMP group in the five-day period, which indicated no toxicity of CMs. When the CMs were introduced into the HA, a large amount of cells proliferated similarly showed in Fig. 6. By the 5th day,
the population of cells increased and these cells were fully attached to the disc. Obviously, the CMs with HA composite have no negative effect on the cell morphology, viability, and proliferation and thus it resulted in an increase in cell growth. After staining, CMs/HA/BMP composite showed cytoskeletal reorganization after 7 days of incubation as shown in Fig. 7. This indicated the double cross-linkers synthesized composite has excellent biocompatibility without toxicity.

**In vivo analysis**

After 8 weeks, the tooth socket was excised from dogs for histological analysis. Tissues were detected with toluidine blue stain in Fig. 8. The tooth socket was fully filled with fibrous tissue. For the HA group, a small amount of new bone (NB) tissue around the implant materials in Fig. 8B. The bone plates arrangement was irregular. The texture of bone trabecula was remarkably clearer (Fig. 8C) than that of control group. Once rhBMP-2 loaded CMs/HA/BMP were implanted, some blood cells and macrophages started to gather followed by the generation of a few chondrocytes or soft tissues around the materials. The amount of NB increased at 8 weeks, and the NB was well integrated with the materials. There appeared to be a greater amount of new bone formed in the defects implanted with the rhBMP-2 loaded CMs/HA/BMP in Fig. 8C.
Fig. 7 Fluorescence images of the cytoskeletal organization of MC3T3-E1 cells co-cultured with materials after 5 days. (A) Control group, (B) HA, (C) CMs/HA/BMP. MC3T3-E1 cells treated with medium containing 200 μg mL⁻¹ materials. Cells stained for F-actin (red) and nucleus (blue). The bar indicates 50 μm.

Fig. 8 Histological images by HE-stained tissue sections of tooth socket after implanting operation for eight weeks. (A1 and A2) Control group, (B1 and B2) HA, (C1 and C2) CMs/HA/BMP. NB: newly formed bone; M: materials. The bar on A1, B1 and C1 indicates 200 μm. The bar on A2, B2 and C2 indicates 100 μm.
DISCUSSION

The reconstruction of dental segmental bone defects remains an important field of study. There is an increasing demand for bone grafting materials. HA which is the main natural bone mineral is reported to occupy high osteoconductive and osteoinductive properties. The application of rhBMP-2 is hampered by the fast release of protein drugs. The functionality of BMP-2 with low dose effective loading is dependent on the carrier. Due to the biocompatibility and injectability, CMs present a potential drug delivery system in bone tissue engineering.

Some scientists demonstrated the synthesis of capectabine-loaded semi-IPN hydrogel microspheres of chitosan-poly (ethylene oxide-acrylamide) using GA and HCl solution. NaOH solution and GA solution also could be used as the cross-linkers by emulsion cross-linking method. In our delivery system, vanillin and vitriolic ac. acid were used as double cross-linkers. Vanillin showed no significant cytoxic effects at the concentrations present in the CMs in vitro. Vitriolic acid adjusted pH for further cross-linking to reduce the amount of cross-linker.

The CMs showed a loose topography with the rough structure in Fig. 1. Pores were created on the surface of the CMs when the concentration of vitriolic acid increased. It could also be found that CMs with specific surface areas were significantly more efficient.

As chitosan emulsions were introduced to a vanillin solution, specific cross-linking was performed on a blend of HA and chitosan: GA with −C=O at the interface reacts with the −NH₂ group of chitosan in the presence of a reducing cross-linker, then CMs were formed at a very fast rate. Then, these microspheres underwent cross-linking and modification after introduction to a vitriolic acid solution. The presence of vitriolic acid also had an effect on the mechanical properties of the composite system. pH value was maintained with the contribution of the −NH₂ groups of the external CS. Regardless of the mechanism, the addition of vitriolic acid made it possible to tailor both the mechanical properties and degradation rate of the CMs.

RhBMP-2 was released from the CMs/HA/BMP scaffolds continuously for over 28 days and the released kinetics were depicted in Fig. 5. The composite system CMs/HA/BMP exhibited a two-phase release process of the encapsulated rhBMP-2. First, an initial burst from the partially swollen surface CMs, followed then by a slower gradual release as the CMs eventually degraded over time.

The CMs with 5% vitriolic acid were chosen for biocompatibility and in vitro bioactivity studies, and rhBMP-2 was loaded in the CMs. The animal studies were used for evaluation of new bone formation. An in vitro cell toxicities test showed that much more cells are adhered on CMs/HA/BMP than those on HA and analysis indicated that CMs/HA/BMP composites were not cytoxic to MC3T3-E1 cells and possessed excellent biocompatibility. Surface roughness plays an important role in cell adhesion on materials. Cells are able to distinguish subtle differences in surface roughness since a rougher surface is preferred over a smooth one.

The implants were harvested for histological analysis to evaluate the tissue response to the HA, CMs/HA/BMP, and the results of toluidine blue stains of tooth extraction socket are shown in Fig. 8. In the experiments of animal teeth socket implantation, rhBMP-2 loaded CMs/HA/BMP scaffolds were shown to have profound osteogenic activity, stimulating new bone formation. More obvious differences in the bone weight between the experimental groups (HA and CMs/HA/BMP) appeared as the days increased. This showed excellent biocompatibility of CMs/HA/BMP in tissue sections without inflammation or injury. From Fig. 8, it is speculated that the HA scaffold composites displayed good biocompatibility and the CMs/HA/BMP can promote bone formation.

This study developed a rhBMP-2 carrier which consists of HA and CMs for segmental bone repair. CMs can be used for controlling drug release. The principal role of our new composite delivery system was using double cross-linkers rather than toxic cross-linkers to control a sustained release of bioactive rhBMP-2. However, the approach to avoiding the bioactivity loss of rhBMP-2 during the entire procedure is a challenge.

CONCLUSION

In conclusion, this study disclosed CMs were successfully encapsulated rhBMP-2 by double cross-linkers, indicating clear advantages for sustained active delivery. According to in vitro and in vivo experiments, synthesized CMs/HA/BMP shown excellent osteoconductive and osteoinductive performance. It could be used in conjunction with mechanically robust scaffolds for teeth bone repair.

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