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“Cleaning” the Surface of Hydroxyapatite Nanorods by a Reaction-Dissolution Approach

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Abstract

Synthetic nanoparticles are always terminated with coating molecules, which are often cytotoxic and not desired in biomedicine. Here we propose a novel reaction-dissolution approach to remove the cytotoxic coating molecules. A two-component solution is added to the nanoparticle solution; one component reacts with the coating molecules to form a salt whereas another is a solvent for dissolving and thus removing the salt. As a proof of concept, this work uses a NaOH-ethanol solution to remove the cytotoxic linoleic acid molecules coated on the hydroxyapatite nanorods (HAP-NRs). The removal of the coating molecules not only significantly improves the biocompatibility of HAP-NRs but also enables their oriented attachment into tightly-bound superstructures, which mimic the organized HAP crystals in bone and enamel and can promote the osteogenic differentiation of mesenchymal stem cells. Our reaction-dissolution approach can be extended to the surface “cleaning” of other nanomaterials.

Introduction

Bone and teeth are hierarchically structured composites containing rod-like nanocrystalline hydroxyapatite (HAP) as their main inorganic component.¹⁻³ Synthetic HAP has been widely used as a biomaterial for bone regeneration and other medical applications.³ Recently, rod-like HAP nanocrystals (also called HAP nanorods or HAP-NRs) have been chemically synthesized.^{3, 4} Compared with regular synthetic HAP, HAP-NRs show high bioactivity,⁵ improved mechanical properties,³ and morphological similarity to the HAP present in bone and teeth,⁶⁻⁹ suggesting their use as an ideal biomaterial for building artificial bone and dental composites.^{3, 10} High-quality HAP-NRs with uniform sizes and

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tunable aspect ratios can be synthesized by using surfactant molecules^{4, 11-17} as structure-directing agents. However, the resultant HAP-NRs are coated with toxic surfactant molecules.¹⁸ Therefore, they may cause adverse responses *in vivo*, and thus unsuitable for biomedical applications.¹⁹ To overcome such limitation, bio-friendly HAP-NRs have been synthesized using biocompatible molecules, such as amino acids,²⁰ urea,²¹ sodium citrate,²² and N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride.²³ But these methods can neither synthesize monodisperse HAP-NRs nor tune their aspect ratios. Moreover, the surface coatings, including both surfactant and biocompatible molecules, may mask the pristine HAP surface and thus diminish the favorable properties of HAP desired in biomedical applications such as bone regeneration. Therefore, a new approach for the synthesis of high-quality HAP-NRs without surface coating is needed to facilitate their use for bone regeneration.

Here we report a novel reaction-dissolution approach to unmask surfactant-coated high-quality HAP-NRs (Scheme 1). The general concept of this approach is to use a solution made of two components to remove the surfactants from the surface of the HAP-NRs. One component will react with the surface coating molecules and another will then immediately dissolve the resultant reaction products. As a proof of concept, we first synthesized linoleic acid-coated high-quality HAP-NRs following a reported method.⁴ Because NaOH can react with fatty acids to form sodium salts²⁴ that can be rapidly dissolved by ethanol,²⁵ we allow the linoleic acid-coated HAP-NRs to interact with a NaOH-ethanol solvent, resulting in the removal of the surface-coating molecules from HAP-NRs. Since surfactant molecules such as fatty acids are common coating molecules for nanoparticles used in medicine, our approach can be extended to the removal of coating molecules from other nanoparticles. More importantly, the successful removal of coating molecules from HAP-NRs triggers the oriented attachment of HAP-NRs into tightly bound parallel-aligned biomimetic superstructures by a simple water-evaporation process. The resultant HAP-NRs as well as their superstructures are surfactant-free and water-dispersible. When incubated with mesenchymal stem cells (MSCs), a commonly used cell source in bone regeneration,²⁶⁻²⁸ the HAP-NRs superstructures showed the ability to not only support the adhesion and proliferation but also promote the osteogenic differentiation of resident MSCs.

Experimental

Synthesis of HAP-NRs

Briefly, 0.5 g octadecylamine, 4 mL linoleic acid and 16 mL ethanol were mixed under agitation. 7.5 mL $\text{Ca}(\text{NO}_3)_2$ solution (0.5 g in 7.5 mL water) was then added for ion-exchange process, followed by adding 7.5 mL Na_3PO_4 solution (0.3 g in 7.5 mL water) for reaction. The system was sealed and hydrothermally treated at 90 °C in an oven for more than 10 h. The collected HAP-NRs were washed with ethanol for 3 times to remove free surfactants. HAP-NRs with different aspect ratios can also be prepared by varying the ratio between linoleic acid and ethanol or changing the reaction temperature.

Surfactant removal from HAP-NRs by reaction-dissolution method

NaOH-ethanol solution, which was prepared by mixing 480 mL of ethanol and 20 mL of 10 M NaOH in water, was used to suspend HAP-NR pellet. In a sealed bottle, the suspension was agitated at 90 °C for 24 h to remove surfactants from the HAP-NRs. Then the mixture was centrifuged at 10,000 g for 10 min to collect the HAP-NRs as a pellet. Such treatment was repeated 3 times to completely remove surfactant molecules. The final HAP-NR pellet was re-suspended in 100 mL of water for further use.

FTIR characterization

HAP-NRs were washed with water and hexane and dried in an oven at 90 °C for 8 h. About 20 mg HAP-NRs were mixed with 200 mg pure KBr powder and the mixture was then grounded and pressed in a hydraulic press, resulting in a 13 mm sample pellet. The FT-IR analysis was conducted on a Nexus FT-IR Spectrometer from Nicolet.

Formation of HAP-NR superstructures

The surfactant-free HAP-NR aqueous solution was allowed for water-evaporation at 37 °C for one week and the HAP-NRs were slowly self-assembled into HAP-NR superstructures. The assembled HAP-NR superstructures were analyzed under TEM and SEM.

MSC proliferation on HAP-NRs superstructures by MTT assay

Cell culture plates were coated with a layer of poly-lysine. Then aqueous solutions containing different amounts of surfactant-free HAP-NRs superstructures or individual HAP-NRs (0 µg, 3.75 µg, 7.5 µg, 15 µg, and 30 µg) were directly deposited on the modified cell culture plates for 30 min. Thus, films made of superstructures or randomly oriented HAP-NRs were prepared. MSCs were then seeded at a density of 4×10^3 cells/well in 96-well plates in standard growth media (200 µl/well) for 24, 48 and 96 h, respectively. In a control experiment where MSCs were cultured with free HAP-NRs, MSCs were cultured on the cell culture plates pre-coated with a layer of poly-lysine at a density of 4×10^3 cells/well in 96-well plates in standard growth media containing free HAP-NRs (0 µg, 3.75 µg, 7.5 µg, 15 µg, and 30 µg) for 24, 48 and 96 h, respectively. Cellular morphologies were imaged under inverted light microscopy. Cell proliferation was evaluated by a standard 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, where MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. MTT (5 mg/ml, 20 µl/well) was added to the cell culture for an additional 4 h. The cell culture media were then discarded followed by the addition of dimethyl sulfoxide (DMSO, 150 µl/well) and agitated for 30 s to dissolve the crystal completely. The values of optical density (OD) were measured at 490 nm on a plate reader.

Study of MSC differentiation on HAP-NRs superstructures by Immunofluorescence staining

MSCs were seeded on the film of HAP-NRs superstructures or randomly oriented HAP-NRs at a density of 4×10^3 cells/well in osteogenic differentiation media for 2 weeks. In comparison, MSCs were also cultured at a density of 4×10^3 cells/well in osteogenic differentiation media containing free HAP-NRs (15 µg/well) for 2 weeks. The cells were

washed and fixed with 4% paraformaldehyde at 4°C for 30 min, permeabilized using 0.3% Triton X-100 for 5 min and then blocked with 5% goat serum solution for 1 h at room temperature. After blocking, the cells were incubated overnight at 4°C with the primary antibodies targeting osteopontin (OPN). Secondary antibody labeled by TRITC was used for labeling OPN at 1:1000 dilutions in a blocking buffer for 1 h at room temperature. Alexa Fluor 488 phalloidin (1:400 in PBS) and DAPI (4, 6-diamidino-2-phenylindole) were used to stain the actin filaments and nuclei, respectively.

Results and Discussion

Liquid-solid-solution (LSS) method^{4, 29} was used to synthesize HAP-NRs coated with a layer of linoleic acid surfactant molecules with their hydrophilic heads (carboxylate groups) binding with HAP-NRs and hydrophobic tails (long carbon chains) exposed to solvents. Hence, the as-synthesized HAP-NRs (Figure 1) can be dissolved in organic solvents, such as hexane, and form a clear solution (Figure 1c). Transmission electron microscopy (TEM) image shows that the as-synthesized HAP-NRs are uniform in size with a width of ~8 nm and a length of ~170 nm (Figure 1a). The electron diffraction pattern (Figure 1b) matches the typical electron diffraction pattern of polycrystalline HAP,^{30, 31} indicating these synthesized HAP-NRs are crystalline.

To remove the surfactants from HAP-NRs, we performed the reaction-dissolution approach to treat the HAP-NRs in a NaOH-ethanol solution (95% ethanol and 5% 10 M NaOH in water) at 90 °C for 24 h. The successful removal of the surfactants was confirmed by comparing Raman and Fourier transform infrared (FT-IR) spectra of treated HAP-NRs with those of as-synthesized surfactant-coated HAP-NRs (Figure 2). The Raman spectrum of the as-synthesized HAP-NRs (Figure 2, spectrum a) shows typical peaks from both HAP (435 cm⁻¹, 590 cm⁻¹, 961 cm⁻¹, and 1083 cm⁻¹)³² and HAP-surface-coating surfactants (linoleic acid) (1260 cm⁻¹, 1300 cm⁻¹, 1440 cm⁻¹, 1650 cm⁻¹, 2900 cm⁻¹, and 3021 cm⁻¹).³³ In contrast, the Raman spectrum of the treated HAP-NRs (Figure 2, spectrum b) only shows typical peaks from HAP crystals. Similarly, the FT-IR spectrum of the as-synthesized HAP-NRs (Figure S1 spectrum d) shows typical peaks from linoleic acid surfactants (1465 cm⁻¹, 1559 cm⁻¹, 2851 cm⁻¹, and 2923 cm⁻¹)¹¹ while that of the treated HAP-NRs (Figure S1, spectrum a) doesn't. Peaks at the wavenumbers of 1423 cm⁻¹, 1462 cm⁻¹, and 1645 cm⁻¹ in spectrum d are attributed to carbonate impurities in the HAP structure. In fact, the HAP in natural bone contains carbonate ions,^{34, 35} indicating that our HAP NRs mimic the chemical composition of natural bone mineral. Therefore, both Raman and FT-IR spectra confirm that surfactants were completely removed from the as-synthesized HAP-NRs by the reaction-dissolution treatment. The resultant surfactant-free HAP-NRs could be dispersed in water and formed a stable solution, indicating their high dispersion stability in water. TEM imaging shows that the surfactant-free HAP-NRs retained their original morphology and superior monodispersity (Figure 1d) during the treatment. Therefore, the removal of surfactants by the treatment of a NaOH-ethanol solvent does not introduce obvious changes in the size and morphology of HAP-NRs.

In a control experiment, surfactant-coated HAP-NRs were treated with NaOH solution only (0.5M in water) and ethanol solution only (95% ethanol and 5% water) at 90 °C,

respectively. FT-IR analysis of the resultant products (Figure S1, spectra b&c) show that NaOH or ethanol alone cannot completely remove the surfactants from HAP-NRs. The leftover surfactants could still be found from the treated HAP-NRs. These results indicate that both NaOH and ethanol are indispensable for removing surfactants in the reaction-dissolution process.

Fatty acids have also been widely used for the synthesis of high-quality hydrophobic nanoparticles in organic solvent.^{29, 36} However, the hydrophobic coating molecules inhibits their potential biomedical applications. By applying the reaction-dissolution approach, we transferred hydrophobic iron oxide nanoparticles (6 nm) in hexane (Figure S2a) into aqueous phase (Figure S2b) by removing the coating molecule, oleic acid. Fig. S2 shows oleic acid-coated iron oxide nanoparticles are soluble in hexane (Figure S2a) and these nanoparticles became water-soluble after our reaction-dissolution treatment (Figure S2b). This application suggests that this reaction-dissolution approach is a general strategy.

Calcination at a high temperature ($>480\text{ }^{\circ}\text{C}$) is another strategy for removing surfactant molecules from HAP nanoparticles.¹³ $500\text{ }^{\circ}\text{C}$ was reported as the best calcination temperature for this purpose, at which a narrow size distribution of treated HAP nanoparticles could be achieved.³⁷ The FT-IR spectrum of the treated HAP-NRs demonstrated the complete removal of surfactant molecules during calcination at $500\text{ }^{\circ}\text{C}$ for 4 h (Figure S1, spectrum e). However, the TEM image shows that heating at high temperature also greatly changed the morphology and size distribution of synthesized HAP-NRs (Figure S3). On the contrary, our reaction-dissolution method could not only remove surfactant molecules (Figure S1 spectrum d) but also retain the shape and size distribution of HAP-NRs (Figure 1d).

TEM image of the as-synthesized HAP-NRs shows that HAP-NRs tend to parallel align into a cluster (Figure 1a). However, the HAP-NRs in the cluster are separated due to the presence of surface coating molecules. Thus it can be expected that once the surface coating molecules are removed by our reaction-dissolution approach, the HAP-NRs may be attached into tightly-bound superstructures, where HAP-NRs are still parallel-aligned, through a well-known oriented attachment mechanism.^{38, 39} Indeed, after we apply a simple water-evaporation treatment to the solutions of HAP-NRs with surface coating molecules removed, the superstructures are formed (Figure 1f&g). The resultant structures mimic the organization of HAP nanorods in bone and enamel.^{40, 41}

We proceeded to test the cytotoxicity of the high-quality surfactant-free HAP-NRs superstructures against MSCs, isolated from rats following our published protocols.⁴²⁻⁴⁴ We found that the HAP-NR superstructure film could support the adhesion and spreading of MSCs (Figure 3). The MTT assay results indicate that the resident MSCs can both proliferate on the films of HAP-NR superstructures (Figure S4a), randomly oriented HAP-NRs (Figure S4b) and free-standing HAP-NRs (Figure S4c). They showed that surfactant-free HAP-NRs within $0.15\text{ }\mu\text{g/ml}$ are not toxic to MSCs. Moreover, the free HAP-NRs superstructure tended to attach to and aggregate on the surface of MSCs (Figure 3c), indicating an interaction between MSCs and HAP-NRs during culturing and possible internalization of HAP-NRs in MSCs. However, MSCs couldn't adhere, spread, and

proliferate on the films made of as-synthesized untreated HAP-NRs (Figure S5a) or in the media containing free as-synthesized HAP-NRs (Figure S5c). The MTT results (Figure S5b&d) also show that the proliferation of MSCs is not favored by either as-synthesized HAP-NR films or media containing free as-synthesized HAP-NRs. These results suggest that the as-synthesized HAP-NRs are cytotoxic to MSCs while the surfactant-free HAP-NRs are nontoxic, have high affinity with MSCs and can support the adhesion, spreading and proliferation of resident MSCs.

We further investigated the osteogenic differentiation of MSCs under four conditions: (1) on the HAP-NR superstructure films and incubated in the osteogenic differentiation media; (2) on the randomly oriented HAP-NR films and incubated in the osteogenic differentiation media; (3) on the culture plate and incubated in the HAP-NR-containing osteogenic differentiation media; and (4) on the culture plate and in the osteogenic differentiation media without HAP-NR (control). After incubation for 2 weeks, the immunofluorescence results (Figure 4) show that the amount of the osteogenic marker, osteopontin (OPN) produced by MSCs due to the osteogenic differentiation, is decreasing in the order of the above four conditions. Total fluorescence measurements in the immunofluorescence images were also performed with Image J by following a previously published method⁴⁵⁻⁴⁷ to quantitatively evaluate the expression level of OPN (Figure S6). These results indicate HAP-NR superstructures significantly promoted the osteogenic differentiation of MSCs with the highest efficiency among all the groups. Thus, our surfactant-free biomimetic HAP-NRs superstructures show the ability to promote the osteogenic differentiation of MSCs, which is highly desired in their application in bone tissue engineering. We believe that the films made of biomimetic HAP-NR superstructures could promote the osteogenic differentiation of MSCs because their building blocks mimic the assembly structures of HAP crystals in enamels and are more ordered than the randomly oriented HAP-NRs. It is reported that ordered structures favor the osteogenic differentiation of MSCs than their random counterparts.^{48, 49}

Conclusions

In conclusion, this work described a facile reaction-dissolution approach to the removal of surfactant molecules from high quality HAP-NRs. The resultant surfactant-free HAP-NRs are water-dispersible, uniform in size and biocompatible. In addition, they can be assembled into enamel-like and bone-mimicking superstructures, which significantly promote the osteogenic differentiation of MSCs. Hence, our reaction-dissolution approach has enabled the development of new building blocks for bone-building biomaterials. Such method can also be extended to modify other fatty acid-stabilized functional nanoparticles to allow them to be useful in biomedicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) TEM image of as-synthesized HAP-NRs. (b) The electron diffraction pattern taken from these HAP-NRs, which confirms the crystalline structure of HAP-NRs. (c) As-synthesized HAP-NRs are dissolved in hexane to form a clear solution. (d) TEM image of treated surfactant-free HAP-NRs. (e) SEM image of a film made of randomly oriented HAP-NRs. (f) TEM image of HAP-NR superstructures. (g) SEM image of a film made of HAP-NR superstructures.

Figure 2.

Raman spectra of the HAP-NRs before (spectrum a) and after reaction-dissolution treatment (spectrum b). The spectrum a (as synthesized HAP-NRs) shows both typical peaks from HAP (435 cm⁻¹, 590 cm⁻¹, 961 cm⁻¹, and 1083 cm⁻¹) and surfactant molecules (linoleic acid) (1260 cm⁻¹, 1300 cm⁻¹, 1440 cm⁻¹, 1650 cm⁻¹, 2900 cm⁻¹, and 3021 cm⁻¹), whereas spectrum b (HAP-NRs after treatment) only shows typical peaks from HAP crystals.

Figure 3.

Morphology and proliferation of MSCs cultured on HAP-NR films or with free HAP-NRs in the media for 48 h. (a) MSCs adhere to and spread on the film of HAP-NR superstructure (15 $\mu\text{g}/\text{well}$). (b) MSCs adhere to and spread on the film of randomly oriented HAP-NRs (15 $\mu\text{g}/\text{well}$). (c) MSCs cultured in the cell medium containing free HAP-NRs (15 $\mu\text{g}/\text{well}$). The free HAP-NRs are attached and aggregated onto MSCs. (d) Control MSCs after being cultured on the cell culture plate.

Figure 4. Immunofluorescence staining study of osteogenic differentiation of MSCs on HAP-NR films or with free HAP-NRs in osteogenic differentiation media. (a) MSCs cultured on a film of HAP-NR superstructures; (b) MSCs cultured on a film of randomly oriented HAP-NRs; (c) MSCs cultured in HAP-NR-containing medium; and (d) MSCs cultured on cell culture plate (control). Osteopontin (OPN) was labeled with TRITC (red), actin filaments were labeled with phalloidin (green), and nuclei were labeled with DAPI (blue).

Scheme 1.

Schematic illustration of removing surfactant molecules from hydroxyapatite-nanorods (HAP-NRs), assembling them into an HAP-NR superstructure, and forming a film of HAP-NR superstructures for supporting MSCs. a) The as-synthesized HAP-NRs are covered by a layer of linoleic acid surfactant molecules. b) After the treatment by a reaction-dissolution approach, surfactant molecules are removed from HAP-NRs. c) Surfactant-free HAP-NRs are assembled into an enamel-like HAP-NR superstructure through an oriented-attachment mechanism, which is favored due to the removal of surface coating molecules. d) HAP-NR superstructures are deposited onto a polylysine-coated cell culture plate to form a film for supporting the proliferation and differentiation of MSCs.