Development of beta-carotene-loaded poly(lactic acid)/hydroxyapatite core-shell nanoparticles for osteoblast differentiation

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Abstract
Beta-carotene (BC) is a nutrient in vegetables and a precursor of vitamin A. BC has been reported to have anticarcinogenic, antiaging, and antioxidation properties and prevents heart diseases. Recently, BC has gained significant attention due to stimulating effect on osteoblast differentiation. Poly(lactic acid)/hydroxyapatite (PLA/HAP) core-shell nanoparticles have been reported earlier with a load capacity of 250% for water-insoluble substances, using a surfactant-free emulsification method. In this work, PLA/HAP core-shell nanoparticles loaded with BC were prepared, and osteoblast differentiation behavior was evaluated. BC was successfully loaded into PLA/HAP core-shell nanoparticles with diameters of approximately 30 nm. BC-loaded PLA/HAP core-shell nanoparticles stimulated osteoblast differentiation by upregulating collagen type I, osteopontin, and osteocalcin expression. In addition, the gene expression levels of those osteoblasts were significantly higher than those stimulated by PLA/HAP core-shell nanoparticles without BC and cultured in a differential medium (with ascorbic acid and β-glycerophosphate). PLA/HAP core-shell nanoparticles showed satisfactory cytocompatibility because they were attached to the osteoblasts. Consequently, BC was effectively delivered to osteoblasts by nanoparticles. These results suggested that BC-loaded PLA/HAP core-shell nanoparticles could enhance bone formation.

1. Introduction
Biodegradable nanoparticles, with particle sizes up to 200 nm, for drug delivery system (DDS) carriers have received much attention for their enhanced permeability and retention (EPR) effect [1]. These nanoparticles can diffuse into and accumulate in lymph vessels of tumor tissues because of leaky and defective structure of tumor blood vessels [2]. Recently, stimuli-responsive DDS carriers, such as pH-sensitive and photo-responsive are also receiving attention [3]. The tissue around the tumor is slightly acidic with a pH of 6.5, whereas that of normal tissue is neutral (pH 7.2–7.4). Moreover, the cells have a wide range of pH levels in endosomes, lysosomes, and mitochondria (5.5–6.0, 4.5–5.0, and 8.0, respectively); these pH gradients help design DDS nanocarriers that can be incorporated into cells via endocytosis [4]. Hydroxyapatite (HAp) is
a mineral component of bone and teeth and is widely used as a biomaterial because of its biocompatibility and biodegradability [5]. For example, HAp with different morphologies has been used as a bone substitute for biomedical applications [6]. HAp is stable in aqueous solutions at neutral pH and dissolves at acidic pH [5]. Thus, HAp is a potential DDS carrier because it is biodegradable and has pH-sensitive drug release profile. Several DDS carriers using HAp and its composites have been reported [7–11]. HAp nanorods loaded with an anticancer agent were synthesized using coprecipitation and hydrothermal methods, and folic acid was stabilized on their surface. The HAp nanorods had an inhibitory effect on tumor tissue because HAp was degraded under weak-acidic conditions [11]. Kim et al. reported the preparation of porous HAp scaffolds coated with a composite of antibiotic-loaded polycaprolactone and HAp. The drug release behavior of the scaffolds was controlled by the composition of the coating layer. A larger amount of HAp in the coating layer increased its dissolution, thereby enhancing drug release [8].

Poly(lactic acid), poly(glycolic acid), and poly(lactic glycolic acid), abbreviated as PLA, PGA, and PLGA, respectively, are widely used in biomedical applications because of their biodegradability [1]. In addition, their degradation rate in vivo can be controlled by adjusting their composition [12]. Hence, PLA, PGA, and PLGA have been widely used as DDS carriers [12–15]. PLA and PLGA particles have been prepared using an emulsification method. However, the surface of the particles in solution during preparation needs to be stabilized with surfactants, such as poly(vinyl alcohol) [16]. In addition, large amounts of surfactants, which are required to make nanosized particles, tend to remain in the particles [16].

Previously, PLA/HAp core-shell nanoparticles of approximately 30–100 nm were prepared using an emulsification method without a surfactant [17,18]. During their preparation, carboxyl groups in PLA were bonded with calcium ions to stabilize the surface, phosphate ions were bonded with calcium ions to precipitate HAp, and the HAp shell was formed on the PLA core [19]. PLA/HAp core-shell nanoparticles exhibited similar X-ray diffraction (XRD) patterns whereas biological hydroxyapatite exhibited non-sharp patterns [20]. The shell was composed of polycrystalline HAp with low crystallinity. It had a uniform thickness of 5 nm with the crystalline c-axis arrangement of HAp. The XRD peaks assigned to 002 and 004 were sharp and narrow in shape compared with the others [21]. PLA/HAp core-shell nanoparticles indicated a hydrophobic substance load ability and their drug-loading capacity was 250%. The drug release behavior of the core-shell particles showed a good correlation with the Korsmeyer–Peppas model, and the drug release amount increased with decreasing pH of the soaking solution because the HAp shell could easily be dissolved under acidic conditions [22]. PLA/HAp core-shell nanoparticles have potential as DDS carriers with excellent drug-loading capacity and pH sensitivity. Considering these properties of PLA/HAp core-shell nanoparticles, anticancer drug-loaded nanoparticles were prepared, which showed cytotoxic effects against cancer cells because of their cytocompatibility and pH sensitivity [21].

Beta-carotene (BC) is a precursor of vitamin A; it exhibits anticarcinogenic, antiaging, and antioxidative characteristics, and prevents heart diseases [23,24]. Lee et al. reported the potential therapeutic effects of BC on colorectal cancer mediated by the inhibition of M2 macrophage polarization, which could promote the proliferation of cancer cells [25]. Recently, BC has been reported to stimulate the differentiation of mesenchymal stem cells into osteoblasts via upregulating expression of runt-related transcription factor 2 (Runx2), SRY-Box Transcription Factor 9 (SOX9), and osteonectin [26,27]. Nishide et al. reported that BC upregulated the expression of osteopontin (OPN) and alkaline phosphatase (ALP) in mouse osteoblast-like cells (MC3T3-E1) via the retinoic acid receptor (RAR) signaling pathway [28]. Additionally, BC can suppress osteoclast genesis and bone resorption by suppressing key factors in the nuclear factor kappa B (NF-kB) signaling pathway [29]. However, BC is insoluble in water and slightly soluble in edible oils (low lipid solubility). Thus, for food chemistry applications, it has been encapsulated for effective delivery [30,31]. PLA/HAp core-shell particles could be a candidate for BC carriers because of their excellent drug-loading capacity for hydrophobic substances and drug-delivering ability [22]. This work focuses on using BC to enhance bone formation. BC-loaded PLA/HAp core-shell

![Scheme 1. BCx/HAp core-shell nanoparticles: preparation process and schematic structure.](image-url)

Scheme 1. BCx/HAp core-shell nanoparticles: preparation process and schematic structure.
nanoparticles were prepared. The structure of these particles was determined, and their stimulating effect on bone formation was examined using mouse osteoblast-like cells.

2. Materials and methods

2.1. BC-loaded core-shell nanoparticle preparation and characterization

BC-loaded PLA/HAp core-shell nanoparticles were prepared using an emulsification method, as previously described (Scheme 1) [17,18]. Briefly, 20 mg of PLA (Resomer R 202 H, Mw = 10–18 kDa, Sigma-Aldrich) and 0/0.5 mg of BC (Fujifilm Wako Pure Chemical) were dissolved in 4 mL of acetone (Fujifilm Wako Pure Chemical). Calcium and phosphate ion solutions were prepared by dissolving 0.20 M calcium acetate monohydrate (Fujifilm Wako Pure Chemical) and 0.12 M diaminommonium hydrogen phosphate (Fujifilm Wako Pure Chemical), respectively, in 20 mL of ultrapure water (milli-Q, resistivity >18.2 MΩ cm). The PLA and BC solutions were added to 160 mL of ultrapure water, and then 20 mL of calcium ion solution was added. Next, 20 mL of the phosphate ion solution was added dropwise under constant stirring at 25°C. After aging for 72 h, the particles were centrifuged (6000 rpm, 10 min), and the supernatant was removed. As a washing process, the resulting particles were resuspended in 20 mL of ultrapure water and centrifuged to remove the supernatant; washing process repeated twice. Finally, the resulting particles were resuspended at a concentration of 2 mg/mL (denoted as BCx/HAp, \(x = 0/0.5\), where \(x\) is the amount of BC). The supernatant of BCx/HAp removed after aging was filtered with ultrafiltration (Vivaspin 20, Sartorius, 10 kDa MWCO). The filtered supernatant was analyzed using an inductively coupled plasma optical emission spectrometer (Avio500, PerkinElmer) to confirm the calcium/phosphate (Ca/P) ratio of BCx/HAp.

The morphology of BCx/HAp was observed by scanning electron microscopy (SEM, 54300, Hitachi). The BCx/HAp solution was dropped directly onto the sample stage of the SEM, dried overnight at 37°C, and coated with an osmium layer (Neoc-Pro, Meiwafosis). The SEM had an acceleration voltage of 10 kV, and 60 point/3 sights were used to measure the particle diameter using ImageJ software (NIH). Representative markers for measuring particle diameter are shown in supplementary Figure S1. The BC0/HAp suspension was dropped onto a carbon grid, dried at room temperature (approximately 25°C), and observed using transmission electron microscopy (TEM, JEM-2100 F, JEOL) with an acceleration voltage of 200 kV. The HAp and (PLA + BC) weight ratios were evaluated using thermogravimetric analysis (TGA, TG-DTA8122, Rigaku). The weight loss percentage at 500°C was defined as the weight ratio of PLA + BC in BCx/HAp. The crystalline structure of BCx/HAp was analyzed by XRD (SmartLab SE/B1, Rigaku) under the following conditions: Cu Ka radiation (\(\lambda = 1.5418\) Å, 40 kV, 30 mA), 1.0°/min scan rate, and step size of 0.01° in a 2θ range of 5–75°. The BCx/HAp bonding structure was evaluated by Fourier transform infrared spectroscopy (FTIR, FT/IR-4700, JASCO) with attenuated total reflectance attachment (ATR PRO ONE, JASCO). In addition, laser Raman spectroscopy (785 nm, Xplora, Horiba) was also performed in the 500–1300 cm\(^{-1}\) region with an exposure time of 60s and eight accumulations for BCx/HAp and PLA, whereas exposure time of 3 s and eight accumulations were used for BC because of its high sensitivity.

2.2. Cell cultures and evaluation of osteoblasts gene expression levels

Mouse osteoblast-like cells (MC3T3-E1, RCB1126, Riken BRC) were used to evaluate the effects of BCx/HAp on osteoblast differentiation using real-time polymerase chain reaction (PCR, CFX96, BIO-RAD). MC3T3-E1 cells were cultured in alpha-minimum essential medium (a-MEM, Fujifilm Wako Pure Chemical) with 10% fetal bovine serum (FBS, Nichirei) and 1% penicillin-streptomycin solution (Fujifilm Wako Pure Chemical) at 37°C under 5% CO\(_2\). BCx/HAp was sterilized in 70% ethanol, washed twice with phosphate-buffered saline (PBS), and resuspended in the culture medium (200 µg/mL). For the cell viability test, MC3T3-E1 cells were seeded (2.0 × 10\(^4\) cells/mL in 100 µL of culture medium; \(n = 5\)) into 96-well plates and cultivated for 24 h. The medium was replaced with a medium containing BCx/HAp. After an additional 96 h of incubation, the cells were washed with the medium and replenished with 100 µL of a-MEM without phenol red (Fujifilm Wako Pure Chemical), followed by 10 µL of the Cell Counting kit-8 (CCK, Dojindo). After 2 h incubation, 100 µL of the resulting medium was transferred to a new 96-well plate, and the absorbance at 450 nm was measured (infinite F200 PRO, Tecan). The number of cells was determined from a standard curve of cell number versus the absorbance of the resulting medium.

To evaluate the gene expression in osteoblasts, MC3T3-E1 cells (2.0 × 10\(^4\) cells/mL in 1 mL of culture medium; \(n = 3\)) were seeded into 12-well plates. After 24 h of culture, the medium was replaced with a medium containing BCx/HAp and further cultivated for 1, 2, and 4 weeks. The medium was replaced two times per week during the incubation. After culture, the cells were treated with TRIzol Reagent (Invitrogen), and total ribonucleic acid (RNA) was isolated from the cells following the manufacturer’s instructions. Subsequently, complementary
Table 1. Sequence of primers used in this work.

| Primer  | Sequence                     |
|---------|------------------------------|
| β-actin | Forward GCACCGTCAGGCTGAGAAC |
| Reverse | TGGTGAAGACGCCAGTGGA          |
| Col I   | Forward GATACCAAACTGGGCGTGCTG |
| Reverse | GCACCGTCAAGGCTGAGAAC         |
| OPN     | Forward TGGACCTGCTGACTGTAAG  |
| Reverse | GCACCGTCAAGGCTGAGAAC         |
| OCN     | Forward ATGAGGCGCTCACACTCTC  |
| Reverse | GCCGTAGAAGCCGCCATAGGC        |

deoxyribonucleic acid (cDNA) was synthesized (PrimeScript RT Master Mix, Takara Bio) from the isolated RNA for real-time PCR analysis. Finally, cDNA, primers, and SYBR green mix (Taq Universal SYBR Green Supermix, BIO-RAD) were mixed according to the manufacturer’s instructions and examined using the intercalator method. β-actin (Takara Bio), collagen type I (Col I, Takara Bio), OPN (Hokkaido System Science), and osteocalcin (OCN, Hokkaido System Science) primers were used in this work (Table 1).

Gene expression levels were analyzed using the ΔΔCt method, and β-actin was used as a housekeeping gene. Cells cultured in a medium without BCx/HAp were used as negative controls. The positive control was cultured in a differential medium after 1 week of incubation. The medium was adjusted to a final concentration of 50 μg/mL ascorbic acid (Nacalai Tesque), 10 mM β-glycerophosphate (TCI Chemicals), and 50 nM dexamethasone (Fujifilm Wako Pure Chemical) [32,33].

2.3. Fluorescence imaging of the core-shell nanoparticles and osteoblasts

Coumarin 6 (Cou6, Fujifilm Wako Pure Chemical) was selected as a fluorescent marker that could be used to evaluate the cellular uptake of DDS nanocarriers [34]. Cou6 was dissolved in acetone (1 mg/mL) and added to the PLA/acetone solution. Subsequently, Cou6-loaded PLA/HAp core-shell nanoparticles (Cou6/HAp) were prepared according to the process described in the previous section 2.1. Cou6/HAp was sterilized in 70% ethanol, washed 2 times with PBS, and resuspended in the culture medium (200 μg/mL). For comparison, a Cou6-containing medium was prepared by dissolving 10 mg of Cou6 in 1 mL ethanol, sterilizing the solution with a 0.22 μm filter, and adding it to the culture medium at 0.1 vol.% (final concentration: 10 μg/mL in culture medium). The amount of Cou6 in the Cou6-containing medium was approximately the same as in the Cou6/HAp-containing medium (200 μg/mL as the particle, 10 μg/mL as Cou6). MC3T3-E1 cells were seeded (0.5 x 10^5 cells/mL in 500 μL of culture medium) onto a cover glass (φ 15 mm) in a 24-well plate and incubated for 24 h. Next, the medium was replaced with the culture medium (as the control), Cou6-containing medium (as a comparison), and Cou6/HAp-containing medium and cultivated for an additional 24 h. The cells were fixed with 4% formaldehyde (Fujifilm Wako Pure Chemical) in PBS for 20 min and washed three times with PBS-0.05% Triton X-100 (PBST). The cells were then incubated with Alexa Fluor 594 Phalloidin (Invitrogen) and DAPI (4',6-diamidino-2-phenylindole, Fujifilm Wako Pure Chemical), washed with PBST, and mounted in ProLong Gold (Invitrogen). Fluorescent images were captured using a fluorescence microscope (BZ-X800, Keyence).

2.4. Statistical analysis

Statistical comparisons between the two means were performed using a two-tailed unpaired Student’s t-test followed by an F-test for homoscedasticity.

3. Results

3.1. Characterization of BCx/HAp core-shell nanoparticles

SEM images of BCx/HAp and photographs of the particle suspensions are shown in Figure 1 a and c). The particles are spherical, and BC0.5/HAp is orange. The diameters of BC0/HAp and BC0.5/HAp are 29 and 24 nm, respectively, and their distributions are shown in Figure 1 b and d. A TEM image of BC0/HAp is shown in Figure 2. The inner gray sphere and black shell are approximately 30 nm in diameter and 4 nm in thickness, respectively, and the diameter of BC0/HAp is approximately 40 nm. The weight ratio of HAp:(PLA + BC) for BCx/HAp is approximately 1:1 (Table 2); the TGA graph is shown in Fig. S2.

The calcium and phosphate ion concentrations in BCx/HAp supernatant, and the amounts used to precipitate the HAp shells of BCx/HAp are shown in Table 3. The Ca/P ratios of the HAp shells of BC0/HAp and BC0.5/HAp were 1.52 and 1.54, respectively, similar to those of biological HAp [35]. The XRD patterns of BCx/HAp (Figure 3) were in good agreement with those of HAp (ICPDS: 09–432). The bands corresponding to phosphate groups [36] and PLA [37] can be observed in the FTIR spectra of BCx/HAp (Figure 4a). The Raman spectra of BCx/HAp are shown in Figure 4b, and the bands correspond to phosphate groups [38,39], PLA [37], and BC [40]. The FTIR and Raman band assignments are listed in Table 4.

3.2. Behavior of osteoblasts cultured with BCx/HAp

The number of cells cultured with BCx/HAp was slightly smaller than that of the control, and there was no significant difference between cells cultured with BC0/HAp and BC0.5/HAp (Figure 5, and
fluorescence images are shown in Fig. S3). The relative gene expression levels of osteoblasts compared with those of the negative control are shown in Figure 6. The Col I expression levels of osteoblasts cultured with BC0.5/HAp at 2 weeks was significantly larger than those cultured with BC0/HAp as well as positive control (cultured in a differential medium). The expression level of OPN at 1 and 4 weeks in osteoblasts cultured with BC0.5/HAp was significantly larger than in those cultured with BC0/HAp. The OPN level of osteoblasts cultured with BCx/HAp was smaller at 2 weeks than that at 1 and 4 weeks. The OPN level of the osteoblasts cultured with BCx/HAp for 4 weeks was significantly larger than that of the positive control. In the case of OCN, gene expression was not detected at 1 and 2 weeks of culture. Osteoblasts cultured with BC0.5/HAp after 4 weeks of culture indicated significantly larger OCN expression levels compared with those

Table 2. Weight ratios of BCx/HAp.

| Sample code | HAp (%) | PLA + BC(%) |
|-------------|---------|-------------|
| BC0/HAp     | 50      | 50          |
| BC0.5/HAp   | 49      | 51          |

Table 3. Calcium and phosphate ions concentration in the supernatant solutions, and the amount used to precipitate the HAp shells of BCx/HAp. Ca/P ratio of the HAp shells of BCx/HAp.

| Sample code | BC0/ HAp | BC0.5/ HAp |
|-------------|----------|------------|
| Calcium ion / mM | In supernatant | 1.12 | 1.06 |
| Phosphate ion / mM | In supernatant | 0.62 | 0.59 |
| Ca/P of the HAp shell | 1.52 | 1.54 |
4.1 Discussion

The diameter of BCx/HAp was approximately 30–40 nm, with a PLA core diameter of 30 nm and a HAp shell thickness of 4 nm. The sizes were in good agreement with our previous reports on PLA/HAp core-shell nanoparticles with a core of 35–40 nm and a shell thickness of 5–6 nm [19,41]. The inorganic and organic ratio of BCx/HAp was approximately 1:1, and the trends were similar to those reported previously [17,22]. The XRD patterns of BCx/HAp showed peaks corresponding to HAp, and the peaks were not very sharp, similar to the trends observed for biological HAp [20]. FTIR and Raman spectra of BCx/HAp exhibited bands corresponding to HAp and PLA. In addition, the FTIR absorbance of the bands corresponding to PLA in BCx/HAp was not significantly different from those of PLA, and the band positions were not shifted. Thus, it could be concluded that BCx/HAp was composed of a PLA core with a HAp shell. BC0.5/HAp showed Raman bands corresponding to the CH₃ rocking (1005 cm⁻¹) and C-C stretching (1152 cm⁻¹) in BC. The suspension color of BC0.5/HAp was orange, which was derived from BC. Thus, BC was successfully loaded into the PLA/HAp core-shell nanoparticles, and the particles consisted of a PLA core and a HAp shell containing BC.

4.2 Osteoblast viability and osteogenic gene expression

Osteoblast viability after culturing with BCx/HAp was slightly smaller than that of the control. The cell number of BCx/HAp was approximately 8 × 10⁴ cells/cm², and the number was confluent because 5 × 10⁴ cells/cm² of MC3T3-E1 occupied >90% of the culture area [42]. Thus, the cell number difference between BCx/HAp and the control may be negligible. However, excess BC inhibited osteoblast proliferation because of the suppression of DNA synthesis [43]. However, BC0.5/HAp showed no cytotoxic effects up to 96 h and exhibited similar trends as BC-containing fibrous scaffolds [26,27]. Because BC was incorporated in the core-shell nanoparticles, the particles were expected to show sustained release of BC, similar to the information previously reported for our particles containing vitamin K₁ [22].

The Col I expression level of osteoblasts cultured with BC0.5/HAp was approximately 2 × 10⁵ times larger than those cultured with BC0/HAp and the positive control after 2 weeks of culture. Col I is an extracellular matrix (ECM) formation marker during bone formation in the early stages, and its formation is necessary for the deposition of bone minerals [32]. The Col I expression level peaks at approximately 10–20 d in in vitro tests [32], and
similar trends of Col I expression were observed for the cell culture with BC0.5/HAp-containing medium. The OPN expression level induced by BC0.5/HAp was larger than that by BC0/HAp and positive control at 1 and 4 weeks. HAp is well known to stimulate osteoblast differentiation and mineralization [44,45], and Ca$^{2+}$ ions released from HAp upregulate OPN expression via calcium channels in osteoblasts [46]. BCx/HAp indicated good cytocompatibility and the particles were attached to the
Because differential /HAp

Figure 21 osteoblasts (Figure 7), similar to our previous findings [21]. The particles can be endocytosed in cells because of their size and good cytocompatibility. Thus, Ca\(^{2+}\) ions can release inside the cells; the acidic pH ranges of endosomes and lysosomes are 5.5–6.0 and 4.5–5.0 [4], respectively, which can dissolve HAp. Thus, BCx/HAp induced a larger OPN expression level than the positive control. Furthermore, the OPN expression level of BCx/HAp in 2-weeks culture was smaller than in 1 and 4-weeks culture. This might originate from the expression behavior of OPN in osteoblasts. OPN expression shows a biphasic pattern of cell proliferation and mineralization [32,47,48]. The metabolite of BC can act as a ligand for the RAR family of transcription factors [49]. Additionally, BC is reported to induce osteoblast differentiation markers, such as Runx2, OPN, and ALP via the RAR signaling pathway [28,43]. Hence, OPN expression levels were upregulated by BC0.5/HAp but not by BC0/HAp because of the release of BC from the nanoparticles.

OCN is a mineralization marker and is not detected in early stages, such as before day 12 of culture; the increased expression levels of OCN and OPN support the progression of ECM mineralization [32]. In addition, BC has been reported to improve the mineralization of osteoblasts, which was significantly larger than that of osteoblasts cultured in the differential medium [26,27]. The OCN expression level induced by BC0.5/HAp was significantly larger than that by BC0/HAp and the positive control. Thus, BC0.5/HAp releases BC around osteoblasts because

| Frequency/cm\(^{-1}\) | FT-IR | Raman Assignments | FT-IR | Raman |
|------------------------|-------|-------------------|-------|-------|
| 560, 600               |       | O-P-O bending in PO\(_4\) | 34    |       |
| 586                    |       | P-O bond in PO\(_4\)     | -     | 36,37 |
| 750                    | 738   | C = O bending      | 35    | 35    |
| 870                    | 870   | C-COO stretching  | 35    | 35,38 |
| 955                    |       | CH\(_3\) rocking and C-C stretching | 35    |       |
| 1025                   | 958   | PO\(_4\) symmetric stretching | 34    | 36,37 |
| 1005                   |       | CH\(_3\) rocking     | -     | 38    |
| 1045                   | 1042  | C-CH\(_2\) stretching | 35    | 35    |
| 1152                   |       | C-C stretching      | -     | 38    |
| 1080                   | 1090  | COC symmetric      | 35    | 35    |
| 1125                   |       | CH\(_3\) asymmetric | 35    |       |
| 1125                   |       | CH\(_3\) rocking    | -     | 35    |
| 1187                   |       | C-C stretching      | -     | 38    |
| 1190                   |       | COC asymmetric stretching and CH\(_3\) asymmetric bending | 35    |       |
| 1207                   |       | C-C and C = C stretching | -     | 38    |
| 1270                   |       | CH bending, COC stretching | 35    |       |
| 1360                   |       | CH and CH\(_3\) bending | 35    |       |
| 1380                   |       | CH\(_3\) symmetric bending | 35    |       |
| 1452                   |       | CH\(_3\) asymmetric bending | 35    |       |
| 1755                   |       | C = O stretching    | 35    |       |

**Figure 5.** Cell numbers cultured with BCx/HAp medium for 96 h. Error bars represent standard deviation (n = 5, *: p < 0.05, n.s.: no significant difference).

**Figure 6.** Relative expression levels of (a) Col I, (b) OPN, and (c) OCN in osteoblast culture with BCx/HAp containing medium or differential medium (positive control) compared with the negative control. Error bars represent standard deviation (*: p < 0.05, **: p < 0.01).
of its good cytocompatibility, thereby upregulating OCN expression level.

Osteoblasts differentiated in the presence of BC-loaded PLA/HAp core-shell nanoparticles (without the differential medium) showed an upregulated expression of Col I, OPN, and OCN. PLA/HAp core-shell nanoparticles have excellent drug-loading ability, sustain-releasability, and cytocompatibility [21,22]. A concentration of 0.1–10 μM BC directly added to the culture medium could enhance osteoblast differentiation [28,43]. The amount of BC in BC0.5/HAp at 200 μg/mL was approximately 9 nM assuming that all of the BC was loaded onto the PLA/HAp core-shell nanoparticles during synthesis. In our previous work, treatment with anticancer agent-loaded PLA/HAp core-shell nanoparticles markedly reduced cancer cell viability compared with its direct addition to the medium because the particle effectively delivered the agent to the cancer cell [21]. Therefore, BC0.5/HAp also exhibited a stimulatory effect with a lower BC concentration compared with its direct addition to the medium. These results suggest that the PLA/HAp core-shell nanoparticles could enhance bone formation by upregulating osteogenic gene expression levels.

5. Conclusion

BC-loaded PLA/HAp core-shell nanoparticles were prepared using the emulsification method and osteoblast differentiation behavior was evaluated in vitro. BCx/HAp was composed of a PLA core and a HAp shell and was spherical in shape with a core diameter of approximately 30 nm. The Raman bands corresponding to BC were observed in BC0.5/HAp, and the particle suspension was orange. Hence, the successful synthesis of BC-loaded PLA/HAp core-shell nanoparticles was confirmed. BC0.5/HAp has a stimulating effect on osteoblast differentiation (i.e., upregulated Col I, OPN, and OCN expression levels), and its expression level was significantly larger than that of BC0/HAp and the positive control. BC0.5/HAp enhanced osteoblast differentiation with a comparably lower amount of BC than the amount directly added to the medium because of its excellent cytocompatibility and effective BC delivery to osteoblasts.

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Disclosure statement

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Figure 7. Fluorescence images of MC3T3-E1 cells cultured with (a) culture medium (control), (b) Cou6-containing medium, and (c) Cou6/HAp-containing medium. Red: F-actin, blue: nuclei, green: Cou6 and Cou@HAp core-shell nanoparticle.
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