Int J Clin Exp Med 2015;8(2):2628-2634
www.ijcem.com /ISSN:1940-5901/IJCEM0004474

Original Article

Novel impurity-free hexagonal hydroxyapatite nanotubes for local delivery of antibiotics in orthopedic surgery: in vitro release validation

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Received December 5, 2014; Accepted January 25, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: Hydroxyapatite (HA) has been studied recently as a drug carrier in prevent implant infections due to its biocompatibility and osteoconductive properties, but most of these studies failed to control infection. The aim of the present study was to evaluate the potential of this impurity-free novel HA nanotube as a carrier for drug delivery in a controlled manner. Gentamicin was selected as an antibiotic to study the drug-carrier properties of this novel HA nanotube. Gentamicin was introduced into the HA nanotubes through immersion and evaporation process. Gentamicin-loaded HA nanotubes were then placed in phosphate buffered saline (PBS) and drug release profile was then monitored by measuring free genntamicin in the solution. An initial burst release of the drug occurred in the first 24 hours; subsequently, 84.7% of the drug was released from the nanotubes in 9 days. After 13 d, the concentrations of released drug were measured close to 2 μg/ml. The porosity of the gentamicin-loaded HA nanotubes was also observed using a Hitachi s-4800 high-resolution SEM, further confirming the drug-carrier property of HA nanotubes. Our novel bone substitute is an effective prophylactic tool for the local delivery of gentamicin to prevent periprosthetic infections.

Keywords: Drug release, gentamicin, hydroxyapatite, nanotube

Introduction

Millions of orthopedic implants are being placed into humans each year, with over 2.5 millions in US alone. Among them, a close to 4.5% become infected and develop chronic osteomyelitis, which cause severe dysfunction, amputation and removal of implants in patients. Current approaches to treat osteomyelitis mainly depend on the antibiotics delivered to the sites through blood circulation. However, the blood supply to the infected sites is compromised in some patients with osteoyelitis, which prevents sufficient delivery of the antibiotics to the sites. Moreover, systematic toxicity might appear due to the high concentrations of drug in the circulation system over long period of time, and drug-resistant microbial strains can slowly develop under the selective pressure of high concentration of antibiotics. Because of these concerns, local delivery of antibiotics for the treatment of orthopedic infections has become increasingly popular [1-3], owing to the advantage of delivering high concentrations of antimicrobial agents locally to infection sites thereby minimizing patient exposure to systemic toxicity [2].

For a drug carrier to be effective, it must ideally have the ability to incorporate necessary amount of drug through physical, chemical or mechanical approaches. The loaded drug should remain a major amount in/on the carriers until it reaches the specific target sites. The loaded drug will then be released slowly from the carriers in a controlled manner to allow a manageable while effective amount of drug surrounding the target sites. Due to the flexibility and multifunctionality, various types of (bio-) polymers or biomacromolecules have been

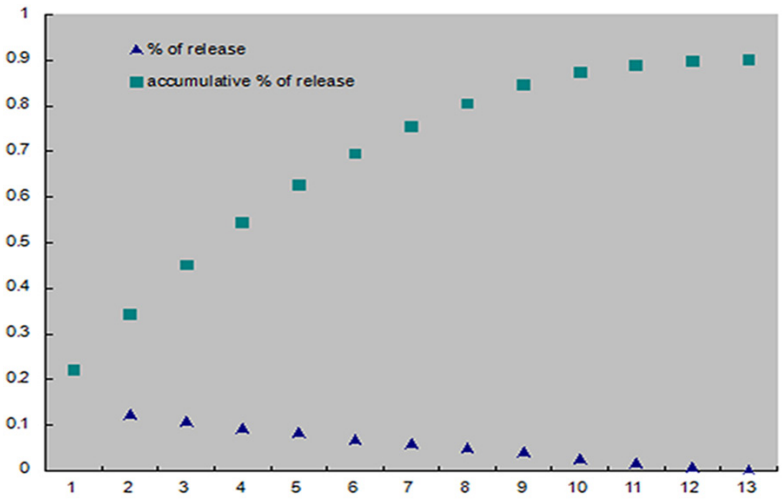

Figure 1. *In vitro* release curves of gentamicin from bone substitute.

developed or utilized for the delivery of antibiotics to in the treatment of bone infection diseases. These include biodegradable alginate carrier system for the treatment of infected bone defects, resorbable poly-L-lactic acid drug carrier for antimicrobial treatment of bone infections, and other polymers such as hydroxyapatite, polymethylmethacrylate and poly (DL-lactide-co-glycolide) [4, 5].

Antibiotic-loaded hydroxyapatite (HA) has been used in orthopedics because of its low density, high mechanical strength and fatigue and wear resistance, excellent corrosion resistance, and its readiness to bond with bones and the surrounding tissues without causing significant cytotoxic effects [6-8]. Many groups have been trying to load HA with different antibiotics to prevent infections, but controlled release hasn't been well achieved in these studies, especially when considering the bone healing is a slow progress which requires 4 to 8 weeks of antibiotic administration, considerably longer than treatment of other diseases.

There is increasing interest in the use of the emerging field of nanotechnology for controlled drug release in recent studies. Considerable efforts have been devoted to chemically modified nano-structure HA to improve its drug-loading ability. Nanoscale carriers are able to deliver high local concentrations of antimicrobials, especially nanotubes which show high drug load capacities and good cell penetration qualities when compared to other nanostructures. Although attempts to fabricate HA with nanoscale structures and morphologies haven been maken by many [9], only a few of them were successful in generating HA nanotubes, and the resulting HA nanotubes were impure [8, 10]. A new HA nanotube was recently developed by us for prophylactic use [8], which adapted a smart biomimetic strategy to fabricate high-purity single-crystalline HA nanotubes using just organoamines as both an inducer and a controller. The resulting pure HA nanotubes were homologous in structure characterized by a hexagonal facet with open tips on the ends. While these nanotubes exhibits high efficient in encouraging cell proliferation, its drug delivery properties haven't been addressed. The aim of the present study was to evaluate the potential of this impurity-free novel HA nanotube as a carrier for drug livery in a controlled manner.

Materials and methods

Synthesis of HA nanotubes

The biomimetic process that leads to the formation of novel highly-pure HA nanotubes has been successfully established in our recent study [8]. With the dropwise addition of organoamines into the $CaCl_2$ and Ca $(H_2PO_4)_2$ mixture under gradually increasing pH values from 4.3 to 9.5, the synthesis process could be divided into two obvious stages. At the first stage where pH value is low (4.3 to 8.2), precipitates formed during the process contained only regular rectangular plates based on the TEM (transmission electron microscopy) observation. Chemical analysis by XRD indicates that these plates were $CaHPO_4 \cdot 2H_2O$ (DCPD). At the moment pH value was increased to 8.2, the process entered into the second stage when these nanoplates suddenly vanished and were replaced by nanofibers showing up. Accompanying the process of this structure switch, chemical composition also underwent the change from DCPD to $CaHPO_4$ (DCP) in these nanofibers. Continuous hydrothermal treatment in the following 6 h led to the conversion of these nanofibers to partially hollow fibers due to the removal of PO_4^{3-}

Table 1. Drug release profile in the gentamicin-loaded HA nanotubes

days	1	2	3	4	5	6	7	8	9	10	11	12	13
Conc. (μg/Ml)	178.2	99.4	87.4	75.3	67.1	55.9	48.6	40.2	33.7	21.3	13.5	6.4	2.1
% of release	0.22	0.122716	0.107901	0.092963	0.08284	0.069012	0.06	0.04963	0.041605	0.026296	0.016667	0.007901	0.002593
Accumulative % of release	0.22	0.342716	0.450617	0.54358	0.62642	0.695432	0.755432	0.805062	0.846667	0.872963	0.88963	0.897531	0.900123

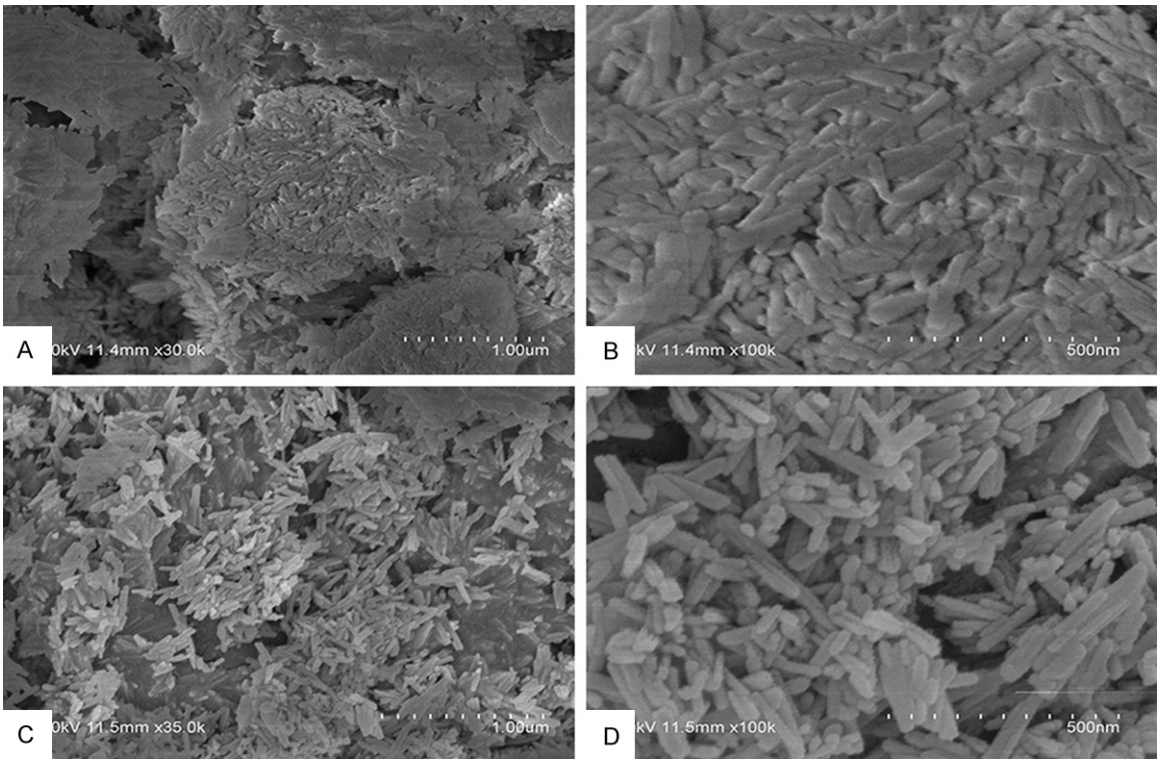

Figure 2. SEM observation of the microporosity of a gentamicin-loaded bone substitute: A, B: Microporosity is filled with gentamicin; C, D: Microporosity is empty after *in vitro* release.

from the inside of these nanofibers. And constant removal of PO_4^{3-} from the inside of the nanofibers due to the sustained hydrothermal treatment eventually led to the formation of nanotubes after around 24 h. With the formation of hollow inside, the surface layer of the DCP fibers also underwent crystallization process due to the adsorption of organoamines onto the surface. The combined internal and external structural change ultimately gave rise to the formation of HA nanotubes with open ends.

Loading antibiotics into HA nanotubes

To load drugs into hydroxyapatite nanotubes, gentamicin was first dissolved in phosphate-buffered saline (PBS). The concentration of gentamicin was kept constant (25 mg/ml). Powdered HA nanotubes were added to gentamicin sulfate solution. To prevent HA nanotube clustering and allow the even distribution of gentamicin inside and on the surface of HA nanotubes, the suspension was first treated with constant sonication for 24 h. After sonication, the suspension was centrifuged to remove the clustered or undissolved HA nanotubes. The remaining samples were then subjected to heat treatment by placing in an oven at 55.8°C for 2 h to remove water residual.

In vitro study of drug release

The drug release profile was tested by first placing the samples in deionized water (DI). Subsequently, 100 mg of the gentamicin-loaded HA nanotubes was introduced into 50 ml of phosphate buffered saline (PBS) (pH 7.4) at 37°C under sterile conditions. The drug release profile was investigated for a period of 13 days. To calculate the amount of drug released, 2 ml of the sample was withdrawn using a pipette every 24 hours and the total volume was restored subsequently with addition of 2 ml fresh PBS. The amount of gentamicin in the collected samples was measured spectrometrically at a wavelength of 274 nm. The actual value of gentamicin loaded in the HA nanotubes was then calculated, and the drug release profile was obtained.

SEM and TEM observations

The structure and morphology of the synthesized HA nanotubes were first characterized using transmission electron microscopy (TEM).

The samples were placed under the observation by a JEOL 2000Fx-II TEM microscopy with a W-source and a point-point resolution of 2 Å operated at 200 kV. To visually evaluate the loading and drug release profile of HA nanotubes, the structure including the porosity of the original and gentamicin- loaded HA nanotubes under different conditions was observed by scanning electron microscopy (SEM), using a Hitachi s-4800 high-resolution system integrated with a FEI Quanta 400 field emission SEM (FEI, Hillsboro, OR) operated at 10 kV.

Results

Structural characterization of HA nanotubes

The structural property of the synthesized HA nanotubes was first characterized using TEM and SEM. A hollow hexagonal facet with open ends was clearly observed in high resolution (HR)-TEM images for the synthesized HA nanotubes [8]. These nanotubes had a length of several micrometers with an inner diameter of 8-50 nm and outer diameter of 20-80 nm. Under lower resolution SEM images (**Figure 1**), the inner structure was difficult to tell, but outer diameter and general length of these HA nanotubes were comparable to what was observed under HR-TEM. The HA nanotubes had a large interface available for interactions with gentimicin or other antibiotics because of the specific inner space provided by their tube-structure at nanoscale.

Controlled drug release from HA nanotubes

To evaluate the local delivery and drug release properties of HA nanotubes, the gentamicin-loaded nanotubes were immersed in sterile PBS, and the released drugs were measured every 24 hours. The **Table 1** displayed the measured concentration of gentamicin for the first 13 days. Based on the measured concentrations, the drug release was calculated by dividing the released drug in 24 hours by the total loaded gentamicin. The drug release profiles of the nanotubes are illustrated in **Figure 1**. An initial burst release occurred in the first 24 hours, followed by a slow release of gentamicin in the following days. 84.7% of the drug was released in 9 days based on the calculation. It can be seen from the **Table 1** that the amount of drug released in the first 24 hours was well above the minimum inhibitory concentration of 0.25-2 ug/ml for pathogens such as *Staphylococcus aureus*. Although the measured concentrations of gentamicin was quite higher than the toxic level of 12 ug/ml at the first few days, these high concentrations were considered to be limited to the local surroundings in the clinical applications due to the poor circulation of blood in the implant surrounding. The concentration level in the whole blood circulation system is expected much lower due to the slow diffusion therefore a large dilution of small amount of gentamicin into the blood.

After 13 d, all of the drug concentration values were higher than 2 μg/ml (**Table 1**). The concentrations measured were higher than the minimal bactericidal inhibitory concentration even after long-term release, which indicates that HA nanotubes can be really effective in the slow and controllable release of antibiotics necessary for infection control in the bone recovery process.

It has been reported that under physiological conditions and in pure water or PBS buffer, the phosphate groups will predominate at HA surface. This yields net negative charged HA surface which exhibits strong binding interactions with positively charged gentamicin, one of the major mechanisms that likely contribute to the high loading as well as the controlled release of gentamicin onto the HA nanotube surfaces in the present study. Since the gentamicin was likely absorbed onto the HA nanotube surface through electrostatic interactions, drug load and release will essentially cause a change in the surface charge and/or structure, which can both be tracked by examining the surface through SEM. A comparison of the SEM pictures at the same magnification before (**Figure 2A** and **2B**) before and after drug release for 48 h in PBS buffer (**Figure 2C** and **2D**) confirmed that the gentamicin present in the microporosities had been eluted. The cloudy surface observed in the HA nanotubes with gentamicin has disappeared with a sharper surface structure after the release of gentamicin. A closer look of HA nanotubes indicates that these microporosities are empty in **Figure 2D**.

Discussion

Local delivery of antimicrobials is desirable for management of orthopedic infections because of the poor circulation of blood in the infected

tissues which makes it very difficult to for the antibiotics to reach adequate therapeutic levels in the affected regions.

Among biocompatible materials, HA is of interest because it is chemically similar to human bone and is thought to be osteoconductive. Hydroxyapatite not only provides a perfect base to enhance osseointegration but also prevent the contact of implant wear particles with the interface, thus avoids the activation of immune response and bone resorption surrounding the surgical sites [11, 12]. HA with various 3-dimensional structures have been shown to retain antibiotics on its surface; therefore, some researchers have designated these materials for prophylactic use. Because the nanotubes have a larger surface area and enhanced surface interactions relative to commonly used microparticles, it is thought that they could absorb more drugs on the surfaces. Also, due to its hollow interior structure, the loads can be better controlled in the release process due a slow diffusion process when compared to the drug loaded on the surface. The HA nanotubes also have other advantages such as biocompatibility and easier to penetrate the tissues and cells, which is extremely important for long-term sustainable delivery for bone healing.

In the present study, gentamicin was selected as the model drug since it is extensively used for antibiotic prophylactic therapy in orthopedic surgery [13, 14]. Furthermore, the efficacy of gentamicin corresponds to its concentration against bacteria, which is normally high under local release.

To our knowledge, no study in the current literature has described the use of HA nanotubes as antibiotic carriers. Our investigation describes the synthesis and characterization of gentamicin-loaded HA nanotubes intended as a drug delivery system. Moreover, this is the first in vitro study in which HA nanotube structures are utilized for drug elution applications.

The diameter of the obtained novel HA nanotubes was uniform, with an inner of 8-50 nm and an outer of 20-80 nm. Antibiotic loading may occur either through loading of the nanotubes by penetration of the antibiotics into the tubes, or through surface adsorption of antibiotics onto/into the nanotubes, or more likely through both for our HA nanotubes. The diameters of the nanotubes could affect the adsorption of the antibiotics because these gentamicin-loaded samples with various sizes showed a heterogeneous spread of gentamicin when checked by SEM.

In our work, the drug release was nearly immediate; however, longer release times for prophylactic applications have also been observed. Porous structures of the nanotubes to provide a large surface could improve the control of drug release because of the different origins and modifications.

According to SEM images, gentamicin was stored in the micropores of the HA nanotubes. During the impregnation process, the gentamicin solution penetrated the micropores of HA nanotubes through capillarity. At the drying stage, the solvent gradually evaporated away. Because of the higher capillary pressure in the micropores, evaporation ends in the micropores, leaving gentamicin in dry form.

Several parameters, such as the height and diameter of the nanotube, may affect the gentamicin release profile. Further studies will be performed to compare the different release rates obtained by modulation of these parameters with our present results.

Conclusion

This study introduced gentamicin into the hydroxyapatite (HA) nanotube bone substitute, and validated the nanotube release profile in vitro in a drug-release model. The results demonstrate that our novel bone substitute is an effective prophylactic tool for the local delivery of gentamicin.

Acknowledgements

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript. This work has received support from the Key Program of Science and Technique Development Foundation in Jiangsu Province (BE20116004), the Peak Talents Foundation in Jiangsu Province (2012-WS-092), and Medical Science and Technology Development Foundation, Nanjing Department of Health (ZKX1-2016, YKK13079). We acknowledge Dr. Dongya Li, Dr. Han Yuan, Dr. Xiao-xiao Xie, and Dr. Xiaobo Wang for their assistance on data collection.

Disclosure of conflict of interest

None.

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