INTRODUCTION
With the advance of biomaterial and surgical techniques, permanent implant prosthesis has been frequently used in orthopedics and dentistry over the past century. Successful implant surgery is highly depending on uninterrupted union of the implant surface material, living bone, or osseointegration. In orthopedic settings, osseointegration can be complicated by insufficient bone quantity due to congenital bone defects, trauma, or malignant tumor invasion. Similarly, in dental medicine, bone losses are typically induced by periodontal disease, trauma and edentulism, which then lead to disability. In order to achieve bone fixation, local augmentation procedures have been used to replace the deficit and increase the bone volume available.

Generally speaking, bones have the ability to regenerate in the presence of some sort of scaffold. As is known to all, the ideal augmentation materials should be biocompatible, osteoinductive, bioreabsorbable to enable bone regeneration, volume stable, resistant to epithelial cells, easy handling, predictable and inexpensive. Unfortunately, few materials have been reported to meet these criteria. For dental implants, autogenous bone grafts harvested from ramus, chin, or iliac crest are recognized as the gold standard for bone grafting. However, they require a secondary surgical site inevitably, which may result in patient discomfort and/or infection. Therefore, it is urgent to develop alternative graft materials with similar osteoinductive capacity.

Natural nacre, mother of pearl, is comprised of crystal platelets of polygonal CaCO₃ aragonite and an organic water-soluble matrix (WSM) consisting of polysaccharides, water-soluble proteins and glycoproteins. The mechanism of natural nacre formation is similar to that of bone tissue formation. As a result, several studies have been carried out to investigate the biocompatible, biodegradable and osteoconductive properties of nacre in-vivo to improve predictability of long-term osseointegration.

For the stimulation of bone regeneration, release of polypeptides, proteins and glycoproteins from WSM is required, which then promotes differentiation and mineralization, the calcium-rich aragonite surface was required to activate the osteoblast and deactivate osteoclasts. Unfortunately, release of WSM from natural nacre is still a challenge due to solid and compact surface. In this study, nano-nacre particles were prepared with an aim to increase the release of organic matrix and calcium. The prepared particles with a smaller diameter than the intact aragonite crystal of nacre contributed to the controlled release of organic matrix and calcium.

MATERIALS AND METHODS
Preparation of nano-nacre particles
Freshwater pearl mussels, Hyriopsis cumingii, were intensively crushed into the nano-nacre particles (Chinese Patent ZL 200910228605). For the preparation of nano-nacre particles, the peals were grinded into powder, and were mixed through a high speed clipper.
Then the powder was suspended in the nano-grinding facility, followed by complete grinding. Particles (20–200 nm) were obtained, and then dried under low temperature. For structural analysis, generated nano-nacre particles were placed on the sample plate of scanning electron microscope (SEM), followed by drying in vacuum and staining with gold ions twice for 3 min. Finally, the samples were visualized using a field emission SEM (FESEM, JEOL JSM-7500FA, Tokyo, Japan).

**Determination of controlled release of calcium and polypeptide**

For spectroscopy analysis, aqueous suspension solution of nano-nacre particles (1 g/L) was obtained after diluting with deionized water. The supernatants were then extracted on day 1, 2, 3, 6, 12, 24, and 36, respectively. Then the extracted samples were traced with UV-visible spectroscopy (Bio-Rad, Hercules, CA, USA) at 193.90 nm to measure the concentration of released polypeptides. Meanwhile, an atomic absorption spectroscopy (Bio-Rad) was used to measure the concentration of released calcium ions.

**Preparation of nano-nacre/type I collagen composite scaffold (NN-ICS) and type I collagen Scaffold (ICS)**

Type I collagen (Sigma-Aldrich, St. Louis, MO, USA) was fully dissolved in 0.05 M NaOH solution. Nano-nacre suspension was then added to the collagen solution and sufficiently dispersed with a weight ratio of 2:1 (nano-nacre: type I collagen). The solution was then centrifuged for 10 min at 10,000 rpm. Upon removal of the supernatant, the remaining mixture was lyophilized to prepare the spongy NN-ICS scaffold. Control ICS scaffold was prepared using a similar process except adding the nano-nacre particles. Both NN-ICS and control ICS were sterilized by γ-ray irradiation with an intensity of 1.5 Mrad.

To characterize the inorganic components, NN-ICS sheet was fixed on the X-ray Diffraction (XRD; PANalytical X’ Pert PRO, Almelo, the Netherlands) observation plate and scanned by X-rays at a speed of 3 degrees per min. The step width was 0.01mm and pipe flow was 35 mA.

**Cell culture and morphology**

MC3T3-E1, purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China), were cultured on minimum essential medium (MEM; Gibco, Waltham, MA, USA) supplemented with 1% sodium pyruvate, 10% fetal bovine serum, 100 μg/mL streptomycin and 100 μg/mL penicillin. Cells were incubated in an incubator with 5% CO₂ at 37°C. Sterilized NN-ICS and control ICS scaffolds were shaped into plates with a size of 10×10×3 mm. MC3T3-E1 pre-osteoblasts were seeded on scaffold plates at the density of 2×10⁴ cells per plate. Scaffolds grown with MC3T3-E1 pre-osteoblasts were harvested on day 1, 7, and 14, and then were fixed in 2.5% glutaraldehyde for 30 min. The scaffolds were rinsed with PBS and distilled water, and then were dried. Finally, the scaffolds were submitted for scanning under SEM.

MC3T3-E1 pre-osteoblasts proliferation was determined using MTS assay (Biotech, Los Angeles, CA, USA) according to the manufacture instructions. MC3T3-E1 pre-osteoblasts were cultured on NN-ICS and control ICS for 24 and 72 h. Then the cells were harvested from the wells and analyzed on a micro plate reader (Model 680, Bio-Rad) at a wavelength of 490 nm.

**Reverse transcription PCR**

Pre-osteoblasts osteogenic MC3T3-E1 expression was determined using reverse transcription PCR. MC3T3-E1 cells were cultured for 7 and 14 days with either NN-ICS or control ICS. Total RNA was extracted at defined time points. The purity of total RNA was assayed using commercial quantitative kit (Invitrogen, Waltham, MA, USA). PCR amplification reaction was carried out with the specific primers in Table 1 using the following parameters: 94°C for 5–10 min, 94°C for 40 s, 55–60°C for 45 s, 72°C extension for 60 s, 30 cycles of 72°C for 10 min. The PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide (Mini-Protean 3, Bio-Rad). PCR products were visualized by gel imaging system (GELDOC2000, Bio-Rad).

**Western-blot assay**

Western blot analysis was used to determine the expression of MC3T3-E1 pre-osteoblast protein (e.g. alkaline phosphatase and type I collagen). MC3T3-E1 cells were cultured for 7 and 14 days with NN-ICS or ICS. After washing with PBS, cells were subjected to cold cell lysis buffer (Cell Signaling, Danvers, MA, USA). Proteins were separated on SDS-PAGE and transferred

<table>
<thead>
<tr>
<th>Gene</th>
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<td>222</td>
</tr>
<tr>
<td></td>
<td>A 5’-CACCAGGGTCACCTTTCGCACC-3’</td>
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to a PVDF film for 90 min. The film was blocked using 5% fat-free dry milk for 2 h, followed by incubating with the primary antibodies including rabbit anti-ALP (Abcam, Eugene, OR, USA) and mouse anti-COL-1 (Abcam) overnight at 4°C. Subsequently, samples were washed with TBST and incubated with the corresponding secondary antibodies for 1 h at room temperature. Film probed with rabbit anti-mouse GAPDH (Abcam) served as control. Afterwards, the samples were washed again with TBST, and visualized in the Image J software (Bio-Rad).

Statistics analyses
All experiments were repeated at least in triplicate. All data were expressed as mean±standard error of the mean. Statistical analyses were carried out using two-way analysis of variance (ANOVA) with the SPSS software for multiple comparisons. A value of $p<0.05$ was considered to be statistically significant.

RESULTS
Characterization of nano-nacre particles
After mechanical crushing of natural nacres, nano-nacre particles consisted of calcium carbonate crystal was obtained, which showed a significant reduction in size compared with the natural nacre particles (19.81±8.32 nm vs. ~6 mm, Figs. 1A and B). Cumulative release of polypeptide and calcium ions from nano-nacre particles was measured indirectly using UV-visible spectroscopy or atomic absorption spectroscopy, respectively. For calcium ion, there was an initial burst release in the first five days, accounting for almost 80% of the total release (Figs. 1C). Similarly, burst polypeptide release was also observed in the first six days, which accounted for 67% of the total amount (Figs. 1D). After burst release, constant linear release was noticed in calcium ion and polypeptide, respectively.

Characterization of NN-ICS
For the morphological assessment of NN-ICS, SEM revealed a porous spongy structure with a pore size of approximate 100–200 μm (Fig. 2A). Inorganic components in NN-ICS were detected using XRD, which showed that the main inorganic component in the matrix was calcium carbonate. The content of impurities and other inorganic salts was extremely low (Fig. 2B).

MC3T3-E1 pre-osteoblasts cytology and molecular biology
On day 1, the profile of MC3T3-E1 pre-osteoblasts cultured on the surface of NN-ICS was normal, and showed rapid full-extension throughout the available area. In addition, cells also exhibited functional activation and cell-cell microvilli contacts were formed within 7 days. On day 14, the majority of cells demonstrated typical osteoblast morphology with obvious synapse on the NN-ICS (Fig. 3A).

As there were few morphological differences between cultured cells, MTS proliferation assay was conducted to determine the cellular proliferation. NN-ICS promoted adhesion and proliferation of the pre-osteoblasts. The OD value in the NN-ICS group was significantly higher.

![Fig. 1](image1.png)  
**Fig. 1** Photograph of natural nacre showing an average diameter of approximately 6 mm (A). SEM image of nano-nacre particles showed disappearance of typical brick crystal structure of nacre (B). Graphs showing cumulative release of calcium ions (C) and polypeptides (D) in the initial 36 days.
SEM micrographs indicated proliferation and differentiation of MC3T3-E1 pre-osteoblasts on NN-ICS and ICS on day 1, 7 and 14, respectively. Graphs showed significant increase of cell metabolic activity in MC3T3-E1 pre-osteoblasts cultured on NN-ICS compared to ICS at both 24 and 72 h. ***p<0.001

Fig. 2  SEM image of NN-ICS presented a high porosity network structure. The pores size of NN-ICS were approximately 100–200 μm (A). The XRD pattern of NN-ICS demonstrated that the main inorganic component of NN-ICS was inorganic calcium carbonate. Other inorganic components were in extremely low proportion (B).

than that of the ICS controls at 24 h (p<0.001). At 72 h, the OD value in NN-ICS group was significantly higher than that of the control group (66±3% vs. 41±2%, p<0.001, Fig. 3B).

The expression of ALP gene was approximately 3-fold higher in NN-ICS group than that of ICS group on day 7 (31.8±11.7% vs. 10.0±3.5%) and day 14 (76.8±16.62% vs. 26.8±5.6%), respectively (Figs. 4A and B). Similarly, collagen-1 gene expression was also significantly higher in NN-ICS group compared to that of ICS group (p=0.027). On day 7, COL-1 expression was also about 3-fold higher in the NN-ICS group compared to that of ICS group (16.9±2.3% vs. 6.4±0.7%). On day 14, the expression of COL-1 in the NN-ICS group was about 1.5-fold higher than that of ICS group (26.0±3.4% vs. 18.03±6.2%, Fig. 4C). These data demonstrated that NN-ICS surface contributed to the osteogenetic differentiation and maturity than the ICS surface.

Significant up-regulation was noticed in ALP protein expression in NN-ICS group compared to ICS controls on day 7 (16.3±0.4% vs. 11.5±0.4%, p<0.001, Fig. 4D). The significant increase of ALP and collagen-1 proteins indicated that NN-ICS promoted the osteogenetic differentiation of MC3T3-E1. On day 14, collagen expression was significantly lower in NN-ICS group compared with that of ICS group (14.68±0.6% vs. 17.28±0.3%, p=0.002). These indicated that MC3T3-E1 pre-osteoblasts entered the mature differentiation stage of osteoblast before 14 days culture on the NN-ICS, which was earlier than those on the ICS control group (Fig. 4E).

COL-1 expression in the NN-ICS experimental group was significantly higher than that of the ICS control on day 7 (23.0±0.4% vs. 15.6±0.5%) and day 14 (22.0±0.4% vs. 13.22±0.6%), respectively.

**DISCUSSION**

Predictable osseointegration between prosthetic implants and host bone is still a challenge for orthopedic and dental implant surgery. Restorative techniques and materials to augment local defects generally rely
Fig. 4 MC3T3-E1 pre-osteoblasts were cultured on the NN-ICS and ICS for 7 and 14 days. (A) Expression of ALP, COL-1, and GAPDH gene in pre-osteoblasts cultured on ICS or NN-ICS. (B, C) The expression of ALP and COL-1 gene in cells cultured on NN-ICS was significantly higher than that of the ICS. (D) Expression of ALP, COL-1, and GAPDH protein in pre-osteoblasts cultured in either ICS or NN-ICS. (E) Significant early ALP expression was observed on day 7, followed by a reduced expression on day 14, thereby indicating that MC3T3-E1 pre-osteoblasts on NN-ICS entered the mature stage of differentiation earlier. (F) Expression of the COL-1 protein was significantly higher in the NN-ICS group compared to the ICS group. *p<0.05; **p<0.01; ***p<0.001

Natural nacre exhibits valuable bone-inducing activity. In particular, the WSM of nacre had been reported to increase alkaline phosphatase activity in mammalian bone marrow stromal cells, which promoted osteoblast proliferation and differentiation, as well as extending osteoblasts life span by inhibiting apoptosis. In biological systems, implantation of nacre blocks into sheep trochlea by replacing half of the femora trochlea was reported to be effective for osteochondral repair. However, as natural nacres are solid and compact in structure, only a limited amount of osteoinductive factors are released from the treated surfaces. Some researchers proposed high-temperature hydrothermal methods to facilitate the change of nacre calcium carbonate into other forms of calcium phosphate molecules. However, the extraction temperature (approximate 200°C) would inevitably denature the organic bioactive factors in nacre. In this study, natural nacres were intensively crushed into nanometer particles to fully expose the organic bioactive factors of the nacre crystals, which led to sustained polypeptide and calcium release that was capable of promoting osteoblast activity and inhibiting the activity of osteoclasts. Compared with conventional extraction procedures, our water-soluble extraction method was less aggressive, thereby preserving the organic factors and improving the release of peptides and calcium from the particles.

Optimal scaffolds for cell loading, ingrowth, and tissue-formation are essential issues in synthetic bone-graft applications. Increased pore size would favor both bone growth and nutrition supply, but it would reduce biomechanical strength. In this study,
NN-ICS demonstrated ideal scaffold morphology and exhibited a highly porous structure with a pore size of approximately 100–200 μm. This dimension met the requirement suggested from early studies, where a pore size of 100–150 mm was essential for providing nutrition and exhibited favorable osteoactivity to induce differentiation of the precursor bone cells. In addition, SEM scan on the surface of NN-ICS demonstrated a scatter of nanometer calcium carbonate particles, which would assist the adhesion and early activity of MC3T3-E1 pre-osteoblasts.

Expression of ALP and COL-1 in M3T3-E1 pre-osteoblasts on the NN-ICS was significantly higher than those on the ICS. In particular, expression of ALP and COL-1 mRNA and protein was significantly higher after culturing on NN-ICS on day 7 compared to those cultured on ICS, which demonstrated that NN-ICS promoted proliferation and differentiation of MC3T3-E1. Down-regulation of ALP protein was noticed on day 14. This further indicated that that NN-ICS was capable of inducing osteoinductive earlier compared to the ICS. Meanwhile, the MC3T3-E1 pre-osteoblasts also entered the mature stage of differentiation earlier, which was featured by reduction of ALP expression. In clinical settings, contact between pre-osteoblasts and implant surface is usually established within 1–2 weeks after endosseous implant placement, while further maturation occurs at least 12 weeks. This shift in early osteoblast differentiation would therefore contribute to the early osseointegration of the implant and potentially improve clinical outcome.

In summary, the current study demonstrated that nano-nacre particles exhibited sustained calcium and polypeptide release, which promoted the cellular proliferation, differentiation and activity of MC3T3-E1 pre-osteoblast. The osteoinductivity induced by nano-nacre particles indicate that they may serve as a successful candidate in the growing field of implant surgery.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

There are no interest conflicts and commercial interest in this paper.

REFERENCES