Effects of crystallinity and surface modification of calcium phosphate nanoparticles on the loading and release of tetracycline hydro-chloride

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Abstract. The influences of crystallinity and surface modification of calcium phosphate nanoparticles (nCaP) on their drug loading capacity and drug release profile were studied in the present investigation. The CaP nanoparticles with different crystallinity were prepared by precipitation method under different temperatures. CaP nanoparticles with lower crystallinity exhibited higher drug loading capacity. The samples were characterized by XRD, FT-IR, SEM, TEM and BET surface area analyzer respectively. The drug loading capacity of nCaP was evaluated to tetracycline hydro-chloride (TCH). The internalization of TCH loaded nCaP in cancer cell was observed by fluorescence microscope. nCaP could be stabilized and dispersed in aqueous solution by poly(acrylic acid) surface modification agent, leading to enhanced drug loading capacity. The drug release was conducted in different pH environment and the experimental data proved that nCaP were pH sensitive drug carrier, suggesting that nCaP could achieve the controlled drug release in intracellular acidic environment. Furthermore, nCaP with higher crystallinity showed lower drug release rate than that of lower crystallinity, indicating that the drug release profile could be adjusted by crystallinity of nCaP. nCaP with adjustable drug loading and release properties are promising candidate as drug carrier for disease treatment.

Keywords: calcium phosphate, drug carrier, pH sensitive, nanoparticle, drug controlled release

1. Introduction
Calcium phosphate (CaP) bio-material has been used for several decades in clinic due to its good biological activity and compatibility [1-3]. However, the implantation of calcium phosphate normally requires anti-infection treatments [4,5]. Local drug delivery is an alternative way to treat infection by loading drug on calcium phosphate material [6-8]. The primary advantage of local antibiotic administration is the ability to obtain high local drug concentrations on the surface and in intimate vicinity of the device without increasing systemic toxicity[9]. Calcium phosphate is the major inorganic component of bones and teeth and has abundant adsorption sites (such as Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3-}) on
the surface for drug adsorption [10,11]. In addition, CaP can achieve controlled drug release based on its pH responsiveness. The surface property and specific surface area are critical for drug loading, which are varied with crystallinity. Therefore, it is important to study the influences of crystallinity and surface modification of CaP on the drug loading capacity and drug release profile. 

Herein the drug loading capacity and drug release profile of CaP nanoparticles with different crystallinity were discussed in detail, which were controlled by adjusting reaction temperature. The surface modification was carried out by poly(acrylic acid) (PAA). The drug loading capacity and drug release profile of CaP nanoparticles to tetracycline hydro-chloride (TCH) was evaluated.

2. Materials and methods

2.1. Preparation and characterization of CaP

First, the aqueous solutions of Ca(NO3)2·4H2O with 0.02mol/L and (NH4)2HPO4 with 0.0334mol/l concentration were prepared. Next, 20ml (NH4)2HPO4 aqueous solution was introduced to 20 ml Ca(NO3)2·4H2O aqueous solution under strong stirring. The pH value was adjusted to 9-10 range using NH3·H2O. The reaction solution was maintained for 1h at different temperatures (25°C,37°C,60°C,80°C) while stirring. Then precipitation was separated and washed 3 times by centrifugation. For PAA stabilized nCaP, the washed precipitation was re-dispersed in water with addition of PAA (0.3mg/mL) and the stable nCaP suspension was obtained after ultrasound treatment.

Several instruments were required for the characterization of the prepared materials. In this experiment, powder X-ray diffractometer (XRD, D8 Advance, Germany) was used to determine the phase composition. Fourier transform infrared spectroscopy (FT−IR, Thermo Nicolet 6700, U.S.A.) to obtain information about the function groups and bonding forms. The scanning electron microscope (SEM, Zeiss Ultra Plus, Germany) and high resolution transmit electronic microscopy (HRTEM, JEM2100F, U.S.A.) were used to illustrate the morphologic information and to determine element content. BET surface area analyzer (BET, ASAP 2020M, U.S.A.) was employed to determine the surface area of the prepared material. The internalized TCH loaded nCaP in HepG2 cancer cells was observed using fluorescence microscope (970CRT, China).

2.2. Drug loading

The washed precipitate was re-dispersed in water and drug was added with resulting concentration of 0.05-1 mg/mL which was kept under stirring for 1h. The drug loaded nCaP was separated after removing the unloaded drug by centrifugation. The drug concentration of the supernant was determined using UV-spectrophotometer to calculate the amount of drug loaded on to nCaP. The loaded drug concentration (DC) was calculated by deducing the unloaded drug from the feeding drug. The drug loading efficiency (LE) and drug loading capacity (DLC) were calculated respectively by the following equations: DLC = 100%×M1/(M2+M3), LE =100%×M2/M1, where M1, M2 and M3 are the weight amounts of feeding drug, loaded drug and nCaP. The independent experiment was repeated three times and result was shown as mean ±SD (n=3).

2.3. Drug release

The drug release profile was measured in PBS (pH 7.4) and NaAc-HAc buffer solution (pH 5.0) at 37°C under dark conditions. The drug loaded nCaP was re-dispersed in 5mL buffer solution and put into dialysis tube (3500) with external 80mL buffer solution. At different time points 3mL external buffer solution was taken out for the determination of drug concentration and then re-poured into external buffer solution for the following experiment. The independent experiment was repeated three times and the result was shown as mean ±SD (n=3).

3. Results and discussion

3.1. Characterization of nCaP
Figure 1 illustrates XRD patterns and FT-IR spectra of nCaP synthesized at different temperatures. As shown in figure 1 (A), at 25°C sample was amorphous and no crystal was formed. At 37°C the weak diffraction peaks were observed which were assigned to hydroxyapatite (HAP) crystal. With the temperature increased to 60°C and 80°C the diffraction peaks were becoming sharper, indicating the crystallinity of HAP was enhanced. In addition, the crystal size was increased shown in figure 1 (B). The FT-IR spectra also provide some information about HAP as shown in figure 1 (C). The peaks at 1048cm⁻¹, 958cm⁻¹, 890cm⁻¹ and 605cm⁻¹ were attributed to characteristic vibrations of PO₄³⁻ group. The peak at 3570cm⁻¹ is due to stretching vibration of -OH group in the HAP crystal. The vibration of -OH group at 3570cm⁻¹ rose as temperature increased, indicating the increase of crystallinity.

Figure 2 shows morphology of nCaP prepared at different reaction temperatures (25°C, 37°C, 60°C and 80°C). The corresponding size results obtained by SEM observation are shown in figure 3. The particles obtained at 25°C had spherical shape with size of 39.44±1.81nm. When the temperature was increased to 37°C, particle’s size increased to 69.3±3.82nm. When temperature further increased to 60°C and 80°C, the particles were becoming needle like shape. Moreover, the particle sizes increased a lot, which were 29.58±1.28nm×121±3.25nm (60°C) and 29.07±1.35nm×175.59±5.18nm (80°C). The crystal growth is along the long axis direction, which is consistent with XRD results.
Figure 2. SEM images of the samples.

Figure 3. Crystallite sizes measured by SEM.

Table 1. BET areas and calculated diameter of nCaP.

| CaP   | 37°C  | 60°C  | 80°C  |
|-------|-------|-------|-------|
| specific area (m²/g) | 110.7241 | 119.3882 | 112.6077 |
| diameter (nm)* | 17.1592 | 15.9139 | 16.8727 |

*d=6/ρ·S (d=diameter, ρ=density, S=specific area)
Table 1 shows the specific surface area of nCaP. Indicating that nCaP synthesized at different
temperatures possessed similar specific surface area (110.7241 m²/g, 119.3882 m²/ and 112.6077
m²/g), corresponding to 17.1592nm, 15.1592 nm and 16.8727 nm in diameter respectively.

![Figure 4](image1.png)

**Figure 4.** Size distribution curve of PAA modified nCaP.

After ultrasound treatment with addition of PAA, nCaP can be stabilized and dispersed in aqueous
solution to form stable suspension. As shown in figure 4, the intensity averaged particle size was
92.9nm with a narrow size distribution (PDI:0.19). The agglomeration of nCaP was declined
dramatically after surface modification.

3.2. Drug loading property

![Figure 5](image2.png)

**Figure 5.** The DLC(A), LE(B) and DC(C)
of nCaP to TCH.

Figure 5 interprets the variation of DLC, LE and DC of nCaP to TCH synthesized at different
temperatures. The highest DLC values of nCaP are 9.44±0.21%, 8.99±0.1%, 8.97±0.07% and
5.67±0.07% for 25°C, 37°C, 60°C and 80°C corresponding to the highest LE values of 85.04±0.84%,
83.33±0.52%, 81.02±0.23%, 59.15±1.41% and the highest DC values of 0.1745±0.0045mg/mL, 0.1658±0.0020mg/mL, 0.1649±0.0016mg/mL, 0.1005±0.0013mg/mL respectively. There was no significant difference in drug loading property of samples synthesized between 25°C and 60°C. However, the DLC decreased a lot when temperature further increased to 80°C, showing that low crystallinity could improve the loading of TCH on nCaP. Samples have close specific surface area (table 1) and should possess similar adsorption ability to TCH. But results displayed different adsorption ability. This might be due to much more Ca²⁺ binding sites on the surface of nCaP with lower crystallinity, leading to higher adsorption to TCH via interaction between surface Ca²⁺ of nCaP and amide groups of TCH. In this experiment, the size and shape of nCaP particles generate little influence on drug loading.

Figure 6. DC, DLC and LE of nCaP (25°C) stabilized by PAA to TCH.

Figure 6 depicts the DC, DLC and LE of nCaP stabilized by PAA. Compared to the results of pure nCaP (25°C,Fig.6), loading of TCH on PAA stabilized nCaP was largely promoted, showing highest DLC and DC with 31.7±4.4% and 0.72±0.14 mg/mL. To conclude that PAA surface modification can enhance the adsorption ability of nCaP to TCH by inhibiting the agglomeration of nCaP.

Figure 7. FT-IR spectra of TCH and TCH loaded nCaP.

Figure 7 shows FT-IR spectra of TCH and TCH loaded nCaP. The characteristic vibrations of TCH were observed at 1700cm⁻¹-1200cm⁻¹ and 850cm⁻¹-650cm⁻¹, indicating TCH was successfully loaded...
on nCaP. Moreover, some peaks of TCH were moved after being adsorbed on nCaP, for example, 1672 cm⁻¹, 1616 cm⁻¹, 1528 cm⁻¹ and 1357 cm⁻¹. The bands at 1672 cm⁻¹ and 1528 cm⁻¹ were due to C=O stretching, NH₂ bending and C-N stretching, showing TCH was adsorbed on nCaP via the interaction between amide group of TCH and surface Ca²⁺ of nCaP.

![Fluorescence microscope observation of TCH loading nCaP (A) and intracellular TCH loading nCaP (B).](image)

**Figure 8.** Fluorescence microscope observation of TCH loading nCaP (A) and intracellular TCH loading nCaP (B).

As shown in fluorescence microscope observation (figure 8), the fluorescent signal was observed in HepG2 cancer cells, indicating TCH was transported into cells by nCaP.

### 3.3. Drug release profiles

![Drug release profiles of nCaP with different crystallinity and PAA stabilized nCaP.](image)

**Figure 9.** Drug release profiles of nCaP with different crystallinity and PAA stabilized nCaP.

Figure 9 shows the drug release profiles of nCaP with different crystallinity and PAA stabilized nCaP. Results showed that TCH was released slowly in PBS solution; however the release of TCH was enhanced much in NaAc-HAc buffer solution (pH 5.0). This indicated that nCaP was pH sensitive drug carrier. Furthermore, after 24h, the rate of released drug from nCaP synthesized at 25°C (72.95±9.35%) was higher than that of nCaP synthesized at 80°C (59.64±4.00%), showing that the drug release was influenced by the crystallinity of nCaP. Higher crystallinity corresponded to lower drug release rate. Additionally, PAA stabilized nCaP showed a little faster drug release profile than pure nCaP, indicating that PAA surface modification increased the drug release from nCaP due to its well dispersing property (lower agglomeration). However, PAA stabilized nCaP could result in higher released drug amount than pure nCaP due to its higher drug loading capacity. Therefore, the released drug concentration could be adjusted in a wide range to satisfy the requirement of drug dose for treating disease.
4. Conclusions
The experimental results show that CaP nanoparticles with different crystallinity could be obtained by adjusting reaction temperature. CaP nanoparticles with lower crystallinity exhibited higher drug loading capacity due to much more Ca$^{2+}$ adsorption sites on the surface. CaP nanoparticles could be stabilized and dispersed in aqueous solution by PAA, leading to enhanced drug loading capacity. TCH loaded CaP nanoparticles could be easily transported into cancer cells, indicating drug could be delivered into cells by CaP nanoparticles efficiently. Experimental data proved that nCaP were pH sensitive drug carrier, suggesting that nCaP could achieve the controlled drug release in intracellular acidic environment. Furthermore, nCaP showed different drug release profiles depending on crystallinity and surface modification. Increasing crystallinity could slow drug release rate, indicating the drug release profile could be adjusted by crystallinity. PAA stabilized nCaP showed faster drug release profile than pure nCaP, and led to higher released drug amount depending on its higher drug loading capacity. nCaP with adjustable drug loading and release properties is promising to become candidate as drug carrier for disease treatment.

Acknowledgment
This work was supported by the National Natural Science Foundation of China (51672206), the National Key Research and Development Program of China (2016YFB1101302), the Science and Technology Partnership Program, Ministry of Science and Technology of China (KY201602002), the Wuhan International Science and Technology Cooperation Project (2016030409020217), the Fundamental Research Funds for the Central Universities (2016-YS-014).

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