Synthesis of nanorod apatites with templates at critical micelle concentrations and in vitro evaluation of cytotoxicity and antimicrobial activity

Ssu-Meng Haung, Jian-Chih Chen, Kai-Chi Chang, Chia-Ling Ko, Dan-Jae Lin and Wen-Cheng Chen

1. Introduction

Apatite, which is mainly composed of calcium phosphates, has excellent biocompatibility, bioactivity, non-toxicity, and osteogenesis ability, and they are commonly used as bone substitute materials in medicine [1-5]. Stoichiometric hydroxyapatite (HA) crystals with the molecular formula Ca_{10}(PO_{4})_{6}(OH)_{2} possess properties similar to those of bones and a distinct morphology; they could be incorporated with various organic substances [6,7]. Depending on their crystal growth environment, apatite can have sheet-like, spherical, needlelike, and rod-like morphologies [8-10].

Apatite is generally deposited with intrinsic collagen fibers perpendicular to the surfaces of osteogenesis. Fully developed bundles of nanorod apatite were found between or within collagen fibers and organized in nematic symmetry, with rough cross-sectional widths of 33-65 nm and lengths of 100-1000 nm [11]. Synthetic methods to obtain nanorod apatites are in great demand because these materials mimic natural bone architecture, with numerous hydroxyl groups [12]. Nanorod apatites could be synthesized by various strategies, such as hydrothermal methods [13], surfactant-mediated crystalline growth [14], sol-gel synthesis through phase transition of crystalline β-Ca_{3}(PO_{4})_{2} [15], and ultrasonication with precipitation [16]. All of these strategies allow good control of the size and length-to-width aspect ratio of the resultant nanorod apatites. As precedent studies revealed the cytotoxic and antiproliferative effects of nanorod apatite [8,9,13-17], the biocompatibility of nanorod apatite obtained through various synthetic procedures appears to be strongly related to their surface conditions. However, the synthetic mechanisms through which these nanorods exert their cytotoxic effects remain unknown and must be verified by experiments. Apatite nanoparticles could achieve the substitution of Ca^{2+} ions with other metal ions, such as Cu^{2+}, Zn^{2+}, and Ag^{+}, or the substitution of PO_{4}^{3-} ions by CO_{3}^{2-} ions without changing its initial structure and properties [18,19]. Another feature is the drug-grafting nanorod apatite that could be taken up by the cells and transported to the intracellular compartments [20,21]. Accordingly, even though these nanoparticles are still
controversial in terms of biocompatibility, one of their substantial application nowadays is binding to many antibacterial agents, organic or inorganic, toward developing new compounds with high biocompatibility and antimicrobial properties [18–21].

The synthetic processes of apatite could be roughly classified as dry methods [22], wet processes [23–25], synthetic surfactant templates [26], and high-temperature processes [27]. The shape (e.g. sphere, cylinder, or lamella) and size of apatite differ in accordance with the synthesis method employed. The surfactant-based process could inhibit excessive agglomeration of nanoparticles because the morphology control of nanorod apatite could be achieved by restricting crystal growth. Although many effective surfactant template-assisted methods to synthesize apatite have been widely reported under hydrothermal conditions [28–30], the method used to prepare apatite in the present study was not on the same synthesis as that of surfactants at critical micelle concentrations (CMCs). In the present experiment, four types of surfactants were used as follows. First, a cationic surfactant cetyltrimethylammonium bromide (CTAB) was ionized in an aqueous solution to produce a positively charged monomer. This cationic surfactant could self-assemble into micelles and after reaching the concentration higher than CMC, it could be used as a surface nucleation site for PO₄³⁻ and Ca²⁺ ions when its micelle size reaches 0.7–2.5 nm. The nucleation reaction could lead to the growth of nanoscale HA (nHA) crystals with uniform morphology and size via low-temperature hydrothermal method and precipitation [31,32]. Second, the anionic surfactant sodium dodecyl sulfate (SDS) is commonly used in detergents, foaming agents, and skincare products, and it could be used to synthesize nanorod apatite [33]. Third, Pluronic F-127 is the trade name of poloxamer 407 with a specific triblock copolymer. Poloxamer 407 is a hydrophilic nonionic surfactant of the general class of copolymers known as poloxamers. The hydrophobic polypropylene oxide (PPO) group in the middle of the surfactant molecule links two hydrophilic polyethylene oxide (PEO) groups. Pluronic-based micelles with an estimated diameter of 30–50 nm are spontaneously formed at a concentration equal to or higher than the CMC [34]. Fourth, the zwitterionic surfactant cocamidopropyl betaine (CAPB) is an amphoteric synthetic detergent that is increasingly used in cosmetics and personal hygiene products [35]. The use of nontoxic reagents during manufacturing is necessary to decrease the toxicity and increase the cell viability of the resultant products in vivo [36].

As apatite nanoparticles synthesized without the use of surfactants showed a higher tendency to aggregate than those synthesized with the use of surfactants [37], four types of surfactants, including cationic, anionic, nonionic, and zwitterionic surfactants, were used as templates in the present study. The addition of controlled CMCs of the surfactants in nanorod apatite synthesized as templates through wet-chemical processes under hydrothermal conditions was investigated. The bacteriostatic properties and biocompatibility of the synthesized nanorod apatite were also characterized and compared.

2. Materials and methods

2.1. Materials

CTAB was purchased from Ferak (01739, Berlin, Germany). SDS (≥ 99.0 purity) and Pluronic F-127 (P2443, M_w = 12.6 kg mol⁻¹) with the triblock copolymer PEO–PPO–PEO was purchased from Sigma–Aldrich. CAPB was obtained from the First Chemical Group (20,130,312,042). The different chemical structures of the surfactants are shown in Figure 1a, and the different groups of nanorod apatite synthesized using various surfactant-templated precipitation and their surfactant concentrations at the CMCs are listed in Table 1.

2.2. Synthesis of nanorod apatite

Five groups of nanorod apatite were synthesized by dissolving 0.215 g of calcium nitrate (PanReac, C0150) and a specific amount of each surfactant (Table 1) in 90 mL of deionized water. Then, 0.713 g of diammonium phosphate (HSE Pure Chemicals, KHA1050) and 2.647 g of sodium citrate (PanReac, 0001113173) were added to 70 mL of deionized water. The ionic solutions were vigorously stirred with a magnetic bar for 30 min. The pH of each system was carefully adjusted to 7.0 within 5 min, and the different solutions of surfactants, as indicated in Table 1, at their CMCs were reacted under hydrothermal conditions at 180°C for 12 h. The resulting water vapor pressure was increased to approximately 190 psi (lb/in²). The fine precipitates formed were centrifuged, washed with deionized water and ethanol, dried at 50°C for 12 h, and then stored in a dry box to avoid the effects of humidity before further characterization. The yield ratio of nanorod apatite obtained from hydrothermal synthesis relative to the theoretical yield (w/w) was approximately 30% in all groups.

2.3. Physicochemical analysis

2.3.1. Fourier-transform infrared spectroscopy (FTIR)

The synthesized nanorod apatite was evaluated via FTIR (Nicolet 6700, Thermo Fisher Scientific, Waltham,
Scanning was conducted in the wavenumber range of 650–4000 cm$^{-1}$ to determine the functional groups present in the products.

2.3.2. X-ray diffraction (XRD) analysis
The crystal phases of the obtained apatite were evaluated using XRD (XRD-6000, Shimadzu, Japan) with CuKα radiation at 30 kV and 20 mA. The scan rate was 2°/min, and scanning was conducted in the 2θ range of 20°–60°.

2.3.3. Transmission electron microscopy (TEM)
The morphologies of the nanorod apatite were observed via TEM (JEM-3010, JEOL, Ltd., Japan) at 200 kV.
2.4. Antibacterial abilities

*Staphylococcus aureus* (ATCC No.: 25,923) and *Escherichia coli* (ATCC No.: 10,798) were cultured in Luria–Bertani broth. The bacterial suspensions were diluted to achieve an optical density at 595 nm (OD$_{595}$) between cell numbers of 0.2–1 × 10$^7$ cells/mL of bacteria, which was confirmed with an ELISA reader. The bacterial suspensions of *S. aureus* and *E. coli* were subsequently diluted to achieve an OD$_{595}$ value of 0.2. Then, on the basis of the effectiveness and difference of multiple pre-trial attempts, 2 mL of each suspension was placed in a sterilized tube containing 0.03 g of the as-prepared

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**Figure 1.** (a) Chemical structures of different surfactants used to synthesize nanorod apatite: (a) cetyltrimethylammonium bromide (CTAB), a cationic surfactant; (b) sodium dodecyl sulfate (SDS), an anionic surfactant; (c) pluronic F-127, a nonionic surfactant featuring a polyethylene oxide (PEO)–polypropylene oxide (PPO)–polyethylene oxide tri-block copolymer; and (d) cocamidopropyl betaine (CAPB), a zwitterionic surfactant. (b) Schematic of the surfactant and ion distributions under different surfactant concentrations and reaction conditions. The picture on the left shows the surfactant whose concentration does not reach the CMC, and the middle and right pictures show the ion changes in the surfactant concentration up to the CMC. The right picture shows the nanorod apatite preferentially oriented toward the c-axis, leading to the development of a(b)-planes featuring positively charged Ca$^{2+}$ ions and a c-plane featuring negatively charged PO$_4^{3-}$ and OH$^-$.  

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**Figure 1.** Continued.
nanorod apatite, and changes in bacterial growth were observed during incubation at 37°C for 1–14 days. A 100 μL culture medium suspension was prepared, and the number of bacteria was determined using an ELISA reader by measuring the OD_{595} value (n = 3).

2.5. Cell viability

The L929 cell line was derived from newborn mouse fibroblasts and provided by the National Institute of Health in Taiwan. This cell line was used to test the cytotoxicity of the as-synthesized nanorod apatite. The solute samples were prepared at a sample-to-medium ratio of 1 g/5 mL; here, the apatite samples were immersed in the culture medium for 24 h. In accordance with the cytocompatibility indicated in the ISO 10,993–5:2009 guideline, the cell viability was determined using the extracts of different groups to culture the L929 cells. Assay was carried out in triplicate (n = 3). The cells were seeded in a 96-well culture plate at a density of 1 × 10^4 cells per well in minimum essential medium alpha (Gibco, Invitrogen Taiwan, Ltd., MD) containing 10% horse serum culture medium (Biological Industries, Haemek, Israel) and allowed to attach overnight. The culture medium was removed, and the nanorod apatite extracts were added to the wells. Culture was performed for another 24 h. The presence of metabolically active cells was measured by reducing tetrazolium salt (Cell Proliferation Assay, Biological Industries, Israel) to formazan. Thereafter, the viability of the L929 cells was determined at OD_{492} by using an ELISA plate reader (EZ Read 400, Biochrom, Cambridge, UK). The morphological characteristics of the cells were observed under an optical microscope (IX71, Olympus, Japan).

2.6. Statistical analysis

The mean length, diameter, and aspect ratio of the apatite samples (n = 40) were analyzed using a two-sample t-test and ANOVA for statistical analysis (IBM SPSS Statistics 20 software).

3. Results and discussion

3.1. Preparation mechanism for nanorod apatites

The chemical structures of the various surfactants are shown in Figure 1a, and schematics of the relevant hexagonal growth mechanisms of the different nanorod apatites synthesized from different surfactants at their CMCs are shown in Figure 1b. The apatite in nucleated embryos specifically and preferentially grows into hexagonal crystals, especially under hydrothermal conditions [23–25,28–30,38]. The micellar space limitations and ionic effects in the solution appeared to affect the shapes (a(b)-plane faces) and growth directions (c-axis) of the resulting crystals [31–35]. Nanorod apatite is preferentially oriented toward the c-axis, thus leading to the development of a(b)-planes featuring positively charged Ca^{2+} ions and c-planes featuring negatively charged PO_4^{3−} and OH^− [38,39]. Accordingly, nanorod apatite surfaces exhibit anisotropic charged characteristics, leading to anisotropic adsorption profiles for template molecules (Figure 1). Micelle-templated precipitations were conducted in the presence of surfactant micelles as a nanostructured template. The shape, size, and charges of a micelle could be utilized for preparing nanorod apatites with regulated length-to-width aspect ratio. Therefore, when anionic SDS was used as the surfactant, excess PO_4^{3−} ions could not attach to the micelles easily due to the increased electrostatic repulsion charges, leading to a large length-to-width aspect ratio [37–41].

3.2. Nanorod apatite growth and FTIR spectral analysis

The functional groups of the synthesized nanorod apatite are shown in Figure 2. The C = O stretching vibrations of CO_2 were observed at 1442 and 1663 cm⁻¹ in each spectrum. The absorption bands at 3571 and 632 cm⁻¹ were attributed to OH^−, and the bending bands at 563 and 603 cm⁻¹ were attributed to the O-P-O bending mode of PO_4^{3−} in apatite. The band at 964 cm⁻¹ corresponded to the O-P-O symmetric stretching vibrations, while those at 1028 and 1093 cm⁻¹ corresponded to its O-P-O asymmetric stretching vibrations of PO_4^{3−}. The characteristic peaks of the functional groups of zwitterionic CAPB and anionic SDS were not detected in the corresponding spectra. By contrast, the characteristic bands of the cation CATB template-assisted T-nHA and anion SDS template-assisted S-nHA groups, including peaks at 2853 and 2927 cm⁻¹, were due to the symmetric and asymmetric stretching vibrations of −CH₂, respectively.
Figure 2. FTIR spectra of nanorod apatite prepared using different surfactant templates via hydrothermal synthesis at 180°C for 12 h.

Figure 3. Diffraction patterns of nanorod apatite prepared using different surfactants at their CMCs via hydrothermal synthesis.
3.3. XRD phase identification

The phase analyses of the nanorod apatite are shown in Figure 3. Comparison of the data obtained with the corresponding Joint Committee on Powder Diffraction Standards cards showed that each group features the main characteristic peaks of HA (JCPD 24–0033). Moreover, the characteristic peaks of calcium metaphosphate (CaP₂O₆; JCPD 150,184) and dicalcium phosphate dihydrate (DCPD, CaHPO₄·2H₂O; JCPD 01–0395) could be observed in the S-nHA and P-nHA groups, respectively. Strong negative-end groups of SDS (O-SO₃⁻) and CAPB (COO⁻) may enhance the reaction of these template molecules with Ca²⁺ ions during the formation of apatite with the various Ca/P nanoparticles of DCPA and DCPD [41]. The ordered growth of apatite generally occurs via the phase transition of calcium phosphate precursors, such as octacalcium phosphate (Ca₈[PO₄]₆(OH)₂·5H₂O), DCPA, and DCPD [42]. The FTIR and XRD results indicated that a large amount of apatite and small amounts of DCPD and CaP₂O₆ were formed when synthesis was carried out using (NH₄)₂HPO₄. The characteristic peaks of DCPD and CaP₂O₆ were metastable, and they were produced by the incomplete solid-state reaction during hydrothermal preparation.

3.4. TEM morphological observation

The precipitate dimensions in Figure 4 illustrate the effect of the surfactants at their CMCs on the size of the nanorod apatite. All of the images showed that the nanorods have a large length-to-width aspect ratio and the shapes of the products are strongly associated with the surfactant used for their synthesis. In particular, the surfactant effectively restricted and unified the nucleus growth of the synthesized apatite during precipitation. The XRD, FTIR, and TEM results (Figures 2–4) confirmed that nanorod apatite was formed. Growth appeared to occur via a layering mechanism, and lengthened growth times resulted in the development of planar crystal faces and rod-shaped morphologies at the initial growth stage [13–16].

The average dimensional scales and length-to-width aspect ratios of the nanorod apatite are summarized in Table 2. The length of the nanorod apatite in each group ranged from 50 nm to 80 nm, and their widths ranged from 12 nm to 21 nm in diameter. The nanorod apatites synthesized with the T-nHA and F-nHA templates were more uniform than those obtained from other surfactants, and the nanorods obtained from P-nHA were relatively smaller than the other products. An important factor influencing the properties of CAPB in an aqueous solution is its strong interaction with anions [43]. Zwitterionic surfactants, comprising positive and negative groups in their headgroups, are essentially electro-neutral as monomers, but their micelles preferentially uptake anions similar to cationic surfactant micelles [44].

Nanoparticles are tiny microscopic particles with at least one dimension in the nanometer scale (usually

Figure 4. Morphologies of nanorodapatites prepared using different surfactants at their CMCs via hydrothermal synthesis.
Table 2. Average length, width, and length-to-width aspect ratio of nanorod apatite prepared using different surfactants under hydrothermal conditions (n = 40).

| Variable sample | Length (nm) | By variable group | P-value in a two group t-test | Diameter (nm) | By variable group | P-value in a two group t-test | Length-to-width aspect ratio |
|-----------------|-------------|-------------------|-------------------------------|---------------|-------------------|-------------------------------|-----------------------------|
| nHA/control     | 72.98 ± 9.81| P-nHA             | p < 0.05                      | 19.82 ± 10.47 | P-nHA             | p < 0.05                      | 4.18 ± 1.80                 |
| T-nHA           | 75.76 ± 30.04| P-nHA             | p < 0.05                      | 20.70 ± 4.86  | S-nHA             | p < 0.05                      | 3.70 ± 1.35                 |
| S-nHA           | 68.28 ± 22.30| P-nHA             | p < 0.05                      | 15.95 ± 3.20  | T-nHA             | p < 0.05                      | 4.47 ± 1.87                 |
| F-nHA           | 81.31 ± 24.42| P-nHA             | p < 0.05                      | 21.40 ± 7.67  | F-nHA             | p < 0.05                      | 3.92 ± 0.96                 |
| P-nHA           | 50.49 ± 17.40| nHA/control       | p < 0.05                      | 12.55 ± 3.01  | nHA/control       | p < 0.05                      | 4.24 ± 1.84                 |

100 nm or less) [45]. In emulsion with micelles formed when the surfactant concentration was over the CMCs, the controlled precipitation in a restricted space of nanostructured templates, a significant refined nanorod apatite could be achieved by restricting crystal growth.

3.5. Quantitative test of antibacterial ability

The quantitative results of the bacteriostatic effect of each surfactant against S. aureus and E. coli are illustrated in Figure 5 to achieve an enhanced understanding of the effect of surfactant residues on the antibacterial properties of the resultant nanorod apatite. The sterilization of the groups was confirmed by comparing their performance with that of the negative control group. The OD values of CTAB, SDS, and CAPB were similar to that of the positive group of dimethyl sulfoxide. Thus, these surfactants at their CMCs demonstrated obvious antibacterial activity against S. aureus and E. coli. The OD value of Pluronic F-127 was higher than that of the other groups but remained lower than that of the negative control group. Thus, this surfactant may be concluded to have moderate antibacterial activity compared with the other surfactants.

The antibacterial properties of the synthesized nanorod apatite against S. aureus and E. coli are shown in Figure 6. Each group of nanorod apatite showed antibacterial activity against S. aureus and E. coli on day 1, and quantitative measurements of S. aureus indicated that groups synthesized with surfactant templates have greater antibacterial properties than those synthesized without templates (Figures 2 and 6a). However, except for the T-nHA group, all of the apatite samples demonstrated relatively short-lived antibacterial ability. Indeed, most of the groups lost their antibacterial ability as the incubation time increased. The antibacterial ability of the nanorod apatite against E. coli was measured (Figure 6b), and the T-nHA and P-nHA groups showed obvious antibacterial effects compared with the nHA group (p < 0.05) on day 1. However, on days 2 and 3, only the T-nHA group continued to demonstrate antibacterial activity against S. aureus and E. coli. The antibacterial ability of the T-nHA group was observed until day 14 of culture (Figure 6c). T-nHA revealed stable antibacterial effects against S. aureus up to day 14. By comparison, the antibacterial effect of this group against E. coli was
initially strong and then declined with increasing contact time until day 14 \((n = 3)\). This finding illustrated that the inhibitory ability of the nanorod apatite may be lost with increasing contact time. Comparison of Figure 6c with Figure 6a and 6b indicated that the nanorods may only temporarily inhibit the proliferation of bacteria. The surface effects of the nanorods may increasingly influence their antibacterial effects over time.

The mechanisms through which the different samples exert their antibacterial activity are complex and may include the interplay of the nanosize, shape, length-to-width aspect ratio, and the ions or molecules attracted or bonded to the surface charge characteristics of apatites [46]. Nanorod apatite contains cations of \(Ca^{2+}\) site and anions of \(PO_4^{3-}\) in its structure, and it has high affinity for organic molecules (Figure 1b). When considering the obvious residual effects of cationic/CTAB and \(NH_4^+\) cations on the surfaces of nanorod apatites (Figure 2), the appearance of the antibacterial activity of cationic/CTAB in T-nHA was higher than the cation of \(NH_4^+\) in S-nHA, F-nHA and P-nHA due to that CTAB has the ability to inducing superoxide stress in bacteria and led bacterial cells to a generation state of superoxide and hydrogen peroxide and therefore play a key action of antibacterial surfactant [38,47,48].

### 3.6. Cytotoxicity quantitative and qualitative testing

The control group exhibited 100% cell viability after 24 h of exposure to the apatite extract (Figure 7a). In accordance with ISO 10,993–5: 2009(E), cytotoxicity was considered a cytotoxic effect due the reduction in cell viability by more than 30% of L929 cells after 24 h in comparison with the control group [49]. Accordingly, no significant difference was observed among the negative group of high-density polyethylene (HDPE), control, and nHA/control groups \((p > 0.05)\). Furthermore, only the F-nHA group showed no toxic effect on L929 cells. The cell viability of this group was significantly different compared with that of the T-nHA, S-nHA, and P-nHA groups \((p < 0.05)\). This result indicated that F-nHA does not remarkably inhibit cell growth. As shown in Figure 5, Pluronic F-127 showed weaker antibacterial effects and less cytotoxicity than the other surfactants. Figure 7b consistently indicates that L929 cells
have a spindle-like morphology. Thus, the cells attached to the samples of the negative control, HDPE, nHA/control, and F-nHA groups showed normal proliferation and no signs of cytotoxicity.

The specific influence of various apatite shapes on biocompatibility is unclear. Precedent studies demonstrated the shape-dependent effects of nHA (needles, plates, spheres, and rods) on cytotoxicity [50]. They revealed that needle- and plate-shaped nHA induce the most significant cell-specific cytotoxicity because these nanorod apatites could enhance the production of reactive oxygen species and promote DNA damage measured by γ-H2AX phosphorylation [17]. In the present study, the cell morphology of the F-nHA group was similar to that of the nHA/control group; thus, nanorods synthesized with F-nHA as the surfactant template were not cytotoxic to L929 cells. The results generally indicated that residual surfactants may remain on the nanorod apatite despite rinsing with a large amount of deionized water following synthesis. This factor, in combination with the nanorod apatite morphology, may contribute to the cytotoxicity of the synthesized materials.

4. Conclusion

Nanorod apatites successfully prepared using a simple technique through precipitation using surfactants at their CMCs as synthetic templates under hydrothermal conditions were investigated. The use of different template groups resulted in differences in length-to-width
aspect ratio, antibacterial ability, and biocompatibility. The P-nHA group exhibited the smallest average length and the diameter of homogeneous nanorod apatite, suggesting that the binding of large amounts of residual NH₄⁺ on the apatite surface to the sites of negatively charged PO₄³⁻ ions restricts nanorod apatite growth. Quantitative measurements of the bacteriostatic test demonstrated the combined antibacterial effects of the surfactants and residual cations against S. aureus and E. coli. Only the nHA/control and F-nHA groups showed no cytotoxicity toward L929 cells. Among the samples synthesized, the nanorod apatite of F-nHA obtained with nonionic surfactant as the template showed a short-term antibacterial effect on S. aureus on day 1 and no cytotoxicity. Taken together the results demonstrate a high antimicrobial activity of T-nHA, however, it could be used alone in medical treatment due to the induced cytotoxicity. Therefore, if the focus is on nanorod apatite with good antibacterial effect of T-nHA synthesized in the presence of cationic/CTAB, it must be compounded with other bone repair materials to control the release and thus reduce its cytotoxicity. These samples may also represent a candidate material that could be used in bone restorative applications in the future.

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Disclosure of potential conflicts of interest

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