Versatile Nanocarrier Based on Functionalized Mesoporous Silica Nanoparticles to Codeliver Osteogenic Gene and Drug for Enhanced Osteodifferentiation

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ABSTRACT: To achieve enhanced stimulatory effects on the osteogenic differentiation of stem cells, the combination of dual factors with synergistic bioactivity has been regarded as the most effective and powerful strategy. In this study, polylysine-modified polyethylenimine (PEI-PLL) copolymers with various molecular weight PEI blocks were first synthesized and evaluated focusing on their cytotoxicity and gene transfection efficiency, and the results demonstrated that the synthesized copolymer PEI-PLL-25k (synthesized using 25 kDa PEI) exhibited lower cytotoxicity and higher in vitro transfection efficiency than commercial PEI-25k (Mr = 25 kDa). In order to effectively load and deliver plasmid DNA and osteogenic drug dexamethasone (DEX), PEI-PLL-25k copolymer and arginine-glycine-aspartate (RGD) peptide were successively anchored onto the surface of mesoporous silica nanoparticles (MSNs) to construct the dual-factor delivery system, which allows the surface adsorption of DNA and DEX loading in the mesopores of MSNs. The modification of PEI-PLL-25k copolymer and RGD on nanoparticles was successfully characterized by various techniques. The functionalized MSNs with RGD conjugation on the surface showed good cyto compatibility as evidenced by in vitro cell viability assays and cytoskeleton observation. The dual-factor delivery system could quickly release plasmid DNA (pDNA), while releasing DEX in a sustained manner. When cultured with the vector bearing bone morphogenetic protein-2 (BMP-2) pDNA, the transfected bone mesenchymal stem cells (BMSCs) were capable of expressing BMP-2 protein. With the simultaneous delivery of DEX and the BMP-2 gene, this dual-factor delivery system could significantly enhance the level of osteogenic differentiation of BMSCs, as demonstrated by in vitro results of alkaline phosphatase (ALP) activity, expression of osteo-related genes, and calcium deposition. Therefore, the versatile functionalized MSNs nanocarrier for codelivery of osteogenic gene and drug may be considered as a promising dual-delivery system to synergistically enhance the osteogenic outcomes of stem cells.

KEYWORDS: mesoporous silica nanoparticles, copolymer, dual-factor, simultaneous delivery, osteogenic differentiation

1. INTRODUCTION

In recent years, many types of bioactive nanomaterials have attracted great attention for bone tissue engineering application because of their intrinsic physicochemical properties, including nanohydroxyapatite, bioactive glass, laponite, and silica-based nanomaterials. Particularly, mesoporous silica nanoparticles (MSNs), due to their customizable morphology, particle size, and pore size, as well as excellent biocompatibility, have attracted considerable interests for biomedical applications. To date, MSNs have been focused largely on the construction of versatile theranostic nanoplat- forms for tumor therapy and diagnostics. However, in the field of regenerative medicine, the use of MSNs is still an emerging field, especially for bone tissue engineering. Previous studies have demonstrated that silicon (Si) ions significantly enhanced the cell growth and osteogenic differentiation of BMSCs. Therefore, MSNs are considered favorable for regulating biological response on osteoblasts and provide therapeutic potential in bone tissue regeneration.

However, single use of bare MSNs is difficult to achieve the desired bioactivity and osteogenic ability which motivates researchers to make great efforts to improve the bioactivity of MSNs. Due to the crucial merit of mesoporous structure, MSNs were employed as vehicles to deliver therapeutic drugs in bone regeneration application. For instance, dexamethasone (DEX) was loaded into MSNs and then incorporated into the polymeric scaffold via electrophoretic deposition, showing promoted osteogenic activities and enhanced therapeutic effect on the calvarial defect. In addition, the stimulatory effect on both osteogenesis and angiogenesis of stem cells by dimethyl-
oxaloylglycine (DMOG) loaded MSNs was demonstrated.\(^{12}\) Apart from the therapeutic molecules, some studies have paid much attention to the loading of osteogenic peptide into MSNs which derived from bone morphogenetic protein, like bone-forming peptide from BMP-7 and BMP-2 derived peptides.\(^{13,14}\) From the results of these studies, the osteogenic peptide-laden MSNs significantly improved the osteogenic ability of osteoblasts, revealing the enhancement on osteogenic activity. Furthermore, osteogenic gene was demonstrated to own the ability of facilitating the osteogenic performance of stem cells.\(^{15}\) To meet the demand of gene delivery, MSNs were commonly decorated to possess the positive charge property and load target gene through electrosstatic interaction.\(^{16}\) Simply, aminated mesoporous silica nanoparticles with positively charged surfaces were used as delivery vehicles for BMP-2 gene.\(^{17}\) However, the cationic polymers were widely employed to modify the MSNs to mediate gene delivery, including polyethylenimine (PEI), polylysine (PLL), polyamidoamine dendrimer, and some copolymers.\(^{16,18-20}\) Previously, polyethylenimine-polylysine (PEI-PLL) copolymer was reported to show better biocompatibility and higher transfection efficiency than pure PEI (\(M_w = 25 \text{kDa}\)).\(^{21}\) The effect of the different ratio of the PLL chain in PEI-PLL on gene transfection efficiency has been extensively explored, while the influence of the molecular weight of PEI in PEI-PLL on transfection efficiency has been rarely reported to our knowledge. Therefore, one of our objectives in this work is to investigate the transfection efficiency of PEI-PLL copolymers synthesized using different molecular weight of PEI. Additionally, it is highly desirable to fabricate functionalized MSNs with PEI-PLL coating for gene delivery. In bone tissue regeneration, induction of osteogenic differentiation of stem cells by delivery of osteogenic gene through a silica-based carrier has already been reported.\(^{17,22}\) However, few studies have investigated the regulatory effect of therapeutic drug and gene codelivered by functionalized MSNs on the osteogenic response of stem cells. Therefore, it is of great interest to design the dual-delivery system of osteogenic drug and gene with MSNs.

Actually, the synergistic effect on osteogenesis through a codelivery of BMP-2 protein and osteogenic drug has been demonstrated previously.\(^{23}\) However, some disadvantages of BMP-2 protein treatment existed, including short half-life, high dose requirement, and high cost. With the employment of BMP-2 gene, these problems were able to be overcome, and it also could provide a promising way to sustainably express the BMP-2 protein at the injured site.\(^{24}\) In this study, we developed a special nanoparticulate osteogenic delivery system to realize codelivery of BMP-2 gene and DEX for achieving enhanced osteogenic ability through synergistic effect. The PEI-PLL copolymers with various molecular weights of the PEI block were extensively explored to determine the final modification onto MSNs via a series of testing techniques including physical characterization, agarose gel electrophoresis assay, and transfection efficiency evaluation. The PEI-PLL with optimized transfection efficiency and RGD peptide were successively anchored onto the surface of MSNs to construct the dual-factor delivery system which allows the surface adsorption of DNA and drug loading in the mesopore of MSNs. Then, the loading capacities and release profiles of pDNA and DEX from delivery system were investigated. Finally, the in vitro cytotoxicity and cellular uptake, as well as the osteogenic function, of modified MSNs bearing BMP-2 gene and DEX were evaluated. To the best of our knowledge, this is the first time the dual-factor delivery system has been constructed for enhanced osteogenic performance by functionalized MSNs with PEI-PLL cationic copolymer and arginine-glycine-aspartate (RGD) motif modification.

2. MATERIALS AND METHODS

2.1. Materials. 
\(\epsilon\)-Benzylsarcosinyl-1-lysine N-carboxyamide (Lys(Z)-NCA) was synthesized according to a previous report.\(^{25}\) Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), succinic anhydride (SA), 3-aminopropyltriethoxysilane (APTES), 1-ethyl-3,3-(dimethylamino)propyl carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), branched polyethylenimine (\(M_w = 25 \text{kDa}, \text{PEI-25k})\), and hydrobromic acid solution (HBr) (33 wt % in acetic acid) were acquired from Sigma-Aldrich (Shanghai, China). Branched PEIs (\(M_w = 10 \text{kDa}\) and 1.8 kDa, denoted as PEI-10k and PEI-1.8k) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). RGD (acylamidine-terminated glycine-arginine-glycine-aspartic acid) and FITC-RGD (FITC labeled glycine-arginine-glycine-aspartic acid) were obtained from Chinatepeptide Co., Ltd. (Shanghai, China). Reporter lysis buffer and Luciferase reporter gene assay kit were received from Promega (Mannheim, Germany). 293T cells (human embryonic kidney cell line) and RAW 264.7 (mouse leukemic monocyte macrophage cell line) were obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). The plasmid pGL3-luc (encoding luciferase), PB[Act-RFP]DS plasmid (encoding red fluorescence protein) and pRES2-Z/Green1-BMP2 (encoding BMP-2 protein) vectors were procured from Changhai YRBio Co., Ltd. (Hunan, China).

2.2. Synthesis and Characterization of PEI-PLL Copolymers. 
The PEI-PLL copolymers were synthesized by ring-opening polymerization (ROP) using PEI as a macrorinitiator and Lys(Z)-NCA as the monomer according to reported protocol.\(^{31,32}\) Briefly, PEI (PEI-25k, PEI-10k, or PEI-1.8k) and Lys(Z)-NCA were respectively added in trifluoroacetic acid (TFA), hydrobromic acid solution, and mixed in a round-bottom flask. The feed ratio of PEI to Lys(Z)-NCA was fixed at 1:100, as listed in Table S1. The mixed solution was maintained at 30 °C and stirred for 72 h. Afterward, the synthesized PEI-Plys(Z) was concentrated and precipitated with excess diethyl ether, followed by filtration and drying under vacuum. Protecting group of benzoyloxybenzoylamine was removed in trifluoroacetic acid (TFA) containing HBr. Finally, the collected product (PEI-PLL-25k, PEI-PLL-10k, or PEI-PLL-1.8k) was purified by dialyzing (molecular weight cutoff, 700 Da, 3500, or 1000 Da) in deionized water and lyophilized.

2.3. Synthesis of Copolymer and RGD-Conjugated MSNs. 
First, aminated MSNs (MSNs-NH\(_2\)) was prepared according to the reported methods.\(^{13,17}\) A 50 mg portion of MSNs-NH\(_2\) was dispersed in 20 mL N,N-dimethylformamide (DMF) and subjected to ultrasonic treatment. A 0.45 g portion of succinic anhydride dissolved in 5 mL DMF was transferred into the suspension, followed by addition with 0.45 mL triethylamine (TEA) to promote the reaction. Finally, the resulting MSNs-COOH was separated by centrifugation and purified by washing repeatedly to remove the TEA and excessive succinic anhydride, dispersed in water or DMF for further use.

To obtain PEI-PLL grafted MSNs, 50 mg MSNs-COOH was dispersed in 20 mL DMF and followed by ultrasonic treatment. Then 32 mg EDC and 19 mg NHS dispersed in 20 mL DMF were added successively anchored onto the surface of MSNs to construct the dual-factor delivery system which allows the surface adsorption of DNA and drug loading in the mesopore of MSNs. Then, the loading capacities and release profiles of pDNA and DEX from delivery system were investigated. Finally, the in vitro cytotoxicity and cellular uptake, as well as the osteogenic function, of modified MSNs bearing BMP-2 gene and DEX were evaluated. To the best of our knowledge, this is the first time the dual-factor delivery system has been constructed for enhanced osteogenic performance by functionalized MSNs with PEI-PLL cationic copolymer and arginine-glycine-aspartate (RGD) motif modification.

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2.4. Characterization. The detailed description of a series of characterization techniques was provided in the Supporting Information. Additionally, the experimental section for evaluation of PEI-PLL copolymers was also included in the Supporting Information, including proton buffering capacity, particle size, zeta potential, pDNA condensation ability, in vitro cytotoxicity, and transfection efficiency.

2.5. Cell Culture. BMSCs were collected and cultured as reported previously.1,12 293T and RAW 264.7 cells were maintained in DMEM medium containing 10% FBS and 1% penicillin/streptomycin, respectively.

2.6. Biocompatibility Evaluation. The cell viability assays of the prepared samples were assessed using Cell Counting Kit-8 (CCK-8) method. Briefly, BMSCs and RAW 264.7 cells were respectively seeded into 96-well plates at a density of 1 × 10^4 cells per well. After 24 h attachment, the medium was removed and treated with new medium containing MSNs-PP or MSNs-PPR at different particle concentration (12.5–800 μg/mL). The loading of DEX by MSNs-PPR is 43 μg/μL. After 24 h incubation, 10 μL serum-free medium containing 10 μL of the CCK-8 solution per well was added. The cell viability was calculated from the measured absorbance.

The effect of MSNs-PPR on the cell morphology and cytoskeleton was investigated by confocal observation. BMSCs were seeded at a density of 1 × 10^4 cells per well in glass bottom dishes. After incubation with 25 or 100 μg/mL MSNs-PPR for 24 h, the culture medium was removed and fixed with 4% paraformaldehyde solution for 15 min at room temperature, followed by permeabilizing with 0.1% Triton X-100 and blocking with 1% BSA. Then the fixed cells were respectively treated with Alexa Fluor 568 phalloidin (Invitrogen, USA) and DAPI for F-actin and nucleus staining. The fluorescent images were captured by confocal laser scanning microscope (CLSM, Carl Zeiss LSM 700, Germany).

2.7. Drug and pDNA Loading. For DEX loading, 0.1 g MSNs-PPR was added into 5 mL ethanol aqueous solution, followed by addition of 10 mg DEX. The suspension was stirred in dark at room temperature for 12 h. To evaporate the solution and facilitate the drug adsorbed into the channel of MSNs, the suspension was placed into the vacuum oven. After that, DEX-entrapped MSNs-PPR was resuspended with PBS solution and collected by centrifugation, then washed twice with PBS (pH 7.2–7.4). All the supernatant was collected and measured using UV–vis spectrophotometry at 242 nm. The loading efficiency of DEX by MSNs-PPR was calculated via the standard curve. The loading of DEX by MSNs-PPR is 43 μg/μg.

2.8. Gel Retardation Assay. The pDNA binding capacity upon DEX@MSNs-PPR was determined by agarose gel electrophoresis. Various weight ratios (1:1, 2.5:1, 5:1, 10:1, 20:1, and 40:1) of DEX@MSNs-PPR/pDNA complexes were prepared in PBS solution and incubation for 1 h. The amount of pDNA in each lane was fixed at 0.5 μg. After that, 10 μL of the complexes were mixed with a 6× agarose gel loading dye solution. Then, 10 μL of the mixture was loaded onto the 0.8% agarose gel containing ethidium bromide (0.1 μg/mL) and run in Tris-acetate-EDTA buffer at 100 V for 40 min. Free pDNA was used as the control.

2.9. In vitro pDNA and DEX Release. To investigate the release behavior of pDNA from DEX@MSNs-PPR, the DEX@MSNs-PPR/pDNA complexes were prepared with 10 μg pDNA and 200 μg DEX@MSNs-PPR in 400 μL PBS for 1 h. After centrifugation, the supernatant was carefully collected and the DEX@MSNs-PPR/pDNA complexes were resuspended with 400 μL PBS. At different time points, the DEX@MSNs-PPR/pDNA complex suspension was centrifuged, and the supernatant was analyzed by the Nanodrop spectrometer to determine the released pDNA amount.

For DEX release investigation, 2 mg DEX@MSNs-PPR/pDNA dispersed in 1 mL PBS solution was placed in dialysis bag (7000 kDa) and then immersed in 4 mL PBS, shaking (100 rpm) at 37 °C. A 2 mL portion of PBS solution was taken out at predetermined time intervals and added with an equal volume of fresh PBS. The cumulative released amount of DEX was determined by UV–vis spectrophotometry measurement at 242 nm.

2.10. Intracellular Uptake Studies. For cellular uptake of DEX@MSNs-PPR, BMSCs were grown at 2 × 10^4 cells per well in 24-well plate for 24 h. Subsequently, the DEX@MSNs-PPR suspend with culture medium (25 μg/mL) was added into the well. The cells were incubated at 37 °C for 4 h, then fixed with 4% paraformaldehyde solution for 30 min. After that, the cell F-actin and nucleus were respectively stained with Alexa Fluor 568 phalloidin and DAPI solution.

For cellular internalization of pDNA, the seeded BMSCs in 24-well plate were incubated with DEX@MSNs-PPR/pDNA complexes. The pDNA was labeled with Cy3 and used amount of pDNA was 1.5 μg per well. After 4 h of incubation, the cells were fixed and stained with Alexa Fluor 488 phalloidin (Invitrogen, USA) and DAPI for F-actin and nucleus staining. The stained cells were visualized and imaged using the fluorescence microscope.

2.11. BMP-2 Expression Measurement. BMP-2 gene-bearing plasmid DNA (pIREs2-ZsGreen1-BMP2 plasmid) was used and delivered into BMSCs. For transfection, BMSCs were seeded at a density of 2 × 10^4 per well and cultured at 37 °C. When the cell density reached to 60–70% confluence, the cells were transfected with DEX@MSNs-PPR or DEX@MSNs-PP/pBMP2 complexes using a fixed dose of 1 μg/well of BMP2 plasmid. After 4 h incubation, the medium in each well was replaced with fresh complete medium containing 50 μg/mL of ascorbic acid and 10 mM of β-glycophosphate. At different time points (3 and 7 days) post-transfection, 100 μL of the supernatant in each sample was extracted. The BMP-2 protein content in the supernatant was measured using a human BMP-2 ELISA Kit (Neobioscience Technology, China).

2.12. ALP Activity Assay. BMSCs were cultured in 24-well plates at a density of 2 × 10^4 per well. After that, the cells were treated with MSNs-PPR, DEX@MSNs-PPR, MSNs-PPR/pBMP2, and DEX@ MSNs-PPR/pBMP2 which were suspended with complete medium containing 50 μg/mL of ascorbic acid and 10 mM of β-glycophosphate. After 7, 14, and 21 days, the ALP value was quantified following the protocol of Alkaline Phosphatase Assay Kit (Beyotime Institute of Biotechnology, China). The ALP activity was expressed relative to the total protein that measured by a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China).

2.13. Quantitative RT-PCR Analysis. After BMSCs cultured with different samples and incubated for 7 and 14 days, the expressions of osteo-related genes, including runt-related transcription factor 2 (RUNX2), collagen I (Col 1), osteopontin (OPN), and osteocalcin (OCN) were determined by quantitative real-time RT-PCR. The total cellular RNA was extracted using Trizol reagent. Then the first strand cDNA was synthesized using a Hieff First Strand cDNA Synthesis Kit (Yeasen, China) according to the manufacturer’s instructions. Subsequently, real-time PCR was performed using the Fast Real-Time PCR System (Applied Biosystems 7500, USA) by the Hieff qPCR SYBR Green Master Mix (Low Rox Plus) Kit (Yeasen, China). The relative transcript quantities were calculated using the ΔΔCt method and normalized to the housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the samples. The primer sequences used in this study are listed in Table S2.

2.14. Alizarin Red S Staining. Calcium mineralization of BMSCs was evaluated by alizarin red S staining. After treated with different
samples for 21 days, cells were fixed with 10% formalin and then treated with alizarin red S working solution with pH value around 4.1–4.3. Before observation, each well was washed with deionized water for removing the excess alizarin red S. The calcium deposition stained with the red color was imaged using the microscope. In order to quantify the calcium deposition, 10% acetic acid was used to dissolve the alizarin red S. After complete dissolution, the supernatant was collected and mixed with 10% ammonium hydroxide. Finally, the absorbance of the samples was measured at 405 nm on a microplate reader.

2.15. Statistical Analysis. Experiments were performed in triplicate, and data are reported as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey’s method. The values of *P* < 0.05 and **P* < 0.01 were considered as statistically significance difference.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of PEI-PLL. ROP reactions were conducted to synthesize the PEI-PLL copolymers with PEI as the initiator and Lys(Z)-NCA as the monomers in chloroform solution, as shown in Figure 1. Then the PEI-PLL copolymers can be achieved by deprotection of benzoxycarbonyl group in PEI-Plys(Z) using hydrobromic acid. Different copolymers were synthesized by using different molecular weight of PEI in the synthesis process. The results from 1H NMR spectra agree with the expected structures of copolymers, confirming that PEI-PLL copolymers have been successfully synthesized (Figure S1). Furthermore, the compositions of PEI and PLL in different PEI-PLL copolymers can be calculated by the results of 1H NMR spectra, as summarized in Table S1.

The chemical structure of synthesized PEI-PLL was further characterized by FTIR spectroscopy (Figure 2A and B). Compared to the pure PEI, PEI-Plys(Z) showed the typical absorption bands at 1652 and 1538 cm⁻¹, which were ascribed to the vibration of C=O (amide I band) and N−H (amide II band).27 Due to the presence of a benzoxycarbonyl group in Plys(Z), the bands at 746 and 699 cm⁻¹ attributed to plane bending vibration of benzene ring and 1704 cm⁻¹ assigned to the C≡O stretching vibration are also appeared.27,28 For PEI-PLL copolymers, the bands at 1652 and 1538 cm⁻¹ were still existed, but no bands at 746 and 699 cm⁻¹ were found, which demonstrated the complete deprotection of the benzoxycarbonyl group from the PEI-Plys(Z). Besides, for comparison of PEI-PLL-25k, PEI-PLL-10k, and PEI-PLL-1.8k, there was an increase in the absorption bands at 1652 and 1538 cm⁻¹ because of the increased weight percentage of formed PLL chains (Figure 2B). These results of FTIR spectra confirm the formation of PEI-PLL copolymers.

The buffering capacity of polycations is responsible for the escaping of the complexes from intracellular endosomes, which were studied by acid−base titration. Due to the existence of multiamine structure (i.e., primary, secondary, and tertiary amino groups), all native PEIs showed remarkable buffering capacity in the pH range from 11 to 2 (Figure S2), which resulted in the proton sponge effect in endolysosomal pH range and thus allowed their PEI/DNA complexes to escape into the cytoplasm.29 From the acid−base titration profiles (Figure 2C), it was clearly seen that the buffering capacities of these PEI-PLL copolymers were lower than that of their native counterparts. At the buffering pH region of 5.1−7.4, the buffering abilities of PEI-PLL-25k and PEI-PLL-10k had a comparable level and were slightly better than PEI-PLL-1.8k. This is likely because the PEI-PLL-1.8k possesses the highest content of PLL and its primary amino groups are completely protonated at pH 9.30 Although the buffering ability was reduced after PLL modification for every sample, they still display a considerable level.31

Agarose gel electrophoresis was conducted to determine the pDNA condensation by the complexes. As displayed in Figure 2D, the native PEI-25k showed a strong binding ability, with complete complexation at the weight ratio of higher than 0.2:1. In comparison, the three copolymers exhibited decreased DNA binding ability than PEI-25k, which were fully retarded at the weight ratios of 0.6, 0.8, and 0.6 for PEI-PLL-1.8k, PEI-PLL-10k, and PEI-PLL-25k, respectively. Here, we find that the profiles of pDNA mobility by PEI-PLL-1.8k and PEI-PLL-25k were almost the same, showing the preferable retardation of pDNA migration than PEI-PLL-10k.

The particle size and zeta potential of formed complexes were measured by dynamic light scattering (DLS). As shown in Figure 2E, the particle size was decreased with the increasing weight ratio. When the weight ratio was above 1:1, the particle size of the complexes turned to below 200 nm. It is known that the particle size of the complexes was closely associated with the cellular uptake. At the current size range of formed complexes, they are considered to be portable for cellular internalization.32 Figure 2F shows the zeta potential of complexes at different weight ratios. The zeta potential value of each sample increased with the increasing weight ratio. At the weight ratio of 1:1, the zeta potentials of the complexes were all positive. At a higher weight ratio than 1:1, the four complexes showed the positive zeta potential and reached the highest value under 25 mV.

3.2. Cytotoxicity and Transfection Efficiency Evaluation of PEI-PLL Copolymers. The cytotoxicity of these PEI-25k/pDNA and PEI-PLL/pDNA complexes with various weight ratios were assessed by CCK-8 method (Figure 3A). Clearly, there were no significant differences among these PEI-PLL/pDNA complexes at the tested concentrations. However, they showed little toxicity to the cells at a weight ratio of 40:1, with the viability still above 70%. In contrast, the PEI-25k/pDNA showed significantly higher cytotoxicity than those PEI-
PLL/pDNA complexes, whose cell viability were less than 40% against 293T cells.

The PEI-PLL copolymer-mediated gene transfection was first evaluated with reporter gene assay in the 293T cells. As shown in Figure 3B, the transfection efficiency of each sample was dramatically dependent on the complex ratio. In addition, the optimum weight ratios for the transfection efficiencies of PEI-25k, PEI-PLL-10k, and PEI-PLL-25k were fixed at 2.5:1, whereas the optimum weight ratio for PEI-PLL-1.8k was fixed at 5:1. Under the optimum transfection conditions, the transfection efficiency of PEI-PLL-25k was the highest one among these copolymers and also higher than that of PEI-25k in 293T cells. To clarify this point, the transfection efficiencies of all complexes were further visualized by observation of RFP-positive cells using fluorescence microscope. From the fluorescence images in Figure 3C, 293T cells transfected with PEI-PLL-25k mediated RFP gene delivery at ratio of 2.5:1 showed the most red fluorescence spots, indicative of the highest transfection capacity. As detected by flow cytometry (Figure S3), the quantitative analysis also found that PEI-PLL-25k was more effective in mediating gene transfection compared with PEI-25k. Consequently, these results confirmed that the copolymers PEI-PLL-25k had higher transfection efficiency than other counterparts. Furthermore, with regard to lower cytotoxicity, PEI-PLL-25k would be a promising gene carrier and thus used currently in the following investigation.

3.3. Preparation and Characterization of MSN-PPR.

The synthetic procedures to fabricate a multifunctional drug delivery system were schematically depicted Figure 1B. PEI-PLL-25k acted as an important component in the prepared MSNs-PPR and was responsible to the interaction with nucleic acid. Following the transformation from terminal amino group

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Figure 2. Characterization of synthesized PEI-PLL copolymers. (A, B) FTIR spectra. (C) Buffering capacity of different samples in 150 mM NaCl solutions. (D) Agarose gel electrophoresis assay, (E) particle size, and (F) zeta potential of different vector/pDNA complexes at various weight ratios.
to terminal carboxyl groups on the nanoparticles, PEI-PLL-25k can be successfully conjugated onto MSNs-COOH surface via amidation reaction. Then, a second amidation reaction was carried out to achieve the RGD peptide functionalization. As shown in Figure 4A, the TEM image of MSNs-NH₂ clearly revealed the spherical particle shape with highly ordered mesoporous structure. The type IV isotherm observed in the nitrogen adsorption−desorption isotherms also confirmed the presence of mesopores in the MSNs-NH₂ sample (Figure 4D).7 A specific surface area of 621.6 m²/g and an average pore size of 2.7 nm were calculated by Brunauer−Emmett−Teller (BET) and Barrett−Joyner−Halenda (BJH) analysis, respectively. After being modified, the particle surface was covered with a layer of polymers and thereby did not exhibit well-defined mesostructure (Figure 4B). Additionally, the modified sample MSNs-PPR was found to have a hydrodynamic size centered at 199.1 nm, showing a slight increase compared with bare MSNs-NH₂ (171.5 nm) because of the formed hydrated layer by polymers (Figure 4C).

The successful functionalization of both PEI-PLL polymer and RGD peptide on nanoparticles was modestly verified by series of characterization techniques including FTIR spectra, zeta potential and TGA. As depicted in Figure 5A, the characteristic peaks for each sample were displayed in the FTIR spectra. For MSNs-NH₂, the peak at 1558 cm⁻¹ was attributed to N−H asymmetric bending vibration. After grafting with carboxylic acid groups, two new peaks at 1412 and 1710 cm⁻¹ assigned to C=O stretching vibrations were appeared in the MSNs-COOH sample. However, the characteristic peak at 1710 cm⁻¹ indexed as C=O stretching vibration in the carboxyl group was disappeared and a new peak at 1470 cm⁻¹ attributed to C−N stretching vibration was

**Figure 3.** (A) Cell viabilities of 293T cells after transfection with different vector/pDNA complexes at various weight ratios for 24 h. ** Significant difference compared to PEI-PLL-1.8k, PEI-PLL-10k, and PEI-PLL-25k, respectively. (B) Luciferase gene expression and (C) fluorescent microscopic images of red fluorescence protein (red color) expression in 293T cells after transfected with different vector/pDNA complexes at various weight ratios for 48 h. Magnification: 10× objective. **p < 0.01.
emerged, which indicated the successful conjugation of PEI-PLL on MSNs.26 The spectrum of the RGD-modified sample gave the enhanced peak at 2949 cm\(^{-1}\) and a new peak at 2841 cm\(^{-1}\) ascribed to the C–H stretching vibration of peptide,

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**Figure 4.** TEM images of (A) MSNs-NH\(_2\) and (B) MSNs-PPR. (C) Size distribution curves of MSNs-NH\(_2\) and MSNs-PPR. (D) Nitrogen adsorption–desorption isotherm and pore diameter distribution curve (inset) of MSNs-NH\(_2\).

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**Figure 5.** Characterization of prepared nanoparticles. (A) FTIR spectra, (B) zeta potential, and (C) TGA curves of MSNs-NH\(_2\), MSNs-COOH, MSNs-PP, and MSNs-PPR. (D) UV–vis spectra of MSNs-PPR before and after centrifugation. (inset, left) Digital photo of MSNs-PPR dispersed in water. (inset, right) Supernatant after centrifugation.
which confirmed the successful functionalization of RGD. Figure 5B shows the zeta potential changes of different samples. It could be seen that MSNs-NH₂ showed a positive potential of +45.2 mV and then changed to be a negative potential of −36.8 mV after carboxylation. After modifying with PEI-PLL and RGD, the zeta potential of MSNs-PPR was reversed to a positive value and eventually maintained at +32.1 mV. In addition, the weight loss of samples was characterized by thermogravimetric analysis. The weight loss of MSNs-NH₂, MSNs-COOH, MSNs-PP, and MSNs-PPR were calculated to be 21.5, 24.7, 48.2, and 55.0 wt %, respectively, as shown in Figure 5C. Obviously, the weight loss of nanoparticles was increased along with the process of conjugation, revealing the success of modification. For further verification of RGD grafting on nanoparticles, FITC-labeled RGD peptide was utilized to modify the nanoparticles and could be monitored by the UV–vis spectrum. As seen in Figure 5D, as-prepared MSNs-PPR suspension showed a light yellow color and exhibited a characteristic peak of FITC around the wavelength of 495 nm. After centrifugation, however, the supernatant was transparent and the adsorption peak of FITC could not be detected, which provided the convincing evidence for RGD functionalization. These results demonstrated that the final MSNs-PPR production has been successfully prepared.

3.4. Biocompatibility of MSNs-PPR. For exploring the biocompatibility, the cytotoxicity against both BMSCs and RAW 264.7 cells of MSNs-PPR was evaluated by CCK-8 assay. According to the cell viability of BMSCs (Figure 6A), MSNs-PP showed a concentration-dependent cytotoxicity against BMSCs. When the particle concentration was 400 μg/mL, the cell viability was decreased to 81.6% after 24 h incubation. Furthermore, more significant cytotoxicity was measured at the particle concentration of 800 μg/mL. In contrast, MSNs-PPR did not show any obvious cytotoxicity at concentrations of

Figure 6. Cell viabilities of (A) BMSCs and (B) RAW 264.7 cells after treated with various concentrations of nanoparticles for 24 h. (C) CLSM images of BMSCs incubated with MSNs-PPR at particle concentration of 25 and 100 μg/mL for 24 h. The green spots indicated the nanoparticles. Cell F-actin was stained with Alexa Fluor 568 phalloidin (red) and nucleus was stained with DAPI (blue). ***P < 0.01.
12.5−800 μg/mL, which suggested that MSNs-PPR had excellent cytocompatibility. Consequently, in terms of the result of cell viability, the cytotoxicity of MSNs-PPR was lower than that of MSNs-PP, which revealed that RGD functionalization could alleviate the toxic effects of MSNs-PP. This may be resulted from the decreased amino group density on the particles surface by RGD conjugation.\textsuperscript{34} In addition, the cell viability of MSNs-PPR against RAW 264.7 cells reflected the same tendency, showing high cell viability at the particle concentration ranging from 12.5 to 800 μg/mL (Figure 6B).

Also, hemolysis was an accessible index to demonstrate the biocompatibility of MSNs-PPR. From the digital photos in Figure S4, obvious hemolytic effect could be observed after incubated with MSNs-PP at the concentration of 800 μg/mL. By quantitative analysis, more than 10% hemolysis percentages were detected at this particle concentration. By contrast, no apparent hemolytic activity of MSNs-PPR was captured even at 800 μg/mL and the measured hemolysis percentages were

Figure 7. (A) pDNA loading on DEX@MSNs-PPR, 2 μg of pDNA was mixed with various amounts of DEX@MSNs-PPR for 1 h. (B) Loading amount plotted with respect to the pDNA content, which was varied (0.25, 0.5, 1.0, 1.5, 2.0, and 4.0 μg) with a fixed 10 μg of DEX@MSNs-PPR. In vitro release profiles of (C) pDNA and (D) DEX from DEX@MSNs-PPR/pDNA complexes. (E) Fluorescent images of BMSCs treated with DEX@MSNs-PPR. The green spots indicated the nanoparticles. Cell F-actin was stained with Alexa Fluor 568 phalloidin (red) and nucleus was stained with DAPI (blue). (F) Fluorescent images of BMSCs treated with DEX@MSNs-PPR/pDNA complexes. The red spots indicated the Cy3 labeled pDNA. Cell F-actin was stained with Alexa Fluor 488 phalloidin (green) and nucleus was stained with DAPI (blue).
all lower than 1%. Afterward, the toxic effect of MSNs-PPR on cell cytoskeleton was investigated using CLSM observation. As shown in Figure 6C, BMSCs were typically presented in well-spread morphology with an extensive actin network under the condition of normal medium. When the particle concentration of MSNs-PPR increased from 25 to 100 μg/mL, more green fluorescence dots emerged and were distributed surrounding the nucleus within the cells. Furthermore, no obvious changes on the actin network were observed after 24 h incubation, consistently implying the good biocompatibility of MSNs-PPR.

### 3.5. Loading and Release of pDNA and DEX.

The loading capacity of pDNA was measured by incubation of nanoparticles with pBMP2 plasmid at different weight ratios for 1 h. The loaded pDNA increased with the increasing carrier content, as presented in Figure 7A. When the weight ratio reached to 20:1, the adsorbed pDNA content on the carrier was about 0.90 μg. Thereafter, slow increase of loaded pDNA was detected and finally raised to 0.99 μg at weight ratio of 80:1. Next, different amounts of pDNA were used to test the pDNA loading saturation for DEX@MSNs-PPR (Figure 7B). It was found that the maximal loading amount of pDNA was 0.49 μg, while the loaded pDNA (0.47 μg) was close to saturation at pDNA amount of 1.0 μg. Subsequently, agarose gel electrophoresis was utilized to further confirm the pDNA complexation by modified nanoparticles (Figure S5). The image revealed that the migrated pDNA bands in the lane at weight ratio of 20:1 almost disappeared, indicating that most of the pDNA was packaged and retarded. Therefore, the resultant DEX@MSNs-PPR could efficiently complex the pDNA at ratio

![Figure 8](image-url)

**Figure 8.** (A) BMP-2 protein expression in BMSCs after transfection with DEX@MSNs-PPR and DEX@MSNs-PPR/pBMP2 for 3 and 7 days. (B) In vitro ALP activity of BMSCs after treatment with various samples for 7, 14, and 21 days. *P < 0.05 and **P < 0.01 versus the Control and MSNs-PPR groups. Effect of different samples on osteo-related gene (C) RUNX2, (D) OPN, (E) Col I, and (F) OCN expression of BMSCs after incubation for 7 and 14 days. *P < 0.05 and **P < 0.01 versus the MSNs-PPR group.
of 20:1 and such a ratio was selected in the further experiments.

The detachment of pDNA from the carrier was a critical factor to guarantee the expression of target gene, thus the release profile of pDNA was investigated. As shown in Figure 7C, the cumulative release of pDNA reached 23% in the first 4 h, and over 61% of pDNA was released after 96 h. This result suggested that pDNA released from DEX@MSNs-PPR was relatively fast, which was probably attributed to the surface attachment by modified nanoparticles. In this circumstance, it was supposed that more target proteins would be expressed and immediately trigger the biological response. Meanwhile, the release behavior of DEX was also monitored by immersion in PBS solution. From the result of Figure 7D, DEX released from the channel of the carrier into the medium in a gradual release pattern. After 20 days immersion, the total release of DEX was up to 75%, implying a long-term drug release over weeks. As a result, the combination of relatively fast release of pDNA with sustained release of DEX might be efficient to stimulate the osteogenic development of BMSCs.

3.6. Internalization of Nanocomplexes. As the drug was resided in the mesopores of nanoparticles, effective and rapid internalization of nanocarrier into the cells was an issue of great importance to drug delivery. Therefore, the cellular uptake of nanocomplexes was monitored by FITC fluorescence. Figure 7E shows the internalization of DEX@MSNs-PPR by BMSCs after 4 h incubation. It could be seen that large amounts of FITC-labeled nanoparticles were distributed in the cytoplasm in a short time. Additionally, the delivery of pDNA using DEX@MSNs-PPR was observed via Cy3-labeled pDNA. Similarly, the fluorescent images in Figure 7F revealed that a high accumulation of pDNA could be observed inside the cells after 4 h post-transfection. Overall, these results showed that functionalized MSNs-PPR could be a well-designed delivery system for codelivery drug and gene.

3.7. Effect of Dual-Factor Delivery on Biological Response. After confirming the cellular internalization of the nanocomplexes, the expression of BMP-2 protein inside the cells was investigated by quantitative analysis. After 3 and 7 days post-transfection, the intracellular BMP-2 protein was analyzed using a BMP-2 ELISA kit, as presented in Figure 8A. Compared with the group of DEX@MSNs-PPR-treated cells, the BMP-2 expression was obviously detected in the DEX@MSNs-PPR/pBMP2-treated cells because of the delivery of BMP-2 gene by nanocarrier and continuous BMP-2 gene release from the vector within cells.

Next, the osteogenic differentiation of BMSCs after BMP-2 gene transfection was investigated. First, ALP activity was determined by quantitative measurement since it was commonly identified to be an important early marker of osteogenesis. From Figure 8B, the results revealed an increasing ALP activity over the culture time for each group. It could be clearly seen that the ALP activity of cells in DEX@MSNs-PPR and MSNs-PPR/pBMP2 groups were significantly higher than that in control and empty vector groups at all time points. But dramatically, the cells treated with DEX@MSNs-PPR/pBMP2 complexes displayed a higher ALP level than that treated with either DEX@MSNs-PPR or MSNs-PPR/pBMP2 complexes.

Figure 9. Evaluations of mineralization in BMSCs after incubation with different samples for 21 days. (A) Digital photos of alizarin red S staining. (B) Quantitative analysis of mineralized matrix. (C) Schematic illustration of osteogenic differentiation of BMSCs induced by MSNs-based BMP-2 gene and DEX codelivery system. *P < 0.05 and **P < 0.01 against the control group.
on days 14 and 21. Accordingly, the ALP activity in DEX@ MSNs-PPR/pBMP2-treated cells showed the highest level compared with the other groups. The above results demonstrated that simultaneous delivery of DEX and BMP-2 gene into BMSCs could significantly increase the ALP activity, implying the enhanced osteogenic ability of DEX@MSNs-PPR/pBMP2 codelivery system.

The representative bone-related genes expression was analyzed by quantitative RT-PCR, including RUNX2, Col I, OPN, and OCN. As shown in Figure 8C–F, apparent changes in genes expression were detected after the cells incubated with the vectors containing osteogenic factor. We observed that the presence of DEX@MSNs-PPR or MSNs-PPR/pBMP2 in cultured cells significantly promoted the mRNA expression of RUNX2. By contrast, due to the combination of DEX and BMP-2, the RUNX2 mRNA expression in dual-factor system was significantly increased. Also, for the mRNA expression analysis of OPN, Col I, and OCN, a similar trend was observed after those vectors’ incubation. These osteogenic genes expression in the DEX@MSNs-PPR/pBMP2 group significantly increased in comparison with DEX@MSNs-PPR and MSNs-PPR/pBMP2 groups during the culture period. Since the osteogenic differentiation of osteoblasts was commonly affected by the combination of several factors, thereby the osteogenic capability would be enhanced because of the induction from the cooperation of two factors of expressed BMP-2 and released DEX which were evidenced in previous studies.23,36

The mineralized matrix formation was also the indicator that could be employed to validate the osteogenic differentiation of BMSCs induced by nanocomplexes, which was characterized using alizarin red S staining (Figure 9A). The cells were typically presented as red, indicating the formation of calcium deposition. On day 21, much more calcium deposits were generated in the groups of DEX@MSNs-PPR, MSNs-PPR/pBMP2, and DEX@MSNs-PPR/pBMP2 as compared with the groups of control and MSNs-PPR. The amount of formed mineralized matrix in each sample was further examined through the quantitative analysis of dissolved dyes. As displayed in Figure 9B, BMSCs cultured with DEX@MSNs-PPR/pBMP2 exhibited the highest level in calcium content than those cultured with other nanocomplexes, consistently demonstrating the improved stimulatory effects on the osteogenesis of dual-factor delivery system.

In this study, the PEI-PLL copolymers with positive potentials were systematically investigated for gene delivery. With respect to the improved cytoppatibility and transfection efficiency relative to the unmodified PEI-25k and other counterparts, PEI-PLL-25k was consciously selected to act as the important component for surface functionalization of MSNs. Further studies demonstrated that as-prepared MSNs-PPR was able to deliver the osteogenic drug and DNA into the cells efficiently due to the high-efficiency of intracellular uptake. Importantly, the codelivery system for simultaneous delivery of DEX and BMP-2 gene could induce higher osteogenic effects on BMSCs than single-factor delivery system. In that case, the biological response for enhanced osteogenic function of dual-factor delivery system was elaborated briefly, as illustrated in Figure 9C. BMSCs were shown to express integrin receptors on the cell surface, which can be recognized by the RGD peptide.37 Due to the high affinity of ligand/receptor interactions, RGD peptide has been regarded as a promising targeting ligand for nanocarrier decoration to mediate drug or gene delivery, such as polyamidoamine dendrimer, iron oxide nanoparticles, and MSNs.37–39 Here, the RGD motifs on nanoparticles were expected to enhance receptor-mediated endocytosis of the gene vector.15 Additionally, the positive surface charges would further facilitate the cellular uptake via nonspecific electrostatic interactions.40 These reasons led to the efficient internalization of functionalized MSNs and ensured the guests delivery into the cells. After endocytosis, expressed BMP-2 protein from transfected BMP-2 gene triggered the osteoblast differentiation of BMSCs. On the other hand, the encapsulated DEX released from the vector also generated positive effects on BMSCs differentiation. As a result, a synergistic effect of BMP-2 and DEX on the osteogenic development of osteoblasts was highlighted, which benefited from the dual-delivery mode of osteogenic drug and gene.

4. CONCLUSION

In summary, we synthesized polycation and RGD peptide functionalized MSNs as the dual-delivery system for simultaneous delivery of osteogenic drug and gene into stem cells. The gene transfection efficiencies of synthesized PEI-PLL copolymers with different molecular weight PEI blocks were evaluated. It was demonstrated that PEI-PLL-25k displayed the preferable transfection efficiency relative to the other counterparts as evidenced by the results of luciferase gene expression, fluorescent protein expression, and flow cytometry assay. Then PEI-PLL-25k and RGD peptide was able to encapsulate the dexamethasone (DEX) into the mesopores and simultaneously carry pDNA via electrostatic interaction. Meanwhile, in vitro release profiles revealed that the loaded osteogenic drug and gene could release from the functionalized vector continuously. In vitro cell viability and hemolysis assays demonstrated the good biocompatibility of MSNs-PPR. In addition, the pDNA encoding BMP-2 gene can be efficiently delivered into BMSCs and expressed BMP-2 protein. Furthermore, compared with BMSCs treated with single BMP-2 gene delivery and single DEX delivery, BMSCs treated with DEX@MSNs-PPR/pBMP2 bearing BMP-2 gene and DEX displayed higher activities of osteogenic differentiation in terms of ALP activity, osteo-related gene expression, and calcium deposits evaluation. Therefore, the developed MSNs-PPR nanocarrier could be employed as nanoparticulate dual-factor delivery system for triggering an efficient osteogenic effect.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.8b01110.

Detailed experimental section for characterization of PEI-PLL copolymers, including proton buffering capacity, particle size, zeta potential, pDNA condensation ability, in vitro cytotoxicity, and transfection efficiency. Results of 3H NMR spectra of PEI, PEI-Plys(Z), and PEI-PLL, buffering capacities of different molecule weight PEI, transfection efficiency of PEI-PLL-25k determined by flow cytometry, hemolysis assays of RBCs treated with MSNs-PP and MSNs-PPR, agarose gel electrophoresis assay of DEX@MSNs-PPR/pDNA
complexes, and summary of molecular characteristics of different PEI-PLL copolymers (PDF)

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**Notes**

The authors declare no competing financial interest.

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