RETRACTED ARTICLE: Degradable poly-L-lysine-modified PLGA cell microcarriers with excellent antibacterial and osteogenic activity

Hanyang Zhang, Jianhang Jiao and Hui Jin
Department of Orthopedic Surgery, The Second Hospital of Jilin University, Changchun, PR China

ABSTRACT
The surface modification of polymeric materials has become critical for improving the bone repair capability of materials. In this study, we used a poly-L-lysine (PLL) coating method to prepare functional poly (lactic acid–glycolic acid) (PLGA) cell microcarriers, and bone morphogenetic protein 7 (BMP-7) and ponericin G1 were immobilized on the surface of microcarriers. The scanning electron microscopy (SEM), water contact angle measurement, and energy-dispersive X-ray spectroscopy (EDX) were used to analyse the surface morphology of PLL-modified PLGA microcarriers (PLL@PLGA) and their ability to promote mineralization. At the same time, the growth factor binding efficiency and antimicrobial activity of the microcarriers were studied. The effects of microcarriers on cell behaviors were evaluated by cultivating MC3T3-E1 cells on different microcarriers. The results showed that the hydrophilicity, protein adsorption, and mineralization induction capability of the microcarriers were significantly improved by PLL surface modification. The biological experiments revealed that BMP-7 and ponericin G1 immobilized-PLL modified microcarriers can effectively inhibit the proliferation of pathogenic microorganisms while enhancing the ability of the microcarriers to promote cell proliferation and osteogenesis differentiation. Therefore, we believe that PLL-modified PLGA cell microcarriers loaded with BMP-7 and ponericin G1 (PLL@PLGA/BMP-7/ponericin G1) have great potential in the field of bone repair.

Introduction
Clinical studies have shown that the self-repair of the bone tissues cannot effectively occur in patients with large bone defects due to skeletal malformation, trauma, or tumour resection. Large bone defects are often treated with autologous or allogeneic bone grafts in the clinical [1,2]. However, these treatments have some drawbacks such as immune reaction, limited availability, and the transfer of pathogens. With the development of biomaterials science, biodegradable biomaterials show great potential in developing bone grafts to induce bone tissue regeneration. Cell microcarriers are a type of microsphere with a specific diameter for cell growth and attachment, and a large number of cells can be obtained in a short time, which solves the issue of insufficient donors [3–5]. Cell microcarriers can effectively exchange nutrients and metabolic wastes during cell culture because of their large specific surface area, which can effectively promote cell proliferation. Furthermore, cell microcarriers can mimic the three-dimensional cell culture environment in the body, which is beneficial for cell migration and proliferation. More importantly, cell microcarriers can be compressed into different shape for injection into various bone defect sites. To date, many studies have used various synthetic or natural polymeric materials to prepare cell microcarriers for tissue defect repair, including polycaprolactone, poly (lactic acid–glycolic acid) (PLGA), collagen, and chitosan [3,6–8].

Among the various materials, PLGA is often used in the fabrication of cell microcarriers because of its excellent mechanical properties, low immunogenicity, low toxicity, and adjustable degradation rate [9,10]. However, the surface hydrophobic properties, acidic degradation products, and lack of cell binding sites have always limited its application in bone tissue engineering. In order to improve the cell affinity of PLGA, a number of modification strategies have been adopted, such as proteins adsorption, chemical grafting, and plasma treatment [11–13]. However, these methods have several limitations: these strategies are complicated, the results are inconsistent, and the expensive instruments are required. Therefore, there is an urgent need for a simple and low-cost method to modify PLGA microcarriers. Poly-L-lysine (PLL) is a biocompatible cationic polymer, established as a potential compound for promoting cell adhesion, proliferation, and regeneration at the biomaterial interface [14,15]. PLL exhibits excellent safety, water solubility, stability, and antimicrobial properties. Because of the positive interaction between the
positive charge on the PLL surface and the negative charge on the cell membrane surface, PLL is often used as a surface modification method to enhance cell adhesion [16]. Furthermore, PLL exhibits a strong interaction with negatively charged molecules and high biofilm permeability, which has a wide range of applications in the medical field [17,18]. More importantly, this surface modification method is convenient and simple, and shows great application value in biomaterial applications. Therefore, PLL surface modification is an ideal approach to improve the function of PLGA microcarriers. To date, the studies on PLL-modified PLGA microcarriers are rare.

In addition, microcarriers have the ability to induce cells proliferation and differentiation when loading growth factors, peptides, or drugs. Bone morphogenetic protein 7 (BMP-7) is a growth factor that has strong osteoinductivity and plays an important role in promoting bone tissue repair. Recent studies have indicated that BMP-7 can stimulate mesenchymal stem cells to differentiate into osteoblasts, and BMP-7 has been used in the treatment of facial bone defects, large bone defects, and spinal fusion [19–21]. However, as a cytokine, BMP-7 shows the disadvantage that cytokines generally exhibit, i.e. a short in vivo half-life. Furthermore, the conventional method of the combination of BMP-7 into polymer materials requires the use of organic solvents, and the bioactivity of the growth factors can be impaired or reduced. Compared with these traditional methods, surface immobilization method has attracted widespread attention as a new method to bind growth factors. PLL coating has been shown to facilitate the binding of bioactive factors and polymer materials [22]. This surface immobilization method has been applied to the controlled release and delivery of various bioactive factors, such as DNA, drugs, miRNA and nerve growth factor (NGF), [17,23,24]. Therefore, PLL coating not only is a surface modification method to enhance the bioactivity of cell microcarriers but also can provide an effective delivery carrier for growth factors. If a material possesses anti-infective activity while possessing the capability of promote bone repair, cell microcarriers can prevent and treat infection while promoting bone repair, and thus have wide application value. Ponericin G1 is a natural antimicrobial polypeptide extracted from the venom of the ponerine ant P. goeldii and has broad-spectrum antimicrobial activity. Ponericin G1 was used to prepare cell microcarriers with excellent antimicrobial activity.

In this study, functional cell microcarriers were prepared by modifying the surface of PLGA cell microcarriers with PLL, and BMP-7 and ponericin G1 were immobilized on the surface of the microcarriers to enhance its bone repair and antimicrobial capabilities. The surface properties, hydrophilicity, and mineralization induction capacity of the microcarriers were determined, and the BMP-7 binding efficiency was measured. In addition, we systematically studied the effects of PLL@PLGA/BMP-7/ponericin G1 microcarriers on MC3T3-E1 cell proliferation, adhesion, and osteogenesis differentiation.

### Experimental

#### Materials

PLGA (LA/GA = 75/25, Mn = 85 000) was purchased from Nanjing Emperor Nano Material Co., Ltd (Nanjing, China). Polyvinyl alcohol (PVA, Alcoholysis 99.8%–100% (mol/mol)) was purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). Ponerin G1 peptide was purchased from Top-peptide Co., Ltd (Shanghai, China). Poly-l-lysine (PLL) were purchased from Sigma-Aldrich (St. Louis, US). Recombinant human BMP-7 protein was purchased from Abcam (Cambridge, MA, USA). Dichloromethane (DCM) was obtained from Beijing Chemical Works.

#### Preparation of PLGA and PLL@PLGA microcarriers

PLGA microcarriers were prepared via an emulsion–solvent evaporation method. Briefly, 500 mg PLGA were dissolved in 10 mL DCM to prepare the PLGA solution. Then, PLGA solution was poured into a rapidly stirring PVA solution (150 mL, 2% (w/v)) at 400 rpm and then stirred overnight to allow the solvent to evaporate. Finally, the produced PLGA microcarriers were washed with deionized water three times and then dried under reduced pressure. The PLL coating was performed as followed. PLGA microcarriers were firstly immerced in excess .1 M NaOH solution for 30 min at room temperature to introduce COOH groups on the surface of microcarriers and then thoroughly washed with distilled water at least five times to remove the remaining NaOH solution. Subsequently, the microcarriers were immersed in 10 mL .25% (wt/vol) PLL or PLL-g-FITC solution overnight at 4°C and then rinsing in distilled water to remove the excess solution. Finally, the PLL@PLGA microcarriers used for the following experiments were sterilized with ethylene oxide and kept in the vacuum.

#### Surface immobilization of BMP-7 and ponericin G1

In this study, BMP-7 and ponericin G1 were immobilized on the microcarriers via simple dipping. The microcarriers were placed in a 24-well plate. Afterward, 1 mL BMP-7 (500 ng/mL in PBS) and ponericin G1 (300 µg/mL in PBS, pH 8.0) were introduced on PLGA or PLL@PLGA microcarriers. The microcarriers were incubated in the BMP-7 or ponericin G1 solution for 4 h at room temperature on a shaker to prepare BMP-7-immobilized PLL@PLGA microcarriers (PLL@PLGA/BMP-7), ponericin G1-immobilized PLL@PLGA microcarriers (PLL@PLGA/ponericin G1), and BMP-7/ponericin G1-immobilized PLL@PLGA microcarriers (PLL@PLGA/BMP-7/ponericin G1).

#### Characterization of microcarriers

PLGA and PLL@PLGA microcarriers were analysed by the following techniques. A scanning electron microscope (SEM, XL30 ESEM-FEG, FEI) was used to observe the morphology and the surface topography of the microcarriers. The mean diameters of microcarriers were calculated by counting 100 microcarriers from the SEM photographs. To directly observe
were cultured in a LB medium solution at 37 °C, Center of Industrial Culture Collection, China). The bacteria were removed from the SBF and washed with distilled water. The biominalization behaviour was observed with SEM, X-ray diffraction (XRD, D8 ADVANCE, Germany), and the energy-dispersive X-ray spectroscopy (EDX, Philips, XL-30W/TMP, Japan) was used to evaluate the contents of both calcium and phosphor on different microcarrier’s surface.

**In vitro biomineralization test**

The assessment of the in vitro biomineralization of different microcarriers was carried out in the simulated body fluid (SBF) according to the literature [27]. The PLGA and PLL@PLGA microcarriers were immersed in a polyethylene bottle containing 10 mL SBF at 37 °C for 8 weeks. The SBF was refreshed every 2 d. After soaking, microcarriers were removed from the SBF and washed with distilled water. The biominalization behaviour was observed with SEM, X-ray diffraction (XRD, D8 ADVANCE, Germany), and the energy-dispersive X-ray spectroscopy (EDX, Philips, XL-30W/TMP, Japan) was used to evaluate the contents of both calcium and phosphor on different microcarrier’s surface.

**Determination of bound BMP-7**

The PLGA and PLL@PLGA microcarriers were placed in a 24-well plate, and 1 mL BMP-7 (500 ng/mL in PBS) was added into each well. Subsequently, the microcarriers were incubated in the BMP-7 solution for 4 h at room temperature on a shaker. The supernatants were collected respectively. Then, BMP-7-immobilized microcarriers were washed with PBS for two times. All the washing liquid was also collected and mixed with previous supernatants respectively. The amount of BMP-7 in the collected mixed solution was assayed using ELISA. Binding efficiency of BMP-7 to the different microcarriers was evaluated according to the following formula:

\[
\text{Binding efficiency (\%)} = \frac{(W_o - W_b)}{W_o} \times 100
\]

where \(W_o\) and \(W_b\) are weight of BMP-7 before and after incubation of the different microcarriers.

**Antibacterial activity evaluation**

Gram-positive *S. aureus* (ATCC 35696) and gram-negative *E. coli* (ATCC 23282) were selected as bacterial strains (China Center of Industrial Culture Collection, China). The bacteria were cultured in a LB medium solution at 37 °C under a constant shaking rate of 120 rpm for 12 h until they grew to mid-exponential phase. Then, the washed bacterial cells were resuspended in PBS, and the optical density (OD) was adjusted to .5 at 600 nm, approximately corresponding to the concentration of 10^7–10^8 colony forming units ml^-1. The antibacterial activities of different microcarriers were investigated against *S. aureus* and *E. coli*. Five milligram sterilized microcarriers samples were placed in a 24-well plate, and 500 μL of the prepared bacterial suspension was added into each well. After incubation at 37 °C for 3 h, bacterial suspension was collected and the wells were washed three times by 4 mL PBS. All the harvested bacteria cells were centrifuged and resuspended in 3 mL of PBS. Next, 30 μL of the bacteria suspensions were spread on the nutrient agar evenly, and the tested discs were separately placed on agar for 16-h incubation at 37 °C. The colonies formed on the agar plate were then counted with a digital colony counter (HICC-B, WSEEN, China). Loss of bacterial viability was calculated as follows:

\[
\text{Loss of viability (\%)} = \frac{(\text{counts of control} - \text{counts of experimental samples})}{\text{counts of control}} \times 100\%
\]

**Cell adhesion and proliferation**

MC3T3-E1 was used to investigate the biological responses in vitro, which was purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. MC3T3-E1 cells were cultured in Dulbecco-modified eagle’s minimal essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium was changed every 2 d. Cell proliferation of MC3T3-E1 in the microcarriers was assayed using MTT method. Briefly, 1 mL of MC3T3-E1 cells (2.5 × 10^4 mL^-1) was seeded onto different microcarriers, followed by incubation at 37 °C and 5% CO2 for 1, 4, and 7 d, respectively. At the appropriate time point, the microcarriers were incubated in an MTT solution (5 mg mL^-1 in PBS) for 4 h. After the removal of the MTT solution, the converted dye was dissolved in acidic isopropanol (0.04 N HCl-isopropanol) and incubated for 30 min in the dark at 25 °C. Subsequently, 150 μL of the solution was transferred to a 96-well culture plate. The absorbance at 540 nm was recorded using a microplate reader (Infinite M200; Tecan, Switzerland).

For cell adhesion studies, at 4-d post-seeding, the culture medium was discarded, and the unattached cells were washed away with PBS. Then, cells were stained with Calcein-AM (Aladdin, China) for 10 min at 37 °C. Subsequently, the samples were washed three times with PBS, and the samples were observed using a fluorescence microscope (TE2000-U, Nikon).

**Alkaline phosphatase (ALP) activity assay**

ALP activity was determined by quantitation of the p-nitrophenol using p-nitrophenol phosphate substrate (pNPP) solution (Sigma) after being incubated for 7 and 14 d. Briefly, the medium of each well was carefully removed and cells were washed three times with PBS. Then, cells were stained with Calcein-AM (Aladdin, China) for 10 min at 37 °C. Subsequently, the samples were washed three times with PBS, and the samples were observed using a fluorescence microscope (TE2000-U, Nikon).
three replicates. Measurements were normalized by the number of cells from BCA protein assay.

**Mineralization of MC3T3-E1 cells**

After 20 d of culture, calcium (Ca) deposition was determined by alizarin red S (ARS) staining of the MC3T3-E1 cells. After aspirating the medium, the cell-microcarrier samples were washed thrice with PBS and fixed with 4% glutaraldehyde for 1 h. The fixed samples were then rinsed thrice with distilled water and stained with ARS (1% solution in water) for 20 min in 37°C incubator. Calcium quantification was measured using cetylpyridinium chloride (CPC) treatment. The absorbance of ARS at 540 nm was recorded on a multifunction microplate scanner. Furthermore, at time point, cell-microcarrier samples were rinsed with PBS and fixed with 4% glutaraldehyde at room temperature for 1 h. Alcohol gradient drying was performed to remove water from cell-microcarrier samples at increasing ethanol concentrations (50%, 70%, 80%, 90%, 95%, and 100%). Finally, the samples were coated with gold and observed by SEM.

**Gene expression by quantitative PCR (Q-PCR)**

MC3T3-E1 cells cultured on various porous scaffolds at 7 d, and the expression of osteogenesis-related genes was quantitatively assessed using RT-qPCR technique according to the literature [27]. Briefly, after 7 d of culture, cells growing on microcarriers were washed with PBS three times and then suspended in 1 mL cold TRizol Reagent (Life Technologies Co., USA). Total RNA was extracted according to the manufacturer’s protocol. Then, the cDNA was synthesized using a PrimeScriptTM RT reagent kit (Takara Bio, Japan) according to the manufacturer’s instructions. The primers of osteogenic genes are shown in Table 1, comprising anti-runt-related transcription factor 2 (Runx 2), osteopontin (OPN), and collagen-I (COL-1), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (Sangon, Co., Ltd., Shanghai, China). The qPCR amplification was performed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. CT (threshold cycle) values were calculated using the Stratagene MxPro software v4.01 system. This experiment was repeated thrice.

| Gene   | Primer sequences                                      |
|--------|-------------------------------------------------------|
| COL-I  | Forward: CCAACAAGCATGTGCTGTAAGAGG, Reverse: GCAATGCCTGCTGCACTGTGA |
| Runx2  | Forward: GCGGGAATGAGAACTA, Reverse: GAGAAGCTCACCTGTCACTTT |
| OPN    | Forward: TGAGGACAAACGCCAAAGGG, Reverse: GAACTTGCTGTGCTGCTGCAC |
| GAPDH  | Forward: AACTTGGCATTGAGAAGG, Reverse: ACAATATGGGATGAGAACA |

**Immunofluorescent staining for osteopontin**

The expression of bone-specific protein by MC3T3-E1 cells was proved by immunofluorescent staining of osteopontin (OPN) protein. After 7 days of cell seeding, the cells were fixed with formalin for 30 min at room temperature and washed with PBS. For immunofluorescence staining, the cells on each substrate were fixed with 4% PFA in PBS for 15 min at room temperature. After washing with PBS three times, the cells were incubated in .2% Triton X-100 for 10 min and subsequently treated with 3% BSA in 37°C incubator for 30 min. Afterwards, the samples were incubated with bone marker proteins of anti-OPN (Abcam) produced in mouse, at a dilution of 1:500 for 2 h following by the addition of fluorescein isothiocyanate (FITC)-labelled secondary antibody (1:500, Abcam) at a dilution of 1:400 for 30 min. Subsequently, the cells were stained by DAPI (Sigma-Aldrich, USA) for 30 min and observed under a fluorescence microscope (TE2000-U, Nikon).

**Statistical analysis**

All data are presented as the mean-standard deviation. For analysis of multiple groups, the statistic difference was evaluated by variance analysis (ANOVA one-way, Origin 8.0). A value of p < .05 was taken to be significant.

**Results and discussion**

**Characterization of microcarriers**

In this study, we prepared PLL-modified PLGA cell microcarriers. The surface morphology of the PLGA and PLL@PLGA microcarriers was observed via scanning electron microscopy (SEM). As shown in Figure 1, PLGA showed a smooth spherical surface. The shape of the microcarriers did not change dramatically after PLL modification, but the surface roughness of the microcarriers increased; in addition, the tiny pores appear on the surface of microcarriers. This result may be due to the corrosion of the microcarrier surface caused by NaOH treatment during PLL modification. The average size of the microcarriers was 563.4 ± 74.2 μm, and the average diameters of the PLGA and PLL@PLGA microcarriers do not show significant differences. To more clearly observe the PLL distribution on the surface of the microcarriers, we modified the microcarriers with fluorescein isothiocyanate (FITC)-labelled PLL and observed them via fluorescence microscopy. As shown in Figure 1(a-3, b-3), compared with the unmodified PLGA microcarriers, fluorescent PLL (green) was clearly observed on the surface of the PLL@PLGA microcarriers after washing with a large amount of deionised water. The above results demonstrate that the surface of PLGA microcarrier was successfully modified with PLL.

**In vitro biomineralization**

Biomimetic mineralization is often used to evaluate the osteogenic activity of bone implant materials. The structure and chemical composition of the hydroxyapatite (HA) formed...
on the surface of the material should be similar to that of natural bone, which can provide an adhesion interface with bone tissue [28]. If a material can effectively promote HA formation on its surface, it can be considered as a material with superior bone repair capability. Therefore, in this study, PLGA and PLL@PLGA microcarriers were immersed in protein-free simulated body fluid (SBF) for 8 weeks, and the biomineralization of different microcarriers was observed using SEM, XRD and energy EDX. As shown in Supplementary Figure S1, several XRD peaks appeared when microcarriers were incubated with SBF. The diffraction peaks are observed around 25.9°, 31.7°, 32.9° and 39°, which were matching closely with the diffraction peaks of HA at (0 0 2), (2 1 1), (3 0 0) and (3 1 0) crystal planes. The above results confirm that HA particles formed on the surface of the microcarriers. As shown in Figure 2, after the microcarriers were immersed in SBF for 8 weeks, only a small number of HA particles formed on the surface of the PLGA microcarriers. However, the surface of the PLL@PLGA microcarriers showed a larger area of HA particles, and the surface of the microcarriers was almost completely covered by the HA layer. The EDX elemental analysis further validated the above results. As shown in Figure 2(a-3, b-3), the exposed Ca and P content on the surface of the PLL@PLGA microcarriers was significantly higher than that on the PLGA surface. The side chain of lysine contains an amino functional group and is considered a good catalyst for biomineralization. In addition, the strong hydrophilic properties of PLL provide the necessary conditions for biomineralization. A large number of studies have found that PLL coatings can improve the biomineralization induction capacity of materials [29,30]. The above results indicate that the PLL surface modification can promote the HA formation on the polymer surface, which may improve its ability to repair bone defects.

**BMP-7 adsorption and release**

During the application of biomaterials, numerous biological phenomena are related to the protein adsorption. During the bone repair process, proteins are first adsorbed onto the material surface and promote cell adhesion and migration. In addition, when the surface immobilization method is used to combine biomaterials with growth factors, the growth factor adsorption efficiency of the material becomes critical. As shown in Figure 3(A), we used an enzyme-linked immunosorbent assay (ELISA) to determine the BMP-7 loading efficiency on the surface of different microcarriers. After 4 h, the BMP-7 loading efficiency on unmodified PLGA microcarriers was $34.76 \pm 6.57\%$, and the BMP-7 loading efficiency of microcarriers after PLL modification was considerably improved, with a loading efficiency that reached $69.56 \pm 9.544\%$. In
addition, we studied the release of BMP-7 from PLGA/BMP-7 and PLL@PLGA/BMP-7 microcarriers. As shown in Figure 3(B), the BMP-7 release rate from PLGA/BMP-7 reached 92.42 ± 3.05% within 21 days, while the BMP-7 release rate of PLL@PLGA/BMP-7 was only 68.7 ± 7.09%. This result confirms that BMP-7 can be stably immobilized on the surface of the PLL@PLGA microcarriers. Super hydrophilic materials have been found to combine with growth factors more easily than hydrophobic materials, and BMP-7 molecules are more easily bound to the materials through H bonds [31]. In addition, with an isoelectric point of 6.2, BMP-7 has a negative global charge in the build-up conditions (pH 7.4). However, PLL have an isoelectric point of 10.5; therefore, at pH 7.4 most of its amine moieties are protonized, so that PLL are positively charged. Under physiological conditions (pH 7.4), PLL coating has strong capabilities to adsorb BMP-7 via electrostatic interactions between positively charged PLL and negatively charged BMP-7. Therefore, the PLL coating is not only a surface modification method that can improve the bioactivity of cell microcarriers but also an efficient and simple method for conjugation of bioactive factors.

Antimicrobial experiment

During the treatment of bone defects, the prevention of wound infection is an important means to ensure bone tissue repair. There is a growing clinical need for antimicrobial devices, as antibiotics are no longer an ideal solution due to
challenges in reaching the target organisms, especially when associated with a medical device [32]. Therefore, cell microcarriers loaded with active antimicrobial molecules can solve this problem and achieve better treatment effect. In this study, we used PLL@PLGA microcarriers loaded with the active antimicrobial peptide (ponericin G1) to improve the antimicrobial properties of microcarriers. Because PLGA is currently known to have no significant antimicrobial effect, we used PLGA as the control group. Figure 4 shows the viability loss of the bacteria *S. aureus* and *E. coli* after 3-h contact with the different microcarriers. For colony counting, colonies photographs are fed into a colony-counting instrument, and the instrument can mark the colonies and count automatically. According to the bacterial count, the PLL@PLGA microcarriers performed much better than the pure PLGA microcarriers in reducing bacterial number. The corresponding bacterial viability loss of *S. aureus* and *E. coli* reached 32.27 ± 7.5% and 36.3 ± 4.32%, respectively. Furthermore, it was found that bacterial viability further decreased with the addition of ponericin G1 in the microcarriers. Among all the microcarrier, we found that the PLL@PLGA/ponericin G1 microcarriers showed the strongest inhibitory effect and the corresponding bacterial viability loss of *S. aureus* and *E. coli* reached 75.21 ± 9.43% and 81.1 ± 9.4%, respectively, demonstrating that the combined use of PLL surface modification and ponericin G1 has synergistic effect on the antibacterial properties of microcarriers. From the above results, we demonstrated that the PLL surface modification combined with the ponericin G1 can endows the microcarriers with long antimicrobial activity.

**Contact angle measurement**

The hydrophilicity of the materials plays an important role in interacting with cells. It is difficult for cells to adhere on the surface of poor hydrophilic materials. Static contact angle measurement is a common method to evaluate the hydrophilicity of materials. In this study, the static contact angle measurement was used to evaluate the hydrophilicity of different microcarrier. As shown in Figure 5, compared with pure PLGA microcarriers, the PLL@PLGA microcarriers showed decreased contact angles from 99.4 ± 7.43° to 30.5 ± 7.2° due to the exposure of PLL on the surface. After loading with
BMP-7 and ponericin G1, the contact angle was further reduced slightly ($p > .05$). PLL is a positively charged polymer with a large number of amino groups (pKa value of 10.5). Those amino groups that exist on the surface of the microcarriers are largely unprotonated under physiological conditions, which can increase the wettability of the materials. Previous studies have found that the optimal contact angle of a material for cell cultivation ranges from $5^\circ$ to $40^\circ$, and cells can better adhere and grow on materials with such hydrophilic range [33]. Therefore, the above results indicate that PLL@PLGA/BMP-7/ponericin G1 microcarriers can provide a suitable hydrophilic microenvironment to promote cell attachment and proliferation.

**Cell adhesion and proliferation**

The total population of MC3T3-E1 cells growing on the surface of different microcarriers at 1, 4 and 7 d was
investigated using MTT assay. As shown in Figure 6(A), with prolonged cell culture time, the number of cells in different microcarriers was gradually increased. At each time point, it was clear found that the OD values of cell viability showed the lowest level on the untreated PLGA microcarriers. Compared to PLGA microcarriers, the cell proliferation on the PLL@PLGA microcarriers was promoted by the PLL surface modification, which may be due to the large number of positive charges on the microcarrier surface. The results of wettability assessments and SEM analysis in this study indicated that the surface modification with PLL increased the surface roughness and wettability of the microcarriers which are positive cues in cell–material interactions. When the MC3T3-E1 cells were cultured on the BMP-7-immobilied PLL@PLGA microcarriers, the cell proliferation was improved compared with the PLL@PLGA microcarriers at 4 d. However, at 7 d, we found that the cell proliferation in the PLL@PLGA/BMP-7 group was slightly lower than that in the PLL@PLGA group. When the cells proliferated to a certain number, they suppressed their proliferation and started to differentiate to pre-osteoblasts. Therefore, we speculated that the lower OD values of MC3T3-E1 cells on PLL@PLGA/BMP-7 microcarriers represented the beginning of the cell osteogenesis differentiation. After the microcarriers loaded with ponericin G1, there is no significantly different in cell proliferation, demonstrating that ponericin G1 has no significant effect on cell activity while exerting antimicrobial activity.

In order to examine the effect of the different microcarriers on the adhesion of MC3T3-E1 cells, MC3T3-E1 cells were seeded on the surface of the microcarriers. As shown in Figure 6(B), after 4 d of culture, MC3T3-E1 cells were stained (green) and observed under a fluorescence microscope. The staining results showed that a large number of adherent cells were present on the surface of microcarriers, and the cells showed colony growth. Among them, PLL@PLGA microcarriers exhibited better cytoskeleton, indicating that PLL surface modification is beneficial for cell growth and cell–cell communication. Furthermore, there were greater cell quantities and positive cellular interactions on BMP-7 immobilized microcarriers. Meanwhile, it was also found that the immobilization of ponericin G1 had little effect on the adhesion of MC3T3-E1 cells. These data were consistent with the results of the cell proliferation experiments. The above results demonstrate that PLL-modified cell microcarriers loaded with BMP-7 and ponericin G1 can promote cell proliferation and adhesion while maintaining antimicrobial activity.

**Alkaline phosphatase (ALP) activity**

ALP plays a crucial role in the initiation of the mineralization process by cell differentiation, and its activity is used as a marker of the early stage of osteogenic differentiation because its level increases with maturation of osteoblast [34]. Therefore, ALP was chosen to explore the osteoinductive activity of the different microcarriers. As shown in Figure 7, after 7 days of cell culture, there was no significant difference in ALP activity between the PLGA and PLL@PLGA groups. However, after 14 days of cell culture, the ALP activity was significantly higher in the PLL@PLGA group than in the PLGA group. Previous studies have demonstrated that lysine moieties have the potential of increasing osteoblast adhesion, possibly by mimicking the lysine-rich bovine bone proteins structure known to promote osteoblast attachment, growth, and differentiation [29]. Among all the microcarriers, the BMP-7-immobilized microcarriers exhibited the highest ALP activity ($p < .05$), indicating that the osteogenic differentiation of cells was better on the BMP-7-immobilized microcarriers than other microcarriers. After ponericin G1 was immobilized, it is found that there is no significant increase in ALP expression on ponericin G1-immobilized microcarriers, suggesting that ponericin G1 has no significant effect on the cell early osteogenic differentiation.

**Cell mineralization**

The capacity of minerals deposition is a marker for mature osteoblasts, which can be used to conform that cell differentiate to osteoblasts and enter into the mineralization phase.
to deposit mineralize ECM. In this study, levels of calcium deposition were evaluated by extracting Alizarin Red with 10% cetylpyridinium chloride (CPC), which was employed to determine calcium mineralization on the microcarriers. As shown in Figure 8, after 20 days of cell culture, the calcium content in the PLL@PLGA group was significantly higher than the PLGA group, demonstrating that the PLL surface modification is effective method to promote cell mineralisation. Many studies have demonstrated that poly-amino acids, such as poly-lysine and poly-glutamic acid, possess high calcium affinity and can effectively induce osteogenesis differentiation and new bone formation [29,35]. After BMP-7 was immobilized, the calcium content of cells was further increased and significantly higher than other groups ($p < .05$), indicating that the bioactivity of BMP-7 immobilized on the surface of the microcarriers is well maintained and make the microcarriers perform long-term osteoconductivity. A number of studies have shown that poly-amino acids, such as poly-lysine and poly-glutamic acid, possess high calcium affinity and can effectively induce osteogenesis differentiation and new bone formation [29,35]. After BMP-7 was immobilized, the calcium content of cells was further increased and significantly higher than other groups ($p < .05$), indicating that the bioactivity of BMP-7 immobilized on the surface of the microcarriers is well maintained and make the microcarriers perform long-term osteoconductivity. A number of studies have shown that BMP-7 can significantly induce cell osteogenesis differentiation as well as enhance bone formation [36]. Interestingly, compared with the PLL@PLGA microcarriers, ponericin G1-immobilized microcarriers exhibited the higher calcium content. As a peptide, we speculate that ponericin G1 can enhance the calcium affinity of the material and provide some functional groups, which can improve the nucleation of the HA. To better investigate the effect of different microcarriers on cell mineralization, we observed MC3T3-E1 cells by SEM. As shown in Figure 8, more apatite particles were observed on the surface of PLL@PLGA microcarriers than the PLGA group. After BMP-7 was loaded, the cells grown on the surface of BMP-7-modified microcarriers had increased mineralized nodule formation. The above results confirmed that the PLL@PLGA microcarriers loaded with BMP-7 and ponericin G1 can significantly enhance the osteogenesis differentiation of MC3T3-E1 cells.

**Expression of osteogenic differentiation-related genes and proteins**

Some key genes and functional proteins will be expressed regularly during the osteoblastic differentiation. At different time points, the levels of genes or proteins can be used to evaluate the degree of osteogenic cell differentiation. For example, Runx2 is an early differentiation marker, whereas OPN is usually expressed at the middle/late stage of differentiation, and the COL-1 gene represents the degree of extracellular matrix formation [37]. In this study, MC3T3-E1 cells were cultured on different microcarriers for 7 days, and the quantitative of the osteogenesis-related gene expression (COL-1, Runx2, and OPN) was analysed using RT-qPCR. As shown in Figure 9, the COL-1 and OPN expressions in the PLL@PLGA group were significantly higher than the PLGA group ($p < .05$), demonstrating that the PLL coating has a pronounced effect on extracellular matrix formation and late osteogenic differentiation. This may be due to the ability of
cell microcarriers and applied in tissue repair and cell therapy. Various materials have been used to prepare large number of cells for the field of cell therapy, but also to obtain a large number of cells in a short time with minimal cell damage. As shown in Figure 10, the OPN protein expression level was significantly higher in the PLL@PLGA microcarriers compared to the PLL-PLGA group. In addition, cell microcarriers loaded with BMP-7 and ponericin G1 have good antimicrobial activity against a wide range of bacteria, including S. aureus and E. coli. Furthermore, the BMP-7 on the surface of the microcarriers showed higher expression levels of Runx2 and OPN than those of pristine microcarriers. These data further confirm that the BMP-7 on the surface of the microcarriers can effectively promote osteogenic differentiation of cells. However, after loading with ponericin G1, there is a significant increase in Runx2 and OPN expression, and the OPN expression was only slightly increased. Therefore, we speculated that ponericin G1 has little effect on the osteogenic differentiation of MC3T3-E1 cells. To better explore the effect of different microcarriers on osteogenic differentiation of cells, the protein expression of OPN by the MC3T3-E1 cells grown on different microcarriers was evaluated by immunofluorescence staining. As shown in Figure 10, the OPN protein expression in the PLL@PLGA microcarriers was significantly higher than in the PLGA group. In addition, the highest OPN protein expression was found on the BMP-7-immobilized microcarriers. According to the antimicrobial activity, cell growth, calcium deposition, and osteogenic differentiation-related gene expression results, the PLL-modified PLGA microcarriers loaded with BMP-7 and ponericin G1 can effectively promote cell adhesion, proliferation, and osteogenic differentiation while maintaining strong antimicrobial activity.

Extensive bone defect, originated from trauma, tumour resection, infection, and skeletal abnormalities, put a great need for bone implants and other treatments. In recent years, with the continuous development of biomaterials, the bone implants composed of biodegradable biomaterials with good biological activity have provided new approaches for the treatment of bone defects. Cell microcarriers are a type of microsphere with a specific diameter for cell growth and attachment. In 1967, van Wezel first used cell microcarriers for large-scale cell culture in vitro, and it is found that this method can obtain a large number of cells in a short time. Furthermore, the cell microcarriers not only provide a large number of cells for the field of cell therapy but also can be directly applied on bone defect site as microcarriers/cells compound. Various materials have been used to prepare cell microcarriers and applied in tissue repair and regeneration, such as chitosan, gelatine, and PLGA. In addition, cell microcarriers can selectively combine with bioactive factors to promote tissue regeneration for different damage sites. PLL is a type of cationic polymer with excellent biocompatibility and can effectively promote cell adhesion and osteogenic differentiation. Furthermore, its large number of functional groups and positively charged could potentially affect interactions with biological macromolecules via H bonding or electrostatic interactions. Therefore, in this study, we used PLL to modify the surface of PLGA cell microcarriers, and the ponericin G1 and BMP-7 were immobilized on the surface of microcarriers. It is found that the hydrophilicity and mineralization induction ability of the modified PLGA microcarriers was greatly improved, and the BMP-7 binding efficiency was considerably improved. Biological experiments showed that PLL-modified PLGA microcarriers can effectively promote cell proliferation, adhesion, and osteogenic differentiation. More importantly, the BMP-7 on the surface of the microcarrier maintained long osteogenic osteoinductivity, and the ALP activity, mineral deposition, and the osteogenic differentiation-related gene expression were significantly improved.

During the application of bone implants, the clinical demand for the antimicrobial properties of materials is growing because of antibiotics are no longer an ideal antibacterial way, especially when an implant is involved. Thus, we applied an antimicrobial peptide, ponericin G1, which has broad-spectrum antimicrobial activity against fungi and bacteria, to improve antibacterial activity of cell microcarriers. Ponericin G1 is peptides isolated from the venom of the predatory ant Pachycondyla goeldii. Ponericin G1 has similarity with cecropin-like peptides, which can lyse bacterial cellular membranes and can also inhibit proline uptake as well as cause leaky membranes. Previous have found that the ponericin G1 has strong activity against a wide range of bacteria, including S. aureus, and has low haemolytic activity. Our results showed that ponericin G1-immobilized microcarriers exhibited enhanced antimicrobial activity against S. aureus and E. coli. Furthermore, it is found that the combination use of PLL surface modification and ponericin G1 had a synergistic effect in terms of inhibiting the growth of pathogenic microorganisms. More importantly, the ponericin G1 has no negative effect on the biological properties of the microcarriers. Therefore, we speculate that PLGA microcarriers loaded with BMP-7 and ponericin G1 have good

![Figure 9](image-url). Real-time qPCR analysis of the osteogenesis-related genes COL-I, Runx2, and OPN after the MC3T3-E1 cells were cultured for 7 and 14 days on different microcarriers: (a) PLGA, (b) PLL@PLGA, (c) PLL@PLGA/BMP-7, (d) PLL@PLGA/ponericin G1, and (e) PLL@PLGA/BMP-7/ponericin G1. *p < .05, n = 3.
prospects for application in bone repair and human bone implant preparation. In future work, we will further study the biological properties of PLL@PLGA/BMP-7/ponericin G1 for obtaining an ideal bone implant.

Conclusions

In summary, PLGA microcarriers were modified using PLL surface modification method, and BMP-7 and ponericin G1 were immobilized on the surface of microcarriers. The obtained results demonstrated that hydrophilicity and mineralization induction ability of microcarriers increased upon surface modification with PLL, which can improve better cell function. Furthermore, after BMP-7 and ponericin G1 were immobilized, the microcarrier exhibited a strong ability to promote cell osteogenic differentiation, and the ALP activity, osteogenic differentiation-related genes expression, and cell mineralization were significantly improved. More importantly, PLL surface modification and ponericin G1 can greatly improve the antimicrobial activity of the cell microcarrier and enhance the clinical application value of microcarriers. The obtained results highlight the potential of PLL@PLGA/BMP-7/ponericin G1 microcarriers as a promising biomaterial for bone tissue regeneration.

Authors’ contributions

H.J. directed the work, designed experiments and analysed the data. H.Z. designed experiments, conducted experiments, analysed the data, and wrote the paper. J.J. analysed the results and improved the work. All authors reviewed the manuscript.
Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This study was supported by a grant from Jilin Scientific and Technological Development Program [20160101109JC].

References
[1] Stevenson S. Enhancement of fracture healing with autogenous and allogenic bone grafts. Clin Orthop Related Res. 1998;355: S239-S246.
[2] Shrivats AR, McDermott MC, Hollinger JO. Bone tissue engineering: state of the union. Drug Discov Today. 2014;19:781–786.
[3] Li LL, Shi XC, Wang ZL, et al. Preparation of biodegradable polycaprolactone microcarriers with doxorubicin hydrochloride by ultrasonic-assisted emulsification technology. Sensor Mater. 2019; 31:301–310.
[4] Hou Y, Gong YH, Gao CY, et al. Collagen-coated polyclactide microcarriers/chitosan hydrogel composite: injectable scaffold for cartilage regeneration. J Biomed Mater Res. 2008;85A:628–637.
[5] Munoz MS, Confalonieri D, Walles H, et al. Recombinant collagen I peptide microcarriers for cell expansion and their potential use as cell delivery system in a bioreactor model. Jove J Vis Exp. 2018; 132:e57363.
[6] Sun XM, Cheng LY, Zhao JW, et al. bFGF-grafted electropun fibrous scaffolds via poly(dopamine) for skin wound healing. J Mater Chem B. 2014;2:3636–3645.
[7] Haider A, Kim S, Huh MW, et al. BMP-2 grafted nHA/PLGA hybrid nanofiber scaffold stimulates osteoblastic cells growth. Biomed Res Int. 2015;2015:21.
[8] Liu PM, Sun L, Liu PY, et al. Surface modification of porous PLGA scaffolds with plasma for preventing dimensional shrinkage and promoting scaffold-cell tissue interactions. J Mater Chem B. 2018; 6:7605–7613.
[9] Guo XD, Song YL, Zheng QX, et al. Surface modification of biomimetic PLGA(ASP-PEG) matrix with RGD-containing peptide: a new non-viral vector for gene transfer and tissue engineering. J Wuhan Univ Technol. 2006;21:41–43.
[10] Croll TI, O’Connor AJ, Stevens GW, et al. Controllable surface modification of polylactico-glycolic acid (PLGA) by hydrolysis or aminolysis: I. physical, chemical, and theoretical aspects. Biomacromolecules. 2004;5:463–473.
[11] Choi Y, Yagati AK, Cho S. Electrochemical characterization of poly-L-lysine coating on indium tin oxide electrode for enhancing cell adhesion. J Nanosci Nanotechnol. 2015;15:7881–7885.
[12] Zhang DD, Zhang YM, Zheng L, et al. Graphene oxide/poly-L-lysine assembled layer for adhesion and electrochemical impedance detection of leukemia K562 cancer cells. Biosens Bioelectron. 2013;42:112–118.
[13] Miao YL, Yang RR, Deng DYB, et al. Poly(L-lysine) modified zein nanofibrous membranes as efficient scaffold for adhesion, proliferation, and differentiation of neural stem cells. Rsc Adv. 2017;7: 17711–17719.
[14] Verma I, Siddiq S, Pal SK. Poly(L-lysine)-coated liquid crystal droplets for sensitive detection of dna and their applications in controlled release of drug molecules. ACS Omega. 2017;2:7936–7945.
[15] Walsh DP, Murphy RD, Panarella A, et al. Bioinspired star-shaped poly(L-lysine) polypeptides: efficient polymeric nanocarriers for the delivery of DNA to mesenchymal stem cells. Mol Pharm. 2018; 15:1878–1891.
[16] Burastero G, Scarfi S, Ferraris C, et al. The association of human mesenchymal stem cells with BMP-7 improves bone regeneration of critical-size segmental bone defects in athymic rats. Bone. 2010;47:117–126.
[17] Stevenson S. Enhancement of fracture healing with autogenous and allogenic bone grafts. Clin Orthop Related Res. 1998;355: S239-S246.
[18] Shrivats AR, McDermott MC, Hollinger JO. Bone tissue engineering: state of the union. Drug Discov Today. 2014;19:781–786.
[19] Li LL, Shi XC, Wang ZL, et al. Preparation of biodegradable polycaprolactone microcarriers with doxorubicin hydrochloride by ultrasonic-assisted emulsification technology. Sensor Mater. 2019; 31:301–310.
[20] Hou Y, Gong YH, Gao CY, et al. Collagen-coated polyclactide microcarriers/chitosan hydrogel composite: injectable scaffold for cartilage regeneration. J Biomed Mater Res. 2008;85A:628–637.
[21] Munoz MS, Confalonieri D, Walles H, et al. Recombinant collagen I peptide microcarriers for cell expansion and their potential use as cell delivery system in a bioreactor model. Jove J Vis Exp. 2018; 132:e57363.
[36] An G, Zhang WB, Ma DK, et al. Influence of VEGF/BMP-2 on the proliferation and osteogenic differentiation of rat bone mesenchymal stem cells on PLGA/gelatin composite scaffold. Eur Rev Med Pharmacol. 2017;21:2316–2328.

[37] Lai GJ, Shalumon KT, Chen SH, et al. Composite chitosan/silk fibroin nanofibers for modulation of osteogenic differentiation and proliferation of human mesenchymal stem cells. Carbohydr Polym. 2014;111:288–297.

[38] van Wezel AL. Growth of cell-strains and primary cells on micro-carriers in homogeneous culture. Nature. 1967;216:64–65.

[39] Ye L, Ding S, Cui YL, et al. Evaluation of the preparation technology on chitosan-gelatin microcarrier by orthogonal design. AMR. 2011;282–283:133–137.

[40] Wu QH, Patocka J, Kuca K. Insect antimicrobial peptides, a mini review. Toxins. 2018;10:461.