Calcium and phosphorus co-doped carbon dots enhance osteogenic differentiation for calvarial defect repair in situ

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Abstract

Calvarial bone defect remains a clinical challenge due to the lack of efficient osteo-inductive agent. Herein, a novel calcium and phosphorus codoped carbon dot (Ca/P-CD) for bone regeneration was synthesized using phosphoethanolamine and calcium gluconate as precursors. The resultant Ca/P-CDs exhibited ultra-small size, stable excitation dependent emission spectra and favorable dispersibility in water. Moreover, Ca/P-CDs with good biocompatibility rapidly entered the cytoplasm through endocytosis and increased the expression of bone differentiation genes. After mixing with temperature-sensitive hydrogel, Ca/P-CDs were injected in situ into calvarial defect and promoted the repair of bone injury. These Ca/P-CDs provide a new treatment method for the bone repair and expand the application in the biomedical fields.

1. Introduction

Calvarial bone defect represents a frequent pathological issue of orthopedic which can be caused by trauma, congenital malformations, infections, and surgery ^[1–3]. In clinic, the therapeutic effect of calvarial bone defect is unsatisfactory owing to the lack of efficient osteo-inductive agent. In addition, traditional surgical transplantation has serious side effects and limited application including graft selection and immune rejection ^[4, 5]. Therefore, there is an urgent need to develop effective treatment strategy for promoting bone regeneration effectively and restoring bone function.

It is well-known that calcium and phosphorus are important elements in the process of maintaining normal bone differentiation and development. They cooperate with parathyroid hormone (PTH) and calcitonin (CT) to promote the expression of genes related to bone differentiation, and achieve the regulation of bone cell function. In addition, both of elements have an impact on bone metabolism and bone tissue morphology ^[6]. For instance, hydroxyapatite (HA) with a general formula of $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ has extensively applied in bone defect repair due to its chemical similarities with the natural bone ^[7, 8]. However, the uncontrollability of morphology and metabolism in vivo of HA weakens its therapeutic effect. As a new type of nano-biomaterial, carbon dots (CDs) have a ultra-small size (2-8nm), excellent optical properties, good biological safety and stability ^[9, 10]. It has attracted widespread attention in the fields of bioimaging, immunolabeling, molecular tracing, tumor targeting, and drug delivery ^[11]. By modifying the abundant active groups on the surface, CDs can be endowed with different specific functions to meet specific biomedical needs ^[12]. Our research group also has reported a series of heteroatomic doped CDs that possessed distinct functions ^[13–15].

The focus of this research is to synthesize functionalized CDs with calcium and phosphorus doping for bone regeneration and repair of bone injury. The physical and chemical properties of Ca/P-CDs was characterized via a series of methods. Afterwards, the function of osteogenesis induced by Ca/P-CDs was explored using mouse osteoblastic cell line (MC3T3-E1) in vitro. Meanwhile, Ca/P-CDs mixed with thermo-sensitive hydrogel (Pluronic F127) were injected into calvarial defect in mice to investigate the

bone regeneration in situ. Finally, we verified the long-term biocompatibility of Ca/P-CDs through histologic section.

2. Material And Method

2.1 Materials

Phosphoethanolamine, calcium gluconate, diethylenetriamine pentaacetic acid (DTPA), and glycine were purchased from Aladdin Reagent Company (Shanghai, China). Fetal bovine serum and Dulbecco's minimum essential medium (DMEM) were procured from Hyclone (Logan, UT, USA). Lyso tracker and Mito tracker were purchased from Beyotime Biotechnology (Beijing, China). qPCR kits were purchased from Sigma (NY, USA). ICR mice were purchased from Center for Experimental Animals of Jiangsu University. Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) was obtained from R&D Systems Europe (United Kingdom). All of these chemical agents were of analytical grade and were utilized without further purification.

2.2 Synthesis of P-CDs, Ca-CDs and Ca/P-CDs

Three types of CDs were synthesized using different precursors by a one-pot hydrothermal method, as described in the literature previously with a little modification ^[16]. P-CDs: 0.4 g of phosphoethanolamine and 0.40 g of DTPA were dissolved in 20 ml of double-steaming water and stirred continuously for 10 min to form a transparent solution. The solution was transferred to a reaction kettle and heated in a muffle furnace at 200°C for 4 h. After the system was cooled to room temperature naturally, the liquid was poured into a 50 ml centrifugal tube and the black impurities are removed by centrifuging at 2000 rpm for 15 min. The prepared solution was then dialyzed against water for 5 days in a cut-off dialysis bag (MWCO = 3 kD, Solarbio Company, Beijing, China). The dialysate was collected and freeze-dried with vacuum freeze dryer. Thus, P-CDs powder was obtained and stored for further characterization. The Ca-CDs and Ca/P-CDs were prepared using calcium gluconate and DTPA, and phosphoethanolamine and calcium gluconate as precursors, respectively.

2.3 Morphological and chemical characterization of Ca/P-CDs

The morphologies of the Ca/P-CDs were observed by high-resolution transmission electron microscopy (HRTEM) on a JEM-2100 microscope (JEOL, Tokyo, Japan) under an accelerating voltage of 200 kV. Elemental composition of the Ca/P-CDs was determined by X-ray photoelectron spectroscopy (XPS) on Escalab 250Xi (Thermo Scientific, America). The surface chemical components of Ca/P-CDs were examined using a Fourier transform infrared (FT-IR) spectrometer (Nicolet Nexus 470; GMI, Franklin, IN, USA) ranged from 4000 to 400 cm⁻¹. The crystal structure of Ca/P-CDs was analyzed by x-ray diffraction (XRD) on a Rigaku-D/MAX2500 diffractometer (Japan) with a scanning speed of 4 °/min in the range from 5–90 °. The optical properties of the Ca/P-CDs were obtained with a UV-2450 UV/vis

spectrophotometer (Shimadzu, Japan), and the photoluminescence emission spectra was recorded using a Cary Eclipse Fluorometer (Varian, Palo Alto, CA, USA).

2.4 Cell culture and biocompatibility of Ca/P-CDs

In vitro cytotoxicity of the Ca/P-CDs was determined using the CCK-8 cell viability kit assay (Solarbio, Beijing, China). Briefly, MC3T3-E1 (mouse embryo osteoblast precursor) cells (1×10^4 cells per well) were seeded into a 96-well plate with four replicates in each group. After incubation at 37°C and 5 % CO₂ for 24 h, the different concentrations of Ca/P-CDs (50 µg/mL) in fresh DMEM were used to replace the growth medium and incubated for another 24 h. Then, the cells were washed with PBS, and 10 µL CCK-8 and 90 µL DMEM solution were added to each well. Next, the plates were incubated for 4 h at 37°C and 5 % CO₂. Finally, the absorbance of each well was detected at the emission wavelength of 450 nm using a Synergy HT Multi- Mode Microplate Reader (Bio Tek, Winooski, VT, USA). Nontreated cells (in DMEM) were used as a control, and the relative cell viability (mean $\pm$ SD, n = 3) was expressed as (Abs sample – Abs zero sitting)/(Abs control–Abs zero sitting) $\times$ 100 %. The experiment was repeated three times independently.

2.5 Hemolysis assay

All animal procedures were conducted in accordance with the Management Rules of the Ministry of Health of the People's Republic of China and approved by the Institutional Animal Care and Use Committee of Jiangsu University (permit number: SYXK2018-053). The hemocompatibility of Ca/P-CDs was carried out according to the protocol reported in the literature with slight modification^[13]. In brief, fresh mouse blood was stabilized with heparin sodium and centrifuged (1, 200 rpm, 15 min) to remove the supernatant. The sediment was washed with PBS five times to obtain the mouse red blood cells (MRBCs). Next, the mouse RBCs were resuspended using 0.9 mL PBS containing Gd-doped CDs with different particle concentrations from 50 to 200 mg/mL (water as positive control and PBS as negative control). These samples were incubated for 2 h at 37°C after gentle shaking, thus centrifuged to collect the supernatant at 12,000 rpm for 1 min. The absorbance of samples at 541 nm was recorded by a UV-Vis spectrophotometer (UV-2450). The hemolysis percentages of Ca/P-CDs was calculated using the absorbance compared with the control.

2.6 Cytophagocytosis assay

The internalization of Ca/P-CDs in the cells was observed using laser confocal microscope. The MC3T3-E1 cells were seeded into the 24-well plates that pre-filled with 10 mm coverslips. When the cell density reached 60 %, the MC3T3-E1 cells were administered with the medium containing 200 µg/ml of Ca/P-CDs and further incubated for 4 h. Then, these MC3T3-E1 cells on the coverslips were washed with phosphate-buffered saline (PBS) twice and fixed with 4% paraformaldehyde (PFA). In order to determine the intracellular distribution, these MC3T3-E1 cells stained with Lyso tracker and Mito tracker were observed under a confocal laser scanning fluorescence microscope (Zeiss LSM-710, Carl Zeiss Meditec AG, Jena, Germany)

2.7 Alizarin red staining

Alizarin red staining was chosen to verify the osteogenic differentiation of MC3T3-E1 cells after incubation with Ca/P-CDs. MC3T3-E1 cells were seeded into a 96-well plate at a density of 1×10^4 cells per well with four replicates. After incubation with 50 $\mu\text{g/mL}$ Ca/P-CDs for 21 days, the MC3T3-E1 cells were washed with PBS and fixed with PBS paraformaldehyde (3.6 %) for 1 hour at 4°C. Then, the MC3T3-E1 cells were stained with 1% alizarin red (sigma Aldrich) solution for 30 minutes. Finally, these MC3T3-E1 cells were washed by PBS to remove free alizarin red, and the Ca/P-CDs-induced mineralization was measured by microscope and Image-J software.

2.8 Real-time PCR

The osteogenic induction of MC3T3-E1 cells using Ca/P-CDs was same as the above. When the induction was over, these MC3T3-E1 cells were collected by enzymatic digestion, and their total RNA was extracted by Trizol reagent. RT-PCR was conducted on the ViiA 7 RT-PCR System (Thermo Fisher Scientific) using the QuantiTect SYBR Green Kit (Qiagen, Quanta, France). The primer sequences of the related genes were listed in the Table 1. The expression of each related gene, including alkaline phosphatase (ALP), osteocalcin (OCN), and Runt-related transcription factor 2 (RUNX2), was normalized to the housekeeping gene (β -actin), and fold differences were calculated using the comparative C_t method. The osteogenic markers ALP, OCN and RUNX2, were analyzed.

Table 1
Primer sequences of real-time PCR reactions

Name	Primer	Sequence	Product
ALP	Forward	5'AACCCAGACACAAGCATTCC3'	151
	Reverse	5'GAGAGCGAAGGGTCAGTCAG3'	
RUNX2	Forward	5'AGAGTCAGATTACAGATCCCAGG3'	238
	Reverse	5'TGGCTCTTCTTACTGAGAGAGG3'	
OCN	Forward	5'TGCTTGTGACGAGCTATCAG3'	149
	Reverse	5'GAGGACAGGGAGGATCAAGT3'	
β -actin	Forward	5'TCTTGGGTATGGAATCCTGTG3'	81
	Reverse	5'AGGTCTTTACGGATGTCAACG3'	

2.9 In vivo animal study

Mice calvarial defect model creation and Ca/P-CDs loaded hydrogel implantation

All animal procedures were conducted in accordance with the Management Rules of the Ministry of Health of the People’s Republic of China and approved by the Institutional Animal Care and Use

Four-week-old BALB/c mice were used (body weight ~ 20 g) for the experiments in vivo. Ten mice were randomly divided into two groups with 5 mice in each group. The brief surgical procedure was as follow: Firstly, the mice were anaesthetized with 10% chloral hydrate in normal saline by intraperitoneal injection at 3.5 ml per kg body weight. Their fur on the skull surface was shaved off using an electric razor and this area was sterilized with Iodine. Then, a 1.5 cm long longitudinal skin incision was made on the mouse scalp from the back of the eyes to the skull area using a sterile scalpel. The fascia was stripped laterally to expose the skull. Next, a 5 mm diameter defect was made using an electric driven low-speed (about 1500 rpm) trephine bur on one side of the parietal bone and cooled with normal saline during the whole process. Finally, 20 μ L of Ca/P-CDs loaded F127 hydrogel (100 μ g/mL) was injected into the calvarial defect. After the surgery, the wound was sutured with simple interrupted suture and then the mice were placed separately and fed normally. CT images of the calvarial defect were acquired on a clinical 64-slice multidetector CT scanner (SOMATOM Emotion, Siemens, Bavaria, Munich, Germany). After 8 weeks, the major organs (heart, liver, spleen, kidneys, and lungs) were collected for conventional histocompatibility analysis.

3.0 Statistical Analysis

All experimental data were presented as mean $\pm$ standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) or Tukey's multiple comparison tests between different treatments. $P < 0.05$ was considered statistically significant.

3. Results And Discussion

3.1 Characterization of Ca/P-CDs

The successful preparation of Ca/P-CDs was performed by using calcium gluconate as calcium source and phosphoethanolamine as surface passivation agent based on Scheme 1. The morphology of synthesized Ca/P-CDs was observed using high-resolution transmission electron microscopy (HRTEM). As shown in Fig. 1A, the Ca/P-CDs possessed discrete and quasi-spherical shape without apparent aggregation. The average diameter of Ca/P-CDs was approximately 2.4 nm that could be conducive to cross the physiological barrier and discharge from the body by the kidney. Figure 1B showed that there was no discernible lattice fringes, indicating the amorphous nature of Ca/P-CDs. XRD pattern was used to study the phase structure of Ca/P-CDs. As shown in Fig. 1C, there was an strong and broad diffraction peak at 24.98 $^{\circ}$, which was similar to the characteristic peaks of graphite ($\{002\}$ planes, $2\theta = 26.5^{\circ}$). Subsequently, we used Fourier infrared spectroscopy to study the chemical composition of Ca/P-CDs (Fig. 1D). FTIR spectroscopy of Ca/P-CDs showed the stretching vibration of O-H at 3450 cm^{-1} , C = O at around 1650 cm^{-1} , C-N/C-C at around 1400 cm^{-1} , C-O at around 1100 cm^{-1} , suggesting the abundant hydrophilic groups on the surfaces of Ca/P-CDs.

3.2 Chemical characterization of Ca/P-CDs

The surface chemical structure of Ca/P-CDs was further studied using X-ray photoelectron spectroscopy (XPS). As shown in Fig. 2A, there were five obvious peaks at 350.133, 284.0, 400.0, and 530.6 eV, which indicated that the Ca/P-CDs were mainly composed of calcium, phosphorus, carbon, nitrogen, and oxygen atoms. The N_{1s} spectrum displayed remarkable N-H peaks at 399.9 eV and N-C peak at 399.3 eV, where the incorporation of N atoms could be used as auxochrome group to enhance the optical performance of Ca/P-CDs. The XPS spectrum showed the P_{2p} peak at 133.60 eV and 132.85 eV, Ca_{2p} peak at 350.70 eV, 350.16 eV, 347.30 eV, 346.55 eV and 346.85 eV (Fig. 2E and 2F). It was verified that the Calcium and phosphorus elements were successfully doped into Ca/P-CDs, where both of the elements were essential for bone growth and differentiation.

3.3 Optical characterization of Ca/P-CDs

The optical properties of Ca/P-CDs were characterized by photoluminescence (PL) spectrum and UV/Vis absorption spectrum. Figure 3A showed there was no obvious absorption peak in an aqueous solution of Ca/P-CDs. Meanwhile, the aqueous solution was pale brown and transparent in daylight, but exhibited bright blue fluorescence under UV irradiation (inset, Fig. 3A). The Tyndall effect occurred when a beam of red laser gone through the aqueous solution, indicating that Ca/P-CDs had good dispersibility and colloid stability in water. The quantum yield of Ca/P-CDs was calculated at 11.3 % using quinine bisulfate as a standard. As shown in Fig. 3A, the Ca/P-CDs displayed a broad range of emission wavelengths, resulting in preeminent multicolor fluorescent emission. In addition, the PL spectrum of Ca/P-CDs showed excitation-dependent emission manner, which was well similar to the conventional CDs ^[14, 15]. Moreover, the maximum excitation wavelength at 396 nm and the maximum emission wavelength at 480 nm further confirmed the blue fluorescence performance of Ca/P-CDs (Fig. 3C) .

3.4 Biocompatibility of Ca/P-CDs

Biocompatibility of nanomaterials is of great importance for biomedical applications. In this study, the cytocompatibility of Ca/P-CDs was evaluated by CCK-8 assay through incubation with VSMC and MC3T3-E1 cells. After 24 h incubation with Ca/P-CDs, the cell viability of VSMC and MC3T3-E1 cells was measured. As shown in Fig. 4A, it can be seen that cell viability of two groups did not show significant changes after treatment with Ca/P-CDs at 0, 10, 50, 100, 200, 400, 800 $\mu\text{g/mL}$. Even at 800 $\mu\text{g/mL}$, both of cell viability were still as high as 88% and 91%, respectively.

Furthermore, the hemocompatibility of Ca/P-CDs was assessed by hemolytic assay. As shown in Fig. 4B, there was strong and sharp absorption peak at 541 nm in the positive control (DI Water group), indicating the presence of the hemolysis of red blood cells. Compared to the negative control (PBA group), the percentages of hemolysis were all less than 3% after treatment with Ca/P-CDs from 10 to 1000 $\mu\text{g/mL}$, indicating that no significant hemolysis occurred. Taken together, these findings clearly suggested that the prepared Ca/P-CDs had negligible cytotoxicity and good biocompatibility.

3.5 Intracellular distribution of Ca/P-CDs after endocytosis

Numerous studies have reported that the conventional CDs readily entered into cytoplasm in living cells due to their ultra small size. However, the intracellular localization of Ca/P-CDs after endocytosis was still unknown. In this study, three fluorescent probes (MitoTracker, ER-Tracker, or LysoTracker) were chosen to investigate the distribution of Ca/P-CDs at the organelle level. As shown in Fig. 5, the presence of strong green fluoresce signal in three groups verified that Ca/P-CDs could be successfully internalized into cytoplasm after 6 hours of co-incubation. In addition, MC3T3-E1 cells had no obvious morphological changes. Furthermore, the red fluorescence from MitoTracker and ER-Tracker highly overlapped the green fluoresce from Ca/P-CDs, respectively. However, this fluorescence signal of colocalization for LysoTracker was relatively weak. According to this findings, we proposed that the Ca/P-CDs entering the cytoplasm were mainly distributed in the mitochondria and endoplasmic reticulum.

3.6 Osteogenic induction of Ca/P-CDs in vitro

The function of osteogenic induction of Ca/P-CDs was explored by the RT-PCR and Alizarin red staining using MC3T3-E1 cells. Three different types of CDs was chosen as control treatments, including bare CDs (B-CDs), Calcium-doped CDs (Ca-CDs), Phosphorous-doped CDs (p-CDs). As shown in Fig. 6A and 6B, the mineralization of MC3T3-E1 osteoblast cell in the Ca/P-CDs treatment group was significantly higher than that of the other group. The function of Ca/P-CDs was further enhanced by the addition of the G. In addition, the Ca-CDs group exhibited better osteogenic induction than the P-CDs group. Meanwhile, the expression of ALP, mature bone markers (OCN) and osteogenic transcription factor (RUNX2) were significantly up-regulated under the same concentration of Ca-CDs, Ca/P-CDs, Ca/P-CDs + GF treatments, among which Ca/P-CDs and Ca/P-CDs + GF group has the highest expression. Ca/P-CDs could promote the expression of bone differentiation-related RNA to be 2–3 times higher than that of the control group (Fig. 6C-D). It can be proved that the Ca/P-CDs could effectively promote the expression of genes related to bone differentiation and promote development and maturity of bone differentiation.

3.7 Osteogenic potential of Ca/P-CDs in vivo

Encouraged by the above results, we evaluated the ability of Ca/P-CDs to promote bone differentiation and development through in vivo experiments. In order to achieve administration in situ, F127 thermal sensitive hydrogel was chosen to load the Ca/P-CDs. After in situ injection, F127 hydrogel loading Ca/P-CDs could form a gel quickly in the calvarial defect due to the body temperature rising. After 8 weeks of treatment, CT scans were performed to observe the healing of the skull defects. As shown in Fig. 7A, the calvarial defect of the mice was still observed after single F127 hydrogel treatment. However, the calvarial defect of the mice was gradually restored after Ca/P-CDs loaded F127 hydrogel treatment. The results verified that Ca/P-CDs could effectively promote the repair of bone injury in vivo.

Besides, histological analysis of major organs were used to evaluated the potential long term biosafety of Ca/P-CDs. As shown in Fig. 7B, it showed no visible pathological changes in the lung, liver, spleen, kidney

and heart after 8 weeks treatment with PBS, P-CDs, Ca-CDs, Ca/P-CDs. There was no obvious inflammation or quantum dot deposition in various organs. The literatures and our study had reported that CDs can be excreted out of the body through the kidneys, which reflected the good biological safety of CDs.

4. Conclusion

In this study, we have successfully prepared the Ca/P-CDs with osteogenic potential via introduction of calcium and phosphorus element by one-pot hydrothermal carbonization. The as-prepared Ca/P-CDs had uniform particle size distribution and good dispersibility in water. Furthermore, the Ca/P-CDs exhibited stable optical properties and favorable biocompatibility. After entering into the cytoplasm, the Ca/P-CDs could escape from the lysosome and mainly distributed in the mitochondria and ER. More importantly, these internalized Ca/P-CDs upregulated the expression of bone differentiation-related genes, including ALP, RUNX2 and OCN. Finally, the Ca/P-CDs promoted the development of osteoblasts in vitro and the repair of calvarial defect in vivo. In summary, the study can provide a novel alternative treatment for clinical bone injury repair and expand the applications of CDs in the biomedical fields.

Declarations

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Figures

Figure 1

Characterization of the Ca/P-CDs. (A) TEM images, (B) HRTEM image, (C) XRD pattern, and (D) FTIR spectrum of the Ca/P-CDs.

Figure 2

XPS spectra of the Ca/P-CDs. (A) Survey spectrum, (B) C1S spectrum, (C) N1S spectrum, (D) O1S spectrum, (E) P2p spectrum. (F) Ca2p spectrum.

Figure 3

Optical characterization of Ca/P-CDs. (A) UV-vis absorption spectrum of Ca/P-CDs. Insert figure a represents the aqueous solution of Ca/P-CDs under sunlight and UV irradiation. Insert figure b represents a beam of red laser traverses the aqueous solution of Ca/P-CDs. (B) PL spectrum of Ca/P-CDs with different excitation wavelength from 340 nm to 420 nm respectively. (C) Normalized PL spectra of the Ca/P-CDs. (D) Maximum emission spectrum of Ce-doped CQDs under maximum excitation wavelength.

Figure 4

The effect of Ca/P-CDs with different concentration on cell viability (A) and hemocompatibility (B).

Figure 5

Fluorescent pictures of MC3T3-E1 incubated with Ca/P-CDs (200 µg/mL) using different organelle fluorescent probes.

Figure 6

Evaluation of osteogenic potential of Ca/P-CDs in vitro. Alizarin red staining (A) and quantitative analysis of MC3T3-E1 (B) after the Ca/P-CDs induction. Gene expression of ALP (C) , OCN (D) , and RUNX2 (E) after the Ca/P-CDs induction. B-CDs: bare-carbon dots. Ca-CDs: Calcium-doped carbon dots. P-CDs: Phosphorus doped carbon dots. GF: growth factor (rhBMP-2, 2 ng/mL).

Figure 7

A, CT imagings of calvarial defect repair after Ca/P-CDs treatment. B, HE analysis of major organs after 8 weeks of Ca/P-CDs treatment. The bars represent 20 μ m.

Supplementary Files

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- [Scheme1.jpeg](#)