Synthesis of Calcium Bisphosphonate/Calcium Polyacrylate Spheres for Gene Delivery

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1. INTRODUCTION

The metal phosphonates as representative organic–inorganic hybrid materials have exhibited excellent physicochemical properties for various applications in the fields of adsorption, separation, catalysis, etc., due to their unique tailorable frameworks allowing selection of appropriate metals and phosphonic acids with special organic groups to combine different functionalities into one. During the past 2 decades, many efforts have been made to control the pore structure of metal phosphonates, and a lot of encouraging results have been achieved. However, it is still challenging to precisely control the spherical morphology of metal phosphonates due to their rapid precipitation in solution. So far, most of the research relating to the control of the spherical morphology focuses on aluminum phosphonates, tin phosphonates, and transition metal phosphonates, the reports of spherical calcium phosphonates are scarce.

It is well known that calcium phosphate (CaP) nanoparticles are promising nonviral inorganic vectors for gene delivery due to their capacity of transfection of various mammalian cells in vitro, as well as low immune responses and cytotoxicity. The calcium phosphonates can be regarded as organic-functionalized CaP, wherein the special organic functional groups can endow the materials with positive-charge surface properties, which is the desired property for the application in gene delivery. As far as we know, the application of calcium phosphonates in gene delivery has not been known until now. Thus, for the first time, calcium phosphate spheres with uniform morphology have been designed and synthesized for gene delivery in this article.

Here, the calcium bisphosphonate spheres were synthesized using 1,4-bis(phosphomethyl) piperazine (BPMP) with a piperazine group through the colloid template method. The PAA–Ca\(^{2+}\)–H\(_2\)O colloid spheres, formed by sodium polyacrylate (PAAS) and calcium ions in the solution, were selected as the template because their particle diameter can be precisely adjusted by the complexation time within limits. In addition, the colloidal spheres of PAA–Ca\(^{2+}\)–H\(_2\)O as the template can control the final product with a regular sphere shape and submicrometer size by providing calcium ions for bisphosphonates to form calcium bisphosphonates. Such a submicrosphere morphology effectively inhibits the resulting particles from aggregating into a block during the transfection, as opposed to their nanoscale analogue, due to the low surface energy. In addition, PAA present in the colloidal spheres of the PAA–Ca\(^{2+}\)–H\(_2\)O template is nontoxic and biocompatible, which has been proven by its wide application in bioadhesive and pH-controlled drug-delivery systems.

ABSTRACT: Calcium bisphosphonate/calcium polyacrylate spheres were synthesized by a facile method and applied for the first time as gene vectors for transfection. The colloidal spheres of the PAA–Ca\(^{2+}\)–H\(_2\)O complex, formed by sodium polyacrylate and calcium ions in the solution, were used as template to synthesize a spherical PAA–Ca\(^{2+}\)–BPMP composite (CaBPMP/CaPAA) in the presence of 1,4-bis(phosphomethyl) piperazine (BPMP). The CaBPMP/CaPAA composite exhibits uniform and well-dispersed spheres with a particle size of about 200 nm as expected. The cytotoxicity assays confirm that CaBPMP/CaPAA spheres are quite safe for different cells even at a high concentration of 500 \(\mu\)g/mL. In vitro transfection results show that CaBPMP/CaPAA spheres serving as gene vectors are capable of transferring exogenous genes into different cells with about 25% of transfection efficiency and good reproducibility. The transfection capacity of CaBPMP/CaPAA spheres may be attributed to the controllable sphere morphology, low cytotoxicity, moderate DNA loading capacity, and bioreposable property. The application of calcium phosphonates with adjustable surface properties derived from the different organic groups of phosphonic acid in gene delivery provides a new design idea for gene vectors.
(phosphomethyl)piperazine (BPMP) has been selected to construct calcium bisphosphonate to provide a positive-charge surface property, which effectively improves the gene-loading capacity of the material. In fact, BPMP can provide zirconium bisphosphonates with a cationic surface property through the protonation of the piperazine groups, which has been achieved in our previous study.28,31 Moreover, these pH-sensitive zirconium bisphosphonates have been applied in colon-targeted drug delivery of negatively charged diclofenac sodium, insulin, and nucleic acid because of their pH sensitivity and biocompatibility.29–31 Therefore, the application of calcium bisphosphonate spheres with uniform morphology as a new vector for gene delivery is anticipated. To evaluate the performance of calcium bisphosphonate spheres as gene vectors, DNA loading capacity, DNA retardation assay, DNase I stability assay, and DNA release study, as well as the cytotoxicity and transfection efficiency of the calcium bisphosphonate/calcium polyacrylate composite for different cells in vitro, were investigated in detail.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization. It is demonstrated that PAAS with a medium molecular weight in the aqueous solution containing calcium ions can form colloidal spheres, wherein calcium ions coordinate with the carboxyl groups in polyacrylate instead of acting as counterions.2–5,4,3 Moreover specifically, the carboxyl groups in polyacrylate adopted different coordination modes to coordinate with calcium ions, which caused polyacrylate chains to coil and shrink forming colloidal spheres of the PAA−Ca2+−H2O complex through hydrophobic interaction.32,35–38 Moreover, the reported study discovered that colloidal spheres of the PAA−Ca2+−H2O complex grew from 160 ± 40 to 490 ± 100 nm with the complexation time increasing from 3 min to 24 h.21 Here, the colloidal PAA−Ca2+−H2O spheres were used as a template for the synthesis of the calcium bisphosphonate/calcium polyacrylate composite, wherein the PAA−Ca2+−H2O complex can provide unsaturated coordinated calcium ions for BPMP to further form the PAA−Ca2+−BPMP composite with controlled sphere morphology.

The morphology of the as-synthesized CaBPMP/PAAS composite was first characterized by scanning electron microscopy (SEM), as shown in Figure 1a. As can be seen from Figure 1a, the sample comprised relatively uniform and well-dispersed spheres with the diameter in the range between 150 and 250 nm. The transmission electron microscopy (TEM) image (Figure 1b) also reveals that the resultant CaBPMP/PAAS composite comprised regular spheres with good dispersity. No porosity can be observed in the CaBPMP/PAAS spheres in the TEM image with a higher magnification (Figure 1c), which is in agreement with the result of the N2 adsorption–desorption isotherm (Figure S1 of the Supporting Information). The CaBPMP/PAAS spheres are amorphous due to the low reaction temperature, which can be confirmed by the powder XRD pattern with a broad hump at about 20°, characteristic of the amorphous phase (Figure S2). The dynamic light scattering result shows that the average hydrodynamic diameter of the CaBPMP/PAAS spheres was about 209 ± 33 nm.

The chemical compositions of the CaBPMP/PAAS spheres were characterized by Fourier transform infrared (FT-IR) and solid-state 31P and 13C MAS NMR spectra. From the FT-IR spectrum of the CaBPMP/PAAS spheres (Figure 2d), a broad absorption band centered at 1100 cm−1 can be assigned to Ca−O−P stretching vibration in accordance with that in the FT-IR spectrum of CaBPMP synthesized directly without colloidal spheres of the PAA−Ca2+−H2O complex as the template (Figure 2c), indicating that phosphonyl groups in BPMP have coordinated to the calcium ions on/into the PAA−Ca2+−H2O colloidal spheres. The presence of an absorption band at 983 cm−1 related with free P−OH in Figure 2cd suggests that the surface defect sites exist in CaBPMP regardless of whether PAA−Ca2+−H2O colloidal spheres are used as a template or not. In addition, the strong absorption peaks located at 1560 and 1415 cm−1 are attributed to antisymmetric and symmetric stretching vibrations of the carboxyl groups in polyacrylate, respectively (Figure 2d). The difference (∆ = 145 cm−1) between ν asym(CO2) 1560 cm−1 and ν sym(CO2) 1415 cm−1 in Figure 2d is similar to that of uncoordinated carboxylic acid groups of PAAS,
BPMP can retain its structural integrity during the synthesis process.

2.2. Cytotoxicity of CaBPMP/CaPAA Spheres. The future application of CaBPMP/CaPAA spheres as vectors for gene delivery requires them to exhibit no cytotoxicity or low cytotoxicity. To evaluate the cytotoxicity of CaBPMP/CaPAA spheres, in vitro cytotoxicity assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method were conducted against NIH-3T3 cells and RBL-2H3 cells by measuring the viability of cells. The viability of NIH-3T3 cells and RBL-2H3 cells at different concentrations of CaBPMP/CaPAA spheres is displayed in Figure 5. As can been seen in Figure 5a, the CaBPMP/CaPAA spheres at concentrations below 500 μg/mL exert no effect on NIH-3T3 cells, that is, CaBPMP/CaPAA spheres in such a concentration range (0.5–500 μg/mL) exhibit no cytotoxicity to NIH-3T3 cells. However, the viability of NIH-3T3 cells obviously reduces with the increase in the concentration of the CaBPMP/CaPAA spheres. The ∼14 and ∼25% decrease of NIH-3T3 cells’ viability can be observed for CaBPMP/CaPAA spheres at concentrations as high as 1000 and 2000 μg/mL, respectively, showing that CaBPMP/CaPAA spheres at higher concentrations exhibit cytotoxic effects on NIH-3T3 cells. However, for RBL-2H3 cells, the change trend of cell viability with the increase in the concentration of CaBPMP/CaPAA spheres is similar to that of NIH-3T3 cells, as shown in Figure 5b, that is, CaBPMP/CaPAA spheres also exhibit no cytotoxicity for RBL-2H3 cells until the concentration increases...
the eluent of the GFP pDNA − Figure 6c. The migration of GFP pDNA can be observed for demonstrating that CaBPMP/CaPAA spheres exert protective ratios of 0.5 complex at CaBPMP/CaPAA spheres/GFP pDNA weight release by the GFP pDNA phosphate-buffer using GFP pDNA as a control. As shown in Figure 6a, the CaPAA complex was investigated by agarose gel electrophoresis weight ratio changed from 0.5 to 2.5 (lanes 2 spheres when the CaBPMP/CaPAA spheres/GFP pDNA migration of GFP pDNA was retarded by CaBPMP/CaPAA the strong a CaPAA microspheres possess good DNA-binding ability due to CaBPMP/CaPAA spheres (lane 1). The result suggests that CaBPMP/CaPAA microspheres possess good DNA-binding ability due to the strong affinity between CaBPMP/CaPAA spheres and GFP pDNA through calcium ions as counterions. The CaBPMP/CaPAA spheres after loading with GFP pDNA were characterized by SEM, particle size distribution, and zeta potential. The SEM image of GFP pDNA-loaded CaBPMP/CaPAA spheres shows that CaBPMP/CaPAA spheres can still retain their original sphere morphology after loading with GFP pDNA (Figure S3). The dynamic light scattering result further confirms that the average hydrodynamic diameter of GFP pDNA-loaded CaBPMP/CaPAA spheres was about 256 ± 49 nm, which is slightly larger than that of unloaded CaBPMP/CaPAA spheres (209 ± 33 nm). The $\zeta$-potential of GFP pDNA-loaded CaBPMP/CaPAA spheres was −35.6 mV, which is more negative than that of unloaded CaBPMP/CaPAA spheres (−25 mV). The above results demonstrate that the GFP pDNA molecules twine around the CaBPMP/CaPAA spheres through electrostatic interactions via calcium ions surrounding the CaBPMP/CaPAA spheres and acting as counterions.

The DNA retardation assay of the GFP pDNA−CaBPMP/CaPAA complex was investigated by agarose gel electrophoresis using GFP pDNA as a control. As shown in Figure 6a, the migration of GFP pDNA was retarded by CaBPMP/CaPAA spheres when the CaBPMP/CaPAA spheres/GFP pDNA weight ratio changed from 0.5 to 2.5 (lanes 2−6) in comparison with that of GFP pDNA alone in the absence of CaBPMP/CaPAA spheres (lane 1). The result suggests that CaBPMP/CaPAA microspheres possess good DNA-binding ability due to the strong affinity between CaBPMP/CaPAA spheres and GFP pDNA through calcium ions as counterions. The DNase I stability assay of the pDNA−CaBPMP/CaPAA complex was performed, and the images of agarose gel electrophoresis are displayed in Figure 6b. It is obvious that the naked GFP pDNA was almost completely degraded in the presence of DNase I, which is proved by the fact that the band of GFP pDNA cannot be clearly observed (lane 2). However, the migration of GFP pDNA was observed after the GFP pDNA−CaBPMP/CaPAA complex at CaBPMP/CaPAA spheres/GFP pDNA weight ratios of 0.5−2.5 was digested by DNase I and treated with phosphate-buffered saline (PBS) solution (lanes 3−7), demonstrating that CaBPMP/CaPAA spheres exert protective effects on GFP pDNA against DNase I. The GFP pDNA release by the GFP pDNA−CaBPMP/CaPAA complex is also presented in the agarose gel electrophoresis images, as shown in Figure 6c. The migration of GFP pDNA can be observed for the eluent of the GFP pDNA−CaBPMP/CaPAA complex (lanes 2−6 corresponding to CaBPMP/CaPAA spheres/GFP pDNA weight ratios of 0.5−2.5, respectively). Furthermore, the band of GFP pDNA after release is identical to that of the native GFP pDNA (lane 1), meaning that GFP pDNA can still retain its structure intact after eluting from the GFP pDNA−CaBPMP/CaPAA complex, that is, CaBPMP/CaPAA spheres as gene vectors have no effect on the structure integrity of loaded DNA.

2.4. In Vitro Transfection Using CaBPMP/CaPAA Spheres as Gene Vectors. To value the transfection activity of CaBPMP/CaPAA spheres, green fluorescent protein-coding plasmid DNA (GFP pDNA) as the model gene and two kinds of cells (NIH-3T3 cells and RBL-2H3 cells) as model cells were selected for the research. In vitro transfection experiments for NIH-3T3 cells were performed using different amounts of CaBPMP/CaPAA spheres to explore the optimal ratio of vectors/DNA. The transfection results are summarized in Figure 7, and the corresponding flow cytometry histogram profiles are listed in Figure S4. It is clear that the transfection efficiency was
The transfection efficiency of CaBPMP/CaPAA spheres for NIH-3T3 cells in the presence of CaCl2 shows almost no change in comparison with that in the absence of CaCl2, suggesting that calcium ions have little impact on the transfection activity of CaBPMP/CaPAA vectors. This can be explained by the fact that CaBPMP/CaPAA spheres have a negative $\zeta$-potential of $-26.3$ mV in the synthesis condition, which causes calcium ions to surround CaBPMP/CaPAA spheres acting as counterions in the synthesis system. Thus, the addition of calcium ions in the transfection experiments would not serve as linkers to bind GFP pDNA with CaBPMP/CaPAA spheres, resulting in no further increase of DNA loading of the CaBPMP/CaPAA spheres.

For NIH-3T3 cells, the transfection efficiency using CaBPMP/CaPAA spheres as gene vectors is $25.1\%$, which is $7 \sim 8$ times higher than that using the standard CaP method\textsuperscript{39} and is also higher than that of modified CaP\textsuperscript{39} and CaBPMP/CaPPA (Figure 8). However, the transfection activity of CaBPMP/CaPAA spheres cannot compare with the reported CaP–polymer composites in the previous references\textsuperscript{42,43}, in which a luciferase-expressing plasmid was used for transfection and the luciferase activity instead of explicit transfection efficiency was used to express transfection activity in the transfection results. Hence, a control transfection experiment was conducted using Lipofecter to estimate CaBPMP/CaPAA vector’s transfection activity. The transfection efficiency of Lipofecter (control group) was $39.7\%$ for NIH-3T3 cells, as displayed in Figures 8 and S5a. The CaBPMP/CaPAA vector’s transfection efficiency for NIH-3T3 cells can reach $63\%$ of that of Lipofecter, suggesting that CaBPMP/CaPAA vectors are effective in gene delivery. The transfection capacity of the CaBPMP/CaPAA vector can be further improved if cell-targeting peptides are used during the transfection, as reported in previous references\textsuperscript{34,45}.

The transfection activity of CaBPMP/CaPAA spheres was also investigated using RBL-2H3 cells (Figures 8 and S5b). The transfection efficiency of CaBPMP/CaPAA spheres in the absence of CaCl2 and in the presence of CaCl2 is $23.6$ and $25.9\%$, respectively. It is obvious that the transfection results of CaBPMP/CaPAA spheres for RBL-2H3 cells are consistent with those of NIH-3T3 cells. The CaBPMP/CaPAA spheres exhibit similar transfection activity for NIH-3T3 cells and RBL-2H3 cells in transfection experiments, further demonstrating that the new gene vector of CaBPMP/CaPAA spheres possesses consistent transfection capacity for different cells and good reproducibility. Even so, the transfection efficiency of CaBPMP/CaPAA is not as high as that of bisphosphonate-stabilized CaP nanoparticles (PEG-NP) reported in a previous reference\textsuperscript{39}, which is mainly due to poor DNA-loading capacity for their more negative surface property, lower surface energy, and less surface area derived from a larger particle size.

To further visualize the transfection capacity of CaBPMP/CaPAA vectors, the intracellular GFP expression was observed using a confocal laser scanning microscope (CLSM). As shown in Figure 9C(C2,C3), the green fluorescent signal was obviously observed in NIH-3T3 cells with the GFP pDNA–CaBPMP/CaPAA complex formed by GFP pDNA and CaBPMP/CaPAA vectors, indicating that GFP has been expressed after CaBPMP/CaPAA vectors carried GFP pDNA into the cells. In contrast, the green fluorescent signal cannot be seen in Figure 9A(A2,A3), that is, no expression of GFP was observed in the cells when NIH-3T3 cells were transfected using GFP pDNA alone, which suggests that GFP pDNA

**Figure 7.** Transfection efficiency using different amounts of CaBPMP/CaPAA vectors and $20 \mu$g of GFP pDNA for NIH-3T3 cells: 0 (blank); 5; 10; 20; 30; 40; 50 $\mu$g CaBPMP/CaPAA vectors and 20 $\mu$g of PcDNA 3.1(+). The transfection activity of GFP pDNA in the presence of CaCl2 were also carried out in the presence of calcium ions. The transfection negative control experiment was also conducted by using CaBPMP/CaPAA vectors (50 $\mu$g) and a plasmid (PcDNA 3.1(+), 20 $\mu$g), which does not encode for any fluorescent proteins, to exclude the autofluorescence signals of CaBPMP/CaPAA vectors during transfection.

In view of the fact that calcium ions can enhance the transfection efficiency of CaP/polymer as per the previous reference\textsuperscript{43}, in vitro transfection experiments for NIH-3T3 cells were also carried out in the presence of calcium ions. The transfection activity of GFP pDNA in the presence of CaCl2 was investigated to eliminate the effect of calcium ions on the cytomembrane. A transfection efficiency of $3.5\%$ was obtained using CaBPMP/CaPAA vectors In Figure 9 C(C2,C3), the green fluorescent signal was obviously observed in NIH-3T3 cells with the GFP pDNA (control group) was 39.7% for NIH-3T3 cells, as displayed in Figures 8 and S5a. The CaBPMP/CaPAA vector’s transfection efficiency for NIH-3T3 cells can reach 63% of that of Lipofecter, suggesting that CaBPMP/CaPAA vectors are effective in gene delivery. The transfection capacity of the CaBPMP/CaPAA vector can be further improved if cell-targeting peptides are used during the transfection, as reported in previous references\textsuperscript{34,45}.

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**Figure 8.** Transfection efficiency of different vectors: standard CaP\textsuperscript{39} nano-CaP (CaP/DNA-nanoparticles)\textsuperscript{49} multishell CaP (multishell CaP-DNA nanoparticles)\textsuperscript{40} LNCP (lipid-coated nano-CaP)\textsuperscript{41} CaBPMP/CaPAA spheres for NIH-3T3 cells and RBL-2H3 cells (from left to right as follows: GFP pDNA (blank); GFP pDNA in the presence of CaCl2; CaBPMP/CaPAA; CaBPMP/CaPAA in the presence of CaCl2; Lipofecter liposome (5 $\mu$g of CaBPMP/CaPAA, 20 $\mu$g of GFP pDNA, 3.75 mmol/L CaCl2) ($p < 0.05$)).
cannot enter into the cells without vectors. The result of CLSM visually confirms that CaBPMP/CaPAA vectors are capable of carrying exogenous genes into the cells and that exogenous genes can be further expressed intracellularly.

3. CONCLUSIONS

In summary, CaBPMP/CaPAA hybrid spheres have been successfully synthesized through a facile method using PAA−Ca\(^{2+}\)−H\(_2\)O colloid spheres as a template and characterized by SEM, TEM, powder XRD, particle size distribution, zeta potential, FT-IR, and \(^{31}\)P and \(^{13}\)C MAS NMR. The electrophoresis result indicated that CaBPMP/CaPAA spheres can effectively load, protect, and release genes. MTT assays confirmed that CaBPMP/CaPAA spheres are quite safe for different cells at high concentrations of 500 μg/mL. Although the desired positive-charge surface property of CaBPMP/CaPAA spheres cannot be realized through the protonation of the piperazine group in BPMP under acidic conditions because acid-labile PAA−Ca\(^{2+}\)−H\(_2\)O colloid spheres as a template exhibited good dissolubility at low pH values during the process of protonation, the CaBPMP/CaPAA spheres acting as gene vectors are able to transfer GFP pDNA into different cells with 25% transfection efficiency and good reproducibility. The transfection performance of CaBPMP/CaPAA spheres in gene delivery can be attributed to their uniform spherical morphology, appropriate particle size, and good dispersity, which favor efficient entry into cells through endocytosis. From another point of view, good dissolubility of PAA−Ca\(^{2+}\)−H\(_2\)O colloid spheres as a template at low pH values makes CaBPMP/CaPAA spheres release the loaded gene in the endosome under a weak acidic environment (pH value of about 5.4), which is conductive to gene transfection because intracellular gene release determines subsequent gene expression. The controllable morphology, low cytotoxicity, moderate gene-loading capacity, and different dissolubilities at different pH values cause CaBPMP/CaPAA spheres to become promising vectors for gene delivery. The application of calcium phosphonates with adjustable surface properties derived from the different organic groups of phosphonic acid in gene delivery provides a new idea for the design of gene vectors. Furthermore, it is worth mentioning that bisphosphonates are efficient anticancer drugs and have wide clinical applications at present. Provided that the used bisphosphonates possess excellent anticancer activity, the calcium bisphosphonate/calcium polyacrylate spheres might provide a new modality for delivery of bisphosphonate-based anticancer drugs into cancer cells.

4. EXPERIMENTAL SECTION

4.1. Materials and Methods. All the chemicals except for BPMP were obtained commercially and used directly without further purification. BPMP was prepared by modifications of the reported methods in our laboratory.\(^{46}\) PAAS (M\(_w\) = 5100) was purchased from Sigma-Aldrich. Calcium nitrate tetrahydrate (Ca(NO\(_3\))\(_2\)·4H\(_2\)O), aqueous ammonia (>25%), and anhydrous ethanol were of analytical grade and purchased from Tianjin Chemical reagents. NIH-3T3 (mouse embryonic fibroblast) and RBL-2H3 (basophilic leukemia cells) were obtained from the Chinese academy of sciences. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum...
(FBS) were purchased from GBICO. MTT and DAPI were purchased from Sigma-Aldrich. GFP pDNA was obtained from the Academy of Military Medical Sciences. The lipofector liposomal transfection reagent was obtained from Beyotime.

SEM was performed on a ZXM6360-LV electron microscope (30 kV). TEM was performed using a JEOL JEM-2010 at an acceleration voltage of 0–120 kV. The N2 adsorption−desorption isotherm was obtained at 77 K on a Micromeritics ASAP 2010 automated analyzer. The surface area was estimated according to the BET method, and the pore size distribution was calculated on the basis of the Barrett−Joyner−Halenda method. The powder XRD pattern was recorded on a Bruker D8 Advance powder diffractometer using a Cu Kα radiation of 0.15406 nm wavelength. FT-IR spectra were recorded on a TENSOR 27 FT-IR Spectrometer in the range of 4000−600 cm−1 using KBr pellets. 13C (100.5 MHz) cross-polarization magic angle spinning (CP-MAS) and 31P (161.8 MHz) MAS solid-state NMR experiments were recorded on a BRUKER DRX 400 spectrometer equipped with a magic-angle spin probe in a 4 mm ZrO2 rotor. 13C signals were referenced to tetramethylsilane, and 31P NMR signal was referenced to NH4H2PO4. The experimental parameters were 6 kHz spin rate, 2 s pulse delay, 6 min contact time for 13C CP-MAS NMR experiments; 8 kHz spin rate, 3 s pulse delay, and 10 min contact time for the 31P MAS NMR experiment. MTT assays were performed on a Tecan M200 ELISA reader at 492 nm. The transfection efficiency was obtained at 480 nm using BD Accuri C6 flow cytometry through channel FL1.

4.2. Synthesis. The calcium bisphosphonate/calcium polyacrylate composite was prepared using colloidal spheres of the PAA−Ca2+−H2O complex as the template. In a typical synthesis, the solution of PAAS (8 mmol/L of the repeating units) was first prepared at 30 °C under gentle magnetic stirring, and the pH value of the PAAS solution was adjusted to 11 by aqueous ammonia. Then, 30 mL of the calcium nitrate solution (0.1 mol/L) was added to 355 mL of pre-prepared PAAS solution dropwise at 30 °C under gentle magnetic stirring for 30 min. The obtained solution was bluish, which can be recognized as characteristic for the formation of PAA−Ca2+−H2O colloidal spheres with a submicrometer size (Figure S6). Afterward, BPMP solution (0.1 mol/L, 15 mL) with pH 11 adjusted by aqueous ammonia was added dropwise to the above bluish solution, and the mixture was further stirred for 24 h at 30 °C. The resultant was collected by high-speed centrifugation, washed with pure water and anhydrous ethanol, and dried at room temperature in a vacuum oven. Hereafter, the synthesized calcium bisphosphonate/calcium polyacrylate composite was designated as CaBPMP/CaPAA.

4.3. Cytotoxicity Assays of CaBPMP/CaPAA Spheres. Cytotoxicity was tested using the MTT assay by measuring the cell viability. NIH-3T3 cells or RBL-2H3 cells in the logarithmic growth phase were digested by 0.25% of trypsin and then a DMEM culture medium containing 10% FBS was added to terminate cell dissociation and obtain a cell suspension. The cell suspension (100 μL) was seeded in 96-well plates with a cell density of 8 × 104 cells/well and cultured at 37 °C in a humid atmosphere with 5% CO2 for 24 h. CaBPMP/CaPAA spheres were suspended in the DMEM culture medium containing 10% FBS and 100 IU/mL penicillin−streptomycin. The particle suspension was added into each well, and the final concentration of the particles was adjusted to 0.5, 1, 5, 10, 50, 100, 500, 1000, and 2000 μg/mL. After culturing at 37 °C in a humid atmosphere with 5% CO2 for 48 h, the culture medium was removed and cells were rinsed with PBS solution. MTT solution (100 μL) with a concentration of 0.5 mg/mL was added to each well and incubated for another 4 h. The medium containing MTT was removed and 150 μL of dimethyl sulfoxide was added to each well under shaking for 10 min to dissolve Formazan. Afterward, the supernatant was measured by ELISA at a wavelength of 492 nm. The cell viability was normalized as a percentage of the value of untreated cells. Statistical analysis was carried out with three biological replicates for this study. The results were statistically analyzed by one-way ANOVA and the Duncan’s new multiple range test to determine the significant difference between group means. A p-value < 0.05 was considered statistically significant (SPSS for Windows, version 12.0).

4.4. DNA Retardation Assay, DNase I Stability Assay, and DNA Release Study. The GFP pDNA (1 μg) and GFP pDNA−CaBPMP/CaPAA complexes (the weight ratios of CaBPMP/CaPAA spheres/GFP pDNA were 0.5, 1, 1.5, 2, and 2.5, respectively) were loaded onto 0.8% agarose gels with ethidium bromide (0.1 μg/mL) and were run with a tris-acetate running buffer at 80 V for 50 min. Then, the DNA retardation was observed by agarose gels electrophoresis.

DNase I (3 U, 1 μL) was added to 1 μg of naked GFP pDNA and GFP pDNA−CaBPMP/CaPAA complexes (the weight ratios of CaBPMP/CaPAA spheres/GFP pDNA were 0.5, 1, 1.5, 2, and 2.5, respectively), which was incubated at 37°C for 30 min with shaking at 100 rpm. The DNase I degradation was ended by adding a solution of ethylenediaminetetraacetic acid (250 mM, 4 μL). Then, GFP pDNA was released by incubation with the PBS buffer (5 μL, 0.1 mol/L) at 37°C for 3.5 h with shaking at 100 rpm. The naked GFP pDNA, the degraded GFP pDNA, and the released samples were loaded onto 0.8% agarose gels with ethidium bromide (0.1 μg/mL) and run with the running buffer at 80 V for 50 min.

The GFP pDNA was released from GFP pDNA−CaBPMP/CaPAA complexes (the different weight ratios of CaBPMP/CaPAA spheres/GFP pDNA were 0.5, 1, 1.5, 2, and 2.5, respectively) by incubating with the PBS buffer (5 μL, 0.1 mol/L) at 37°C for 3.5 h with shaking at 100 rpm. The samples including original GFP pDNA and the released GFP pDNA were loaded onto 0.8% agarose gels with ethidium bromide (0.1 μg/mL) and run with the tris-acetate running buffer at 80 V for 50 min.

4.5. In Vitro Transfection Using CaBPMP/CaPAA Spheres as Gene Vectors. Cells from NIH-3T3 and RBL-2H3 cell lines were plated in 12-well plates at a density of 2 × 105 cells/well and cultured in DMEM with 10% FBS at 37 °C in a 5% CO2 humidified atmosphere overnight. Then, the medium was discarded and replaced with fresh DMEM with FBS. The different amounts of CaBPMP/CaPAA vectors (5, 10, 20, 30, 40, and 50 μg) were suspended in 1 mL of the medium containing the same amount GFP pDNA (20 μg), respectively. Then, the suspension with different mass ratios of vector/DNA was added to each well. The GFP pDNA (20 μg) without CaBPMP/CaPAA vectors was used as the blank experiment, whereas CaBPMP/CaPAA vectors (50 μg) and PcDNA 3.1(+) (20 μg) were used as the negative control to exclude the autofluorescence signals of CaBPMP/CaPAA vectors during transfection. The cells were incubated at 37 °C for 4 h. Afterward, the medium was replaced with fresh DMEM medium with 20% FBS and incubated for 24 h. The medium was again replaced with fresh DMEM medium with
10% FBS and incubated for another 24 h. The transfection efficiency of CaBPMP/CaPAA vectors was obtained at 480 nm using flow cytometry. The effect of calcium ions on the transfection efficiency was determined according to the above procedure using CaBPMP/CaPAA vectors (5 μg) and GFP pDNA (20 μg) in the presence of CaCl$_2$ (3.75 mM/mL). The control transfection experiment of Lipofecter liposome was carried out using Lipofecter liposome (50 μL) and GFP pDNA (20 μg) according to the usage instruction for Lipofecter.

### 4.6. Intracellular Expression of GFP Observed by CLSM

The GFP pDNA (20 μg), CaBPMP/CaPAA (5 μg), and GFP pDNA-CaBPMP/CaPAA complex (w/w = 20/5 μg) were incubated with NIH-3T3 cells for 4 h under the conditions mentioned above, respectively. The cells were further incubated another 48 h, followed by washed with PBS (pH 7.4). To label the nucleus, the cells were incubated with DAPI for 10 min. Then, the cells were washed with PBS (pH 7.4), and the intracellular expression of GFP in the cells was observed by CLSM of Olympus Fluov View FV1000.

## Associated Content

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.6b00437.

N$_2$ adsorption–desorption isotherm and powder XRD pattern of CaBPMP/CaPAA spheres, SEM image of GFP pDNA-loaded CaBPMP/CaPAA spheres, flow cytometry histogram profiles for NIH-3T3 cells and RBL-2H3 cells, and photos of solution containing PAAs and calcium ions at different temperatures (PDF).

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### Notes

The authors declare no competing financial interest.

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