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Chitosan-decorated calcium hydroxide microcapsules with pH-triggered release for endodontic applications†

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The treatment of apical periodontitis (AP) remains challenging because traditional root canal therapy (RCT) outcomes are limited by the complexity of the root canal system, drug toxicity, and host immune factors. It is necessary to develop methods to prepare controlled drug release vehicles with improved biocompatibility for treatment of AP. Herein, calcium hydroxide microcapsules with chitosan and ethylcellulose coated (CS–EC@Ca(OH)$_2$ microcapsules) were prepared and the presence of hydrogen bonding between shell materials was observed by FT-IR. Through release assessment and biological assays, we showed that the microcapsules had enhanced controlled-release performance and biocompatibility. In particular, drug release from the microcapsules was pH-triggered. The cumulative release of drugs in pH 5.0 buffer was 8-fold higher than that in pH 7.0 buffer. Furthermore, the microcapsules exhibited prolonged antibacterial activity against refractory strains of *Enterococcus faecalis*. Additionally, the CS–EC@Ca(OH)$_2$ microcapsules reduced inflammation and promoted osteogenesis, which could be beneficial for the healing of AP with bone defects. Therefore, CS–EC@Ca(OH)$_2$ microcapsules improve the innate properties of Ca(OH)$_2$ and hold potential for AP treatment.

1. Introduction

Apical periodontitis (AP) primarily resulting from pulpal infection has become a common endodontic disease. Patients are at risk of gingival swelling because of microbial infection-induced periapical bone destruction. In the clinic, AP is treated by removal of bacteria *via* root canal therapy (RCT).[1] However, it is difficult to completely remove the bacteria because of root canal system complexity. As a result, the infection persists and gives rise to acute osteomyelitis or chronic granuloma. Host factors in the periapical local tissues play pivotal roles in the process of lesion repair.[2] Immune and endocrine diseases such as diabetes may exacerbate the infection and delay the healing of AP bone defects.[3] Hence, effective drugs are used in local AP lesions to control inflammation and promote osteogenesis. Maintenance of extracellular pH is important to balance bone formation and resorption.[4] An alkaline microenvironment of pH 8.0–8.5 is crucial for bone regeneration. In contrast, a local acidic microenvironment can induce inflammation, thereby reducing osteoblast activity.[5,6] Therefore, an ideal material for endodontic application should have no cytotoxicity and should regulate the microenvironment to minimize bacterial growth, suppress inflammation and promote osteogenesis.

pH-Triggered systems have attracted considerable interest for biomedical applications.[7,8] pH-Sensitive drug release systems using different biomaterials in the form of microcapsules, micelles, hydrogels and mesoporous materials have been proposed.[9,10] In particular, chitosan (CS) presents excellent biodegradability and antibacterial activity.[11] CS is derived by *N*-deacetylation of chitin and is a positively charged polymer composed of *N*-acetyl-glucosamine and glucosamine units connected by glucosidic bonds.[12] The presence of free amine groups causes CS to be pH responsive, as it can be ionized in an acidic environment.[13] pH-Triggered CS-decorated composites have been developed for biomedical applications.[14] Such pH-triggered composites are expected to be useful for endodontic treatment.

Calcium hydroxide (Ca(OH)$_2$) is frequently used to treat pulpitis and periapical diseases because of its antibacterial

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† Electronic supplementary information (ESI) available: Primer sequences (5′–3′) for quantitative real-time PCR, SEM image of CS–EC@Ca(OH)$_2$ microcapsules at low magnification, FT-IR spectra of CS@Ca(OH)$_2$ and EC–CS mixture respectively, surface elemental analysis by XPS. See DOI: 10.1039/c5tb01643f

activity and bone formation activity.[15] However, it is associated with burst release that generates a strong alkaline environment that inhibits osteogenesis, although it is beneficial for inhibiting bacteria.[16] In the clinic, Vitapex (a widely used oily vehicle of $Ca(OH)_2$ paste) is used in apical periodontitis cases.[17] Oily vehicles can achieve the slow release of $Ca(OH)_2$ compared to an aqueous vehicle; however, some studies revealed that Vitapex had poor antimicrobial performance.[18,19] Moreover, the cytotoxicity, immunogenicity, and difficulty of removal also limit the application of oily vehicles.[20] To solve these problems, new vehicles for $Ca(OH)_2$ were developed. Ballal *et al.* developed a $Ca(OH)_2$-loaded controlled release gel formulation using different gel bases, such as propylene glycol, poly-ethylene glycol 6000, chitosan, and guar gum.[21] Among these gel vehicles, chitosan exhibited a controlled release for prolonged duration. But there is a lack of further studies on physicochemical properties and biological characteristics. In our previous work, we reported that encapsulation of $Ca(OH)_2$ with EC can decrease its cytotoxicity and enhance its antibacterial activity through the controlled release of $Ca(OH)_2$.[22,23] However, EC@$Ca(OH)_2$ microcapsules display a burst release at the very early stage in neutral media, which can cause a rapid increase in pH, thereby inhibiting alveolar bone regeneration. It is thus necessary to develop a pH-triggered microcapsule system to rapidly release $Ca(OH)_2$ in acidic microenvironments to rapidly increase the pH. Such a system would inhibit bacterial infection while preventing bone resorption. When the microenvironment becomes neutral or slightly alkaline, the release of $Ca(OH)_2$ decreases in response. There are no reports on $Ca(OH)_2$-loaded pH-triggered release systems with reduced cytotoxicity, and enhanced antibacterial activity, anti-inflammatory effect, and mineralization, which would promote the repair of AP.

Herein, we propose a new strategy to synthesize pH-triggered CS–EC@$Ca(OH)_2$ microcapsules. The property of pH-triggered release and cytotoxicity was evaluated, along with the ability of antibacterial activity, anti-inflammation, and mineralization.

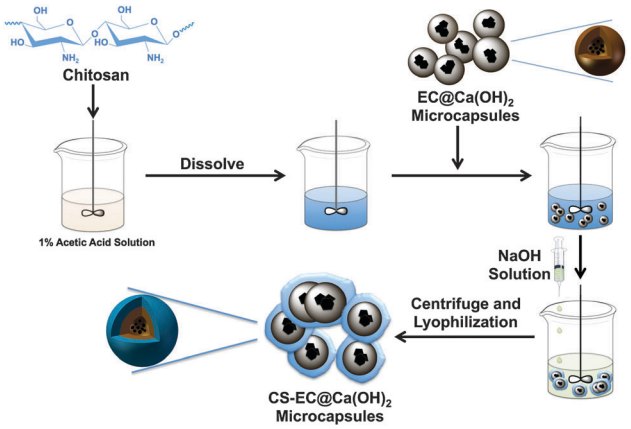
2. Experimental

2.1. Materials

EC and $Ca(OH)_2$ were purchased from Sinopharm Chemical Reagents. The 95% *N*-deacetylated CS was supplied by Aladdin (Shanghai, China). NaOH and acetic acid were provided by Beijing Chemical Works (Beijing, China). All chemicals were of analytical grade, and all aqueous solutions were prepared with de-ionized water.

2.2. Preparation of CS–EC@$Ca(OH)_2$ microcapsules

As illustrated in Scheme 1, the CS–EC@$Ca(OH)_2$ microcapsules were prepared by precipitation of CS onto the EC@$Ca(OH)_2$ microcapsules in an aqueous medium. EC@$Ca(OH)_2$ microcapsules were prepared as previously described.[22] CS (40 mg) was dissolved in 100 ml of aqueous acetic acid solution (1% v/v) using an RW20 digital homogenizer (IKA, Staufen, Hesse-Darmstadt, Germany). The EC@$Ca(OH)_2$ microcapsules (300 mg) were added to the above solution with stirring at 1500 rpm.



Scheme 1 Schematic illustration of CS–EC–$Ca(OH)_2$ microcapsule preparation.

NaOH solution (pH = 13.0) was dropped into the mixture at a rate of 6 ml min^{-1} by means of a peristaltic pump (LSP02-1B, Longer Pump, Baoding, China) to achieve pH = 10.0. CS precipitated onto the EC@$Ca(OH)_2$ microcapsule surface. Eventually, the CS–EC@$Ca(OH)_2$ microcapsules were collected after centrifugation at 2000 rpm for 5 min and completely dehydrated using a lyophiliser (FD-1C, Detianyou Laboratory Equipment Factory, Beijing, China). The microcapsules were subjected to UV sterilization overnight for biological experiments.

2.3. Characterization of the microcapsules

Scanning electron microscopy (SEM, S-4800, Hitachi, Japan) was used to characterize the surface morphology. The microcapsules were dried ambiently and sputtered with platinum. Transmission electron microscopy (TEM, JEM-1011, JEOL, Japan) was used to observe the internal structure of the microcapsules. A Zetasizer Nano ZS90 (Malvern Instruments, UK) was used to measure the particle size of the microcapsules in water.

Thermal gravimetric analysis (TGA) was performed using a Pyris 1 system (PerkinElmer, USA) to determine the composition of the microcapsules at a scanning rate of 10 °C min^{-1} under an air atmosphere at a flow rate of 20 ml min^{-1}. Fourier transform-infrared (FT-IR) spectroscopy was performed using an Equinox 55 system (Bruker, Germany). Surface elements were measured by X-ray photoelectron spectroscopy (XPS, ESCA-Lab250Xi, Thermo Scientific, USA).

In order to determine the drug loading and encapsulation efficiency, CS–EC@$Ca(OH)_2$ microcapsules were completely dissolved in an adequate mixture of 0.1 M HCl and 96% ethanol (3 : 2, v/v) with ultrasonic bath according to the previous study.[24] Then, the Ca^{2+} concentration was determined using a calcium assay kit (Biosino Bio-technology and Science, Beijing, China) and the weight of $Ca(OH)_2$ was calculated based on the molecular weight. The drug loading (%) and encapsulation efficiency (%) of the CS–EC–$Ca(OH)_2$ microcapsules were calculated as follows:

Drug loading (%) = weight of $Ca(OH)_2$ in microcapsules/weight of microcapsules $\times 100\%$

Encapsulation efficiency (%) = weight of $Ca(OH)_2$ in microcapsules/weight of $Ca(OH)_2$ put into operation $\times$ 100%

2.4. pH-Triggered drug release *in vitro*

CS–EC@$Ca(OH)_2$ microcapsules (50 mg) in a dialysis bag were dispersed in 1 ml of acetate buffer (pH = 5.0 or 6.0) or HEPES buffer (pH = 7.0). The dialysis bag was placed in a serum bottle with the respective buffer (100 ml) at 37 °C under shaking at 100 rpm in an incubator (NRY-100C, Nanrong Laboratory Equipment Factory, Shanghai, China). After 200 μl was collected at the desired time interval, an equal volume of fresh buffer was added. Ca^{2+} concentration was measured using a calcium kit as described above.

2.5. Antibacterial activity

Enterococcus faecalis, a Gram-positive bacterium associated with refractory and secondary infections in endodontics,[25] was obtained from the American Type Culture Collection (ATCC, USA, 29212). Brain heart infusion (BHI, BD, USA) broth was prepared with buffer solution instead of distilled water to maintain the pH value during bacteriostatic experiments.[26,27] The buffer solutions were acetate buffer for pH 4.0, HEPES buffer for pH 7.0, and ammonium chloride buffer for pH 10.0. Bacterial suspensions (3×10^8 colony-forming units [CFUs] ml^{-1}; 40 μl) and BHI broth (3 ml) with different pH values were added to a 12-well plate. Transwell inserts (0.4 μm filter, Corning, USA) containing pure $Ca(OH)_2$, EC@$Ca(OH)_2$, or CS–EC@$Ca(OH)_2$ microcapsules (30 mg) and BHI broth (0.4 ml) were placed in the wells. The inserts without drugs served as a negative control. After incubation for 24 h, the inserts were transferred to new wells containing a fresh bacterial suspension (40 μl) and fresh BHI (3 ml) with different pH values. The remaining broth was diluted 10^4 times with fresh broth and inoculated onto BHI agar plates using a spiral inoculation instrument (easySpiral Pro, Interscience, France). The plates were cultured at 37 °C for 24 h in an incubator (Thermo Scientific), and the colonies were counted to calculate the number of CFUs. The antibacterial activity was examined continuously for 7 days, and all experiments were performed in triplicate.

2.6. Cytotoxicity evaluation

MG63 cells (ATCC CRL-1427) were cultured at 37 °C in a humidified 5% (v/v) CO_2 incubator (Thermo Scientific) and seeded in 6-well plates (3×10^4 cells per ml). The culture medium was high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (Gibco). After attachment, the culture medium (3 ml) containing $Ca(OH)_2$ (4.5 mg), EC@$Ca(OH)_2$ microcapsules (5.4 mg), or CS–EC@$Ca(OH)_2$ microcapsules (14.1 mg) was added, ensuring that the $Ca(OH)_2$ content was equivalent. The culture medium without drugs served as a negative control. After culturing, the cells were washed with Dulbecco's Hank's balanced salt solution (D-Hanks) three times, and Cell Counting Kit 8 (CCK-8, Dojindo, Japan) solution was added to each well (1 : 10, v/v) with fresh culture medium. After 2 h of incubation in the dark, the supernatant (100 μl) was transferred to a 96-well cell culture plate. The optical density (OD) of the supernatant was measured using a microplate reader (BioTek ELx808, USA) at 450 nm.

2.7. Gene expression assay for anti-inflammation and mineralization

Pure $Ca(OH)_2$ (6 mg), EC@$Ca(OH)_2$ microcapsules (6 mg), or CS–EC@$Ca(OH)_2$ microcapsules (6 mg) and culture medium (2 ml) were aliquoted onto Transwell inserts. The inserts were placed into a 6-well plate containing culture medium (3 ml) for drug pre-release (0–14 days). MG63 cells were cultured and seeded. After cell attachment, 15 mg of lipopolysaccharide (LPS, L4391, Sigma, USA) in 3 ml of culture medium was added. The Transwell inserts containing pre-released drugs were placed in the wells. After 3 days of culturing, total mRNA was isolated from the cells by using TRIzol (Invitrogen, USA) and converted into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR analysis was performed using SYBR Green (Roche, USA) on an ABI 7500 RT-PCR machine (Applied Biosystems, USA). The primers (5′–3′) for interleukin (IL)-1β, IL-6, runt-related transcription factor 2 (RUNX2) and collagen type I (COL I) are listed in Table S1 (ESI†). The data were analysed according to the comparative CT ($2^{-\Delta\Delta CT}$) method. All measurements were repeated in triplicate. All quantitative data were statistically analysed by one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of CS–EC@$Ca(OH)_2$ microcapsules

In contrast to traditional methods used to prepare CS microcapsules including emulsion crosslinking, spray-drying, ionic gelation and coacervation/precipitation,[28] in the present study, the CS–EC@$Ca(OH)_2$ microcapsules were prepared by precipitating CS onto EC@$Ca(OH)_2$ microcapsules in an aqueous dispersion in the presence of increased pH. No organic solvents, surfactants, or crosslinkers were used during the synthesis. Our method is advantageous because it limits the potential additional cytotoxicity of the microcapsules. The dissolved CS started to precipitate and form a layer on the EC@$Ca(OH)_2$ microcapsule surface when NaOH was added to adjust the pH of the dispersion. Among the parameters controlling size distribution, surface morphology and drug loading of the microcapsules,[29] it is crucial to control the addition rate of the NaOH solution to form a uniform CS layer on the EC@$Ca(OH)_2$ microcapsule surface. The original $Ca(OH)_2$ particles were irregular in shape (Fig. 1a), whereas the EC@$Ca(OH)_2$ microcapsules were spherical in shape (Fig. 1b) and their exterior surface was smooth. TEM imaging (inset of Fig. 1b) showed that the $Ca(OH)_2$ particles were well encapsulated with a layer of EC. After encapsulation with another CS layer, the microcapsules still presented a similar smooth exterior surface (Fig. 1c, Fig. S1, ESI†). After encapsulation of the $Ca(OH)_2$ particles with EC, the mean diameter dramatically increased from 148 nm to 900 nm (Fig. 1d).

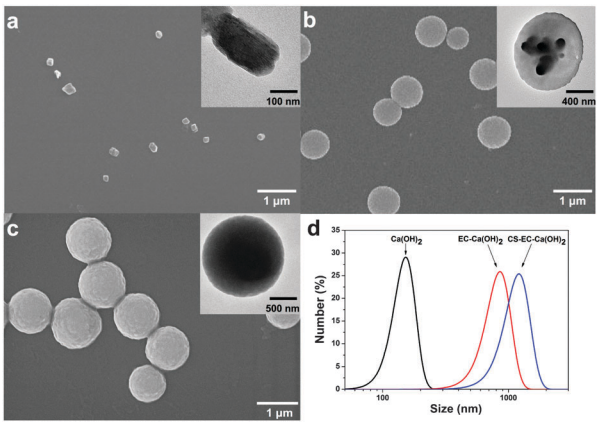


Fig. 1 SEM and TEM (inset) images of three representative samples: (a) $Ca(OH)_2$, (b) EC@$Ca(OH)_2$ microcapsules and (c) CS–EC@$Ca(OH)_2$ microcapsules. (d) Particle size distribution of the samples in water.

TEM imaging (Fig. 1b) indicated that each microcapsule contained more than one particle. Further encapsulation with CS further increased the mean diameter to 1200 nm.

The content of polymers in the microcapsules and their thermal behaviour were measured by TGA (Fig. 2). Pure $Ca(OH)_2$ lost 18% of its weight at 400–500 °C, corresponding to thermal dehydration. The as-prepared EC@$Ca(OH)_2$ microcapsules also presented a weight loss of 15% at 250–330 °C, corresponding to the thermal decomposition of EC. The EC@$Ca(OH)_2$ microcapsules contained about 85% $Ca(OH)_2$ and 15% EC. Progressive weight loss was also observed from the CS–EC@$Ca(OH)_2$ microcapsules at 250–400 °C, corresponding to the thermal decomposition of EC and CS. The CS–EC@$Ca(OH)_2$ microcapsules contained about 56% $Ca(OH)_2$, 10% EC, and 34% CS.

The composition and interaction between the components were characterized using FT-IR spectra (Fig. 3, Fig. S2, ESI†). Pure $Ca(OH)_2$ possesses three characteristic bands at 3643 cm^{-1} (stretching vibration of –OH), 1439 cm^{-1} (–OH in plane vibration) and 876 cm^{-1} (Ca–O–H band). EC possesses a characteristic peak at 1101 cm^{-1} corresponding to the symmetric structural vibration of the ethoxy group. After encapsulation of $Ca(OH)_2$ with EC, the characteristic peak of EC at 1101 cm^{-1} was split into two peaks at

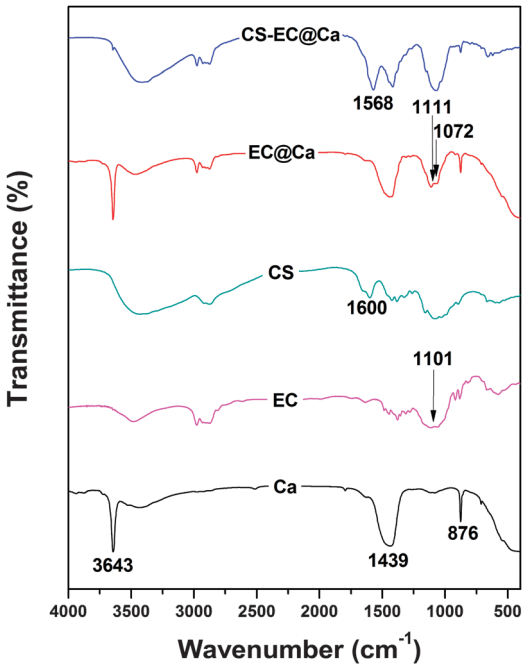


Fig. 3 FT-IR spectra of the representative samples.

1072 cm^{-1} and 1111 cm^{-1}, implying the presence of a hydrogen bond between $Ca(OH)_2$ and EC. The characteristic peak of pure CS at 1600 cm^{-1} was assigned to the C–O band of the amide group. After coating of the EC@$Ca(OH)_2$ microcapsules, the peak at 1600 cm^{-1} was greatly shifted to 1568 cm^{-1}, indicating the presence of a hydrogen bond between EC and CS.

A Ca content of 5.40% was determined for the EC@$Ca(OH)_2$ microcapsules by XPS (Fig. 4, Table S2, ESI†), which indicates that some free $Ca(OH)_2$ is present on the microcapsule surface. After further coating with CS, the Ca content at the microcapsule surface was only 0.34%, indicating that no $Ca(OH)_2$ was present at the surface. Additionally, the N content of 3.39% revealed the presence of CS.

The drug loading (%) and encapsulation efficiency (%) of the CS–EC@$Ca(OH)_2$ microcapsules were 31.8% ± 6.4% and 17.3% ± 5.1%, respectively.

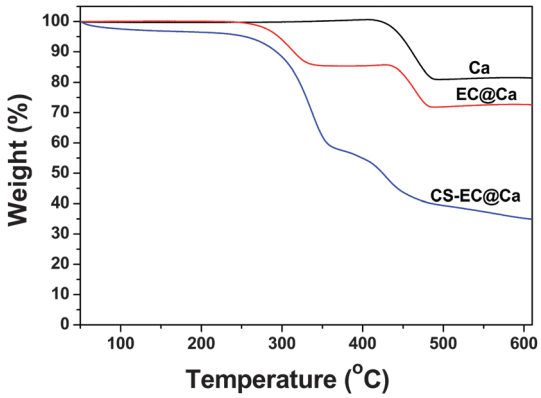


Fig. 2 TGA traces of the representative samples.

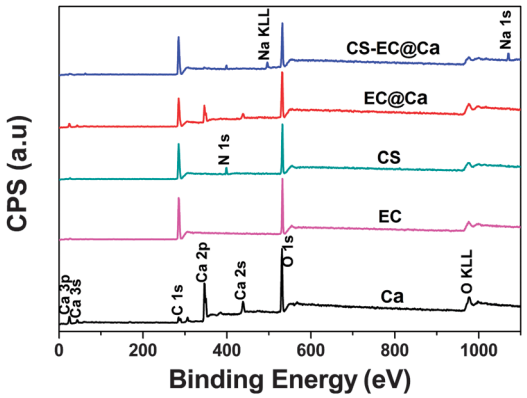


Fig. 4 XPS results of the representative samples.

3.2. *In vitro* release profile

To determine the effect of another CS layer on the controlled release of $Ca(OH)_2$ (Fig. 5), the as-prepared EC@$Ca(OH)_2$ microcapsules were treated under the same conditions used for preparation of CS–EC@$(OH)_2$ but without CS. The residual $Ca(OH)_2$ on the exterior surface of the microcapsules was washed away. At the initial stage, $Ca(OH)_2$ was released in burst mode, which was minimally dependent on pH variation at pH 6.0–7.0. When the pH was decreased to 5.0, the release was greatly accelerated, probably because the EC shell became unstable in the acidic solution.[30,31] In contrast, no burst release was observed from the CS–EC@$Ca(OH)_2$ microcapsules. The release became much slower at pH = 7.0 with a cumulative release of 9% after 120 h.

At pH = 6.0, the release became slightly faster with a cumulative release of 18% after 120 h. A slightly alkaline microenvironment is conducive to regeneration of bone. When the pH was 5.0, the release was dramatically increased, reaching a plateau of 58% within a short period of 24 h. CS is a weakly alkaline polymer with a pK_a of 6.3. At pH = 5.0, which is below the pK_a, the CS chains become positively charged and are easily soluble in water, thus accelerating the release.[32] Therefore, these microcapsules could be used to provide programmable treatment for inflammatory lesions. In an acidic inflammatory microenvironment, the CS–EC@$Ca(OH)_2$ microcapsules can rapidly release $Ca(OH)_2$ to increase the pH. When the pH of the microenvironment is adjusted to be close to neutral, the release automatically decelerates.

The pH-triggered release mechanism was revealed by visualization of the morphological evolution of the microcapsules during release under various conditions. At pH = 7.0, the shell was perforated during release (Fig. 6a). After 24 h, the $Ca(OH)_2$ surface beneath the EC shell coarsened, and the perforated channels became more distinct (Fig. 6b). The EC coating layer was preserved. After 120 h, all of the $Ca(OH)_2$ was released outwardly, and the corresponding hollow EC cages are shown to the left. At pH = 5.0, after 0.5 h of release, the $Ca(OH)_2$ surface took on a highly distinct morphology (Fig. 6c). This is consistent with a faster release of $Ca(OH)_2$. After 24 h, $Ca(OH)_2$ pillars were well grown across the EC shell (Fig. 6d). The interior $Ca(OH)_2$ became highly branched. At pH = 7.0, the CS–EC@$Ca(OH)_2$ microcapsules showed preservation of their core/shell structure after 120 h. In contrast, all of the $Ca(OH)_2$ was released from the EC@$Ca(OH)_2$, which presented a hollow cage morphology. This indicates that CS greatly inhibits the release of $Ca(OH)_2$. At pH = 5.0, some perforated channels were observed after 0.5 h (Fig. 6e). After 24 h, the interior particle surface was coarsened after further release. No pillars were found across the polymer layer (Fig. 6f). The perforated channels became more distinct.

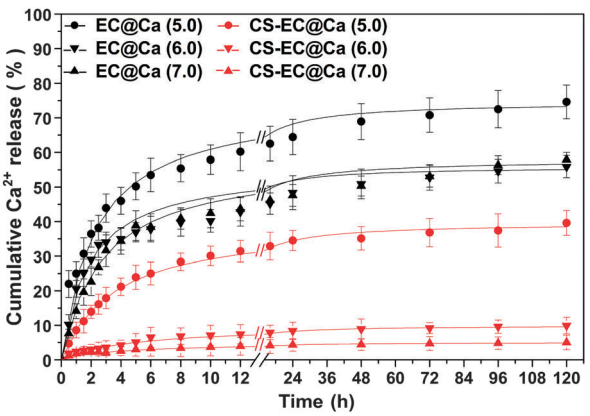


Fig. 5 Cumulative release of Ca^{2+} from the EC@$Ca(OH)_2$ and CS–EC@$Ca(OH)_2$ microcapsules was dependent on pH.

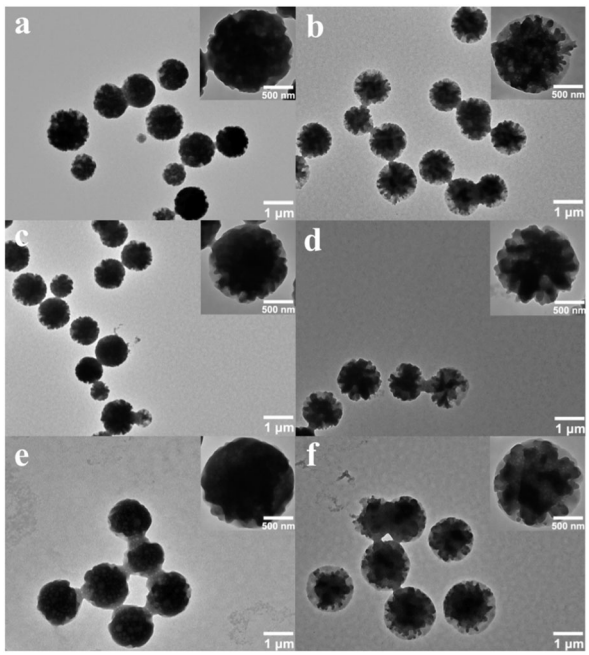


Fig. 6 TEM images of representative microcapsules at low and high (inset) magnifications after release at various pH values and times: (a and b) EC@$Ca(OH)_2$ microcapsules at pH = 7.0 at 0.5 h and 24 h; (c and d) EC@$Ca(OH)_2$ microcapsules at pH = 5.0 at 0.5 h and 24 h; (e and f) CS–EC@$Ca(OH)_2$ microcapsules at pH = 5.0 at 0.5 h and 24 h.

3.3. Antibacterial activity of the microcapsules

$Ca(OH)_2$ exerts antibacterial activity by releasing hydroxyl ions (OH^-) to damage bacterial DNA, proteins and the cytoplasmic membrane.[33,34] In neutral buffer, pure $Ca(OH)_2$ reduced Gram-positive *E. faecalis* levels particularly during the first 1–2 days (black column, Fig. 7b). The antibacterial activity dramatically decreased after day 3 because most of the $Ca(OH)_2$ had been released. In comparison, the EC@$Ca(OH)_2$ microcapsules showed extended antibacterial activity up to 4 days, implying a progressive release of $Ca(OH)_2$. After 5 days, the antibacterial activity decreased. After coating the EC@$Ca(OH)_2$ microcapsules with CS, antibacterial activity of the CS–EC@$Ca(OH)_2$ microcapsules was maintained for up to 7 days. This lasting antibacterial activity is partially attributed to sustained release from the CS–EC@$Ca(OH)_2$ microcapsules, as verified by the accumulative release results. Additionally, the exterior CS possesses bacteriostatic activity against both Gram-positive and

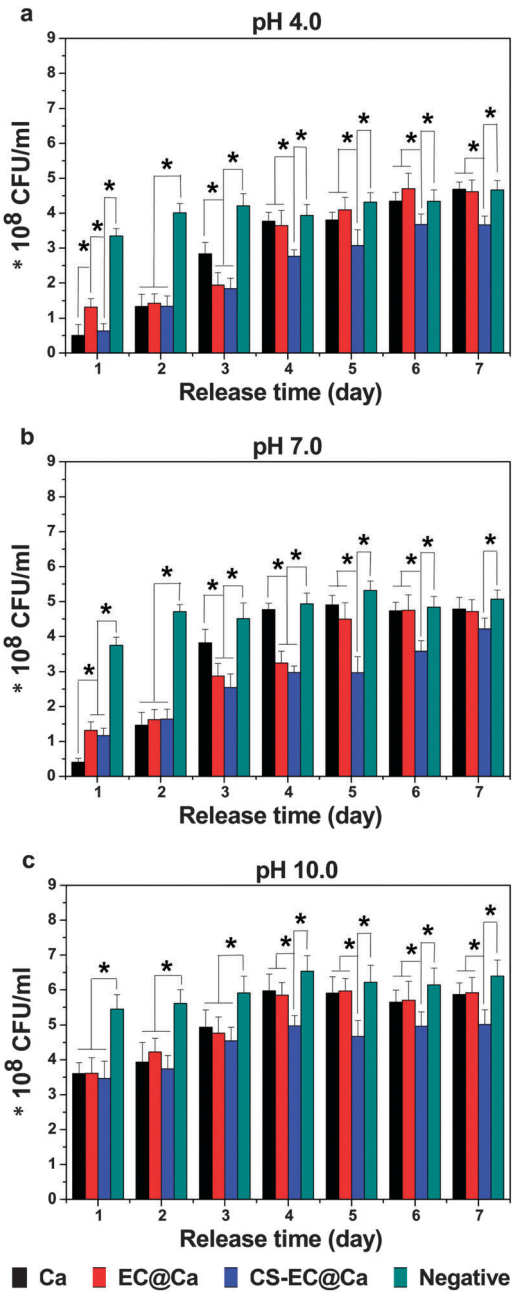


Fig. 7 Antibacterial activity of the three representative samples of $Ca(OH)_2$, EC@$Ca(OH)_2$ and CS–EC@$Ca(OH)_2$ and its dependence on release time. The negative control was bacteria cultured in the absence of drugs. The label * indicates a significant difference between the groups ($p < 0.05$).

gram-negative bacteria.[35,36] The negatively charged cell membrane and positively charged CS molecules could interact with each other through electrostatic attraction, which might result in the loss of membrane structure and release of some intracellular substances. In addition, after the cell wall was being penetrated, CS bound DNA and therefore disrupted protein synthesis. The antibacterial effect can also be promoted by CS-mediated chelation of trace metals, flocculation, and inhibition of enzyme activity.[37] In pH 4.0 buffer solution, CS–EC@$Ca(OH)_2$ microcapsules can inhibit bacterial growth in the early stage because pH-triggered microcapsules release enough $Ca(OH)_2$ in the acidic buffer. In addition, CS–EC@$Ca(OH)_2$ microcapsules can release drugs in a sustained manner to reduce the activity of *E. faecalis* during 7 days, which can reveal the effective antibacterial activity in an acid microenvironment caused by apical periodontitis (Fig. 7a). On the other hand, although the whole activity of *E. faecalis* is higher due to the alkali-resistant property and the less drug release in pH 10.0 buffer, we still can find that the group of CS–EC@$Ca(OH)_2$ microcapsules could effectively and sustainably inhibit bacterial growth compared to other groups. This is because of the controlled drug release and the antibacterial activity of CS (Fig. 7c).

3.4. Cytotoxicity of the microcapsules

MG63 is a bone cell line with remodelling capability and is thus commonly used to test the cytotoxicity of biomaterials.[38–40] In our study, the use of MG63 cells avoided the heterogeneity of odontoblasts or osteoblasts obtained *via* primary culture. As previously reported, high $Ca(OH)_2$ concentrations, for example 1 mg ml^{-1}, damage cells, whereas no toxicity is observed below 0.25 mg ml^{-1}.[16,22] To observe the maximum cytotoxicity, 1.5 mg ml^{-1} pure $Ca(OH)_2$ powder was used as the positive control, and microcapsules containing an equal amount of $Ca(OH)_2$ were selected to evaluate the influence of encapsulation on the cell toxicity. The microcapsules were directly added to the culture medium for 7 days. Compared with the negative control, the cell number was remarkably decreased after treatment with pure $Ca(OH)_2$ after days 3 to 7 (Fig. 8). In contrast, the cell viability remained similar to that of the negative control after treatment with both EC@$Ca(OH)_2$ and CS–EC@$Ca(OH)_2$ microcapsules. Based on the cumulative release results (Fig. 5), the concentration of $Ca(OH)_2$ released by the CS–EC@$Ca(OH)_2$ microcapsules was roughly estimated to be below 0.15 mg ml^{-1}. It is thus understandable that no toxicity was observed at such a low concentration of $Ca(OH)_2$. Additionally, the lack of toxicity *in vivo*

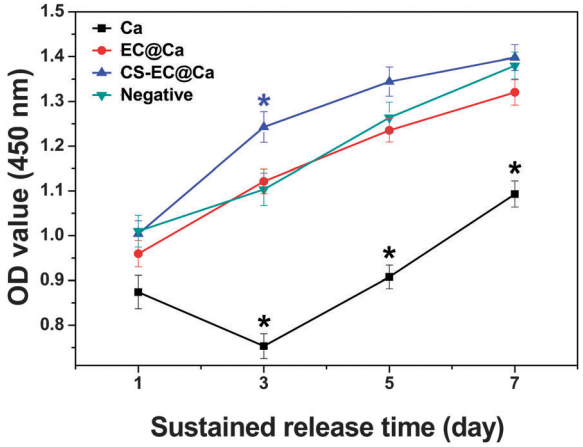


Fig. 8 Cytotoxicity of the three representative samples and dependence on release time. The negative control was MG63 cells cultured in the absence of drugs. The label * indicates a significant difference *vs.* the negative control ($p < 0.05$).

of both EC and CS was also confirmed. In fact, the cell number on day 3 increased in the case of CS–EC@Ca(OH)$_2$ microcapsule treatment. It is thus reasonable to consider that CS may promote cell adhesion and growth.[41,42] Coating of CS onto the original microcapsules is thus effective to improve the biocompatibility of the microcapsules.

3.5. Anti-inflammation and mineralization assay of the microcapsules

The effects of materials on regulating inflammatory response and bone remodelling were assessed based on the expression of pro-inflammatory factors and mineralization markers in MG63 cells.[43–45] In our study, LPS was added to the culture medium to simulate the inflammatory environment of periapical diseases *in vitro*. The gene expression of two pro-inflammatory factors, IL-1β and IL-6, and two mineralization markers, RUNX2 and COL I, was evaluated by qRT-PCR after 3 days of culture with the drugs (Fig. 9).

MG63 cells express IL-1β and IL-6 at high levels after stimulation with LPS. Treatment with Ca(OH)$_2$ significantly decreased the IL-1β and IL-6 expression (Fig. 9a and b), probably as a result of decreased LPS virulence in the presence of Ca(OH)$_2$ after hydrolysis of LPS into fatty acids and amino sugars.[46] At the early release stage, both types of microcapsules displayed similar alteration of inflammatory factor expression compared to pure Ca(OH)$_2$. However, the EC@Ca(OH)$_2$ microcapsules showed strong inhibition of inflammatory mediators for longer durations, for example 5 days, when pure Ca(OH)$_2$ was no longer active. The CS–EC@Ca(OH)$_2$ microcapsules showed further reduction of LPS-induced inflammation up to 14 days.

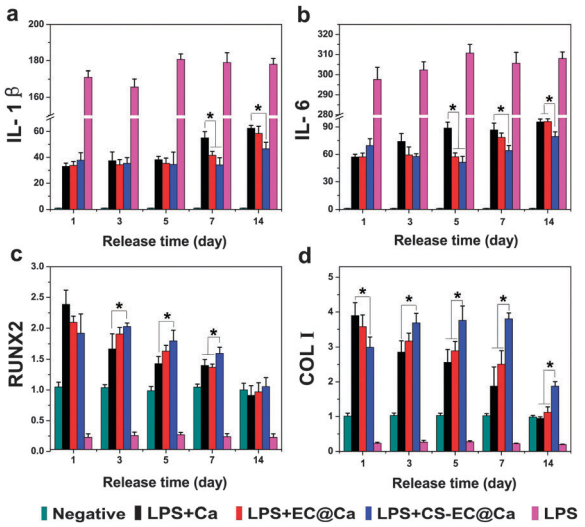


Fig. 9 Gene expression of IL-1β, IL-6, RUNX2 and COL I in MG63 cells during treatment with LPS and varying Ca(OH)$_2$ preparations at specific time intervals. The negative control was MG63 cells cultured in the absence of drugs or LPS. The label * indicates a significant difference among the LPS + Ca(OH)$_2$ group, LPS + EC@Ca(OH)$_2$ group and LPS + CS–EC@Ca(OH)$_2$ group ($p < 0.05$).

RUNX2 is the main osteogenic transcription factor regulating osteoblast differentiation.[47] COL I is an early differentiation marker that is expressed throughout bone formation and facilitates osteoblast differentiation and mineralization *in vitro*.[16] As shown in Fig. 9c and d, the expression of both mineralization factors, RUNX2 and COL I, was greatly decreased following LPS stimulation compared with the negative control. In contrast, their expression was increased after treatment with the three representative drugs. The expression was significantly higher when the cells were treated with both types of microcapsules for a long duration, for example on days 3–7 ($p < 0.05$). In particular, the CS–EC@Ca(OH)$_2$ microcapsules exhibited a more sustainable effect on the up-regulation of COL I, up to day 14. The sustainable release of Ca(OH)$_2$ from CS–EC@Ca(OH)$_2$ microcapsules may induce osteoblast differentiation *via* increased extracellular Ca^{2+} levels by prolonging the phosphorylation and activation of JNK and p38 in the periapical region.[16,48] Furthermore, a slightly alkaline microenvironment can also facilitate the formation of bone under inflammation challenge.[49]

4. Conclusions

A facile approach is reported to synthesize pH-triggered Ca(OH)$_2$ microcapsules by precipitation of CS onto EC@Ca(OH)$_2$ microcapsules in water. In addition to sustained release of Ca(OH)$_2$ from the microcapsules, CS–EC@Ca(OH)$_2$ microcapsules display pH-triggered release. As a result, the antibacterial activity of the microcapsules against refractory strains of *Enterococcus faecalis* is greatly prolonged. Additionally, the CS–EC@Ca(OH)$_2$ microcapsules remarkably reduced inflammation and promoted osteogenesis. The CS–EC@Ca(OH)$_2$ microcapsules are thus promising for the treatment of endodontic diseases.

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