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Physical characterization and osteogenic activity of the quaternized chitosan-loaded PMMA bone cement

Honglue Tan [a,b], Shengrong Guo [d,*], Shengbing Yang [c], Xiaofen Xu [d], Tingting Tang [a,*]

[a] Shanghai Key Laboratory of Orthopaedic Implant, Department of Orthopaedic Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China
[b] Department of Orthopaedic Surgery, Changzhou Wujin Hospital, Jiangsu University School of Medicine, China
[c] Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai, China
[d] School of Pharmacy, Shanghai Jiao Tong University, China

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ABSTRACT

Gentamicin-loaded polymethylmethacrylate (PMMA), widely used for primary cemented arthroplasty and revision surgery for preventing or treating infections, may lead to the evolution of antibiotic-resistant bacteria and dysfunction of osteogenic cells, which further influence the osteointegration of bone cement. In a previous study, we reported that a new quaternized chitosan derivative (hydroxypropyltrimethyl ammonium chloride chitosan, HACC) that was loaded into PMMA significantly inhibited the formation of biofilms caused by methicillin-resistant *Staphylococcus* strains. In the present study, we further investigated the surface morphology, hydrophilicity, apatite formation ability and osteogenic activity of HACC-loaded PMMA. Chitosan-loaded PMMA, gentamicin-loaded PMMA and PMMA without antibiotic were also investigated and compared. The results showed that, compared to other PMMA-based cements, HACC-loaded PMMA had improved properties such as a lower polymerization temperature, prolonged setting time, porous structures after immersion in phosphate-buffered saline, higher hydrophilicity, more apatite formation on the surface after immersion in simulated body fluid, and better attachment and spreading of the human-marrow-derived mesenchymal stem cells. We also found better stem cell proliferation, osteogenic differentiation, and osteogenesis-associated genes expression on the surface of the HACC-loaded PMMA compared to the gentamicin-loaded PMMA. Therefore, this new anti-infective bone cement had improved physical properties and osteogenic activity, which may lead to better osteointegration of the bone cement in cemented arthroplasty.

1. Introduction

The treatment of osteomyelitis and infected arthroplasty is challenging. Local debridement, removal of implants, irrigations, obliteration of dead space, osseous repair, and systemic use of antibiotics for long periods of time are the standard procedures for treating orthopedic infections [1]. Because of the compromised vascularity, the existence of necrotic tissue, and bacterial biofilm formation on the surface of the implant, it is difficult to obtain an effective local antibiotic concentration by systemic administration [1]. Furthermore, intravenous antibiotic therapy using multiple antibiotics with high serum concentrations may carry the risk of systemic toxicity. Therefore, there has been increased interest in local antibiotic delivery systems, which yield higher local antibiotic concentrations [2–4].

Local antibiotic delivery vehicles that have been reported for treating orthopedic infections include polymethylmethacrylate (PMMA), poly(lactic acid) (PLA), poly(lactic acid-co-glycolic acid) (PLGA), poly(DL-lactic acid) ($P_{DL}LA$), calcium phosphate paste, hydroxyapatite, tricalcium phosphate and plaster of Paris [4,5]. However, the most widely studied and applied material is PMMA bone cement, which was first introduced by Buchholz et al. [6]. Antibiotic-loaded PMMA has been proven to be successful for the treatment and prevention of osseous infections, and represents the current gold standard for local antibiotic delivery systems in orthopedic surgery [7]. PMMA bone cement loaded with gentamicin is normally used for total hip and total knee arthroplasties during primary and revision surgeries, and has proven to be an effective practice for preventing and treating infections after total joint replacement [8–12]. In addition, gentamicin-loaded PMMA beads have been one of the most widely used local antibiotics in clinical practice, especially for osteomyelitis therapy [13–15]. However, the local overuse of antibiotics also leads to the evolution of antibiotic-resistant bacteria, which accounts for the failure of anti-infective treatments [11,16]. Thus, it is necessary to find more effective antimicrobial agents to load into PMMA against antibiotic-resistant organisms.

* Corresponding authors. Tel.: +86 21 23271133; fax: +86 21 63137020.
E-mail addresses: srguo@sjtu.edu.cn (S. Guo), tingtingtang@hotmail.com (T. Tang).

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To succeed in orthopedic surgery, implant materials must be anti-infective (discouraging bacterial adhesion) as well as habitable by bone-forming cells (favouring the activity of osteogenic cells) [17,18]. In a clinical study with total hip replacements, the concentration of gentamicin around PMMA bone cement was found to be at levels of 400–600 μg ml^{-1} on the first day [19]. During in vitro studies, the release concentrations of gentamicin-loaded PMMA beads (0.2 g with 4.5 mg gentamicin) were 600 μg ml^{-1} on the first day, 120 μg ml^{-1} on day 10 and 10 μg ml^{-1} on day 80 [14]. These high concentrations, which were enough to prevent or treat the infections caused by susceptible bacterial strains, also far exceeded the critical osteochondrogenesis-affecting level of 100 μg ml^{-1} for osteogenic cells [20]. According to previous studies, gentamicin at high local concentrations reduced the viability, proliferation, and alkaline phosphatase activity of the osteoblasts [21–25], and inhibited the proliferation and differentiation of human bone marrow mesenchymal stem cells in vitro and in vivo [20,26,27], which compromised the bone-healing process. Therefore, finding new bone cements based on PMMA that have anti-infective properties without adverse effects on the host osteogenic cells should be a major concern.

In previous studies, we successfully synthesized a new water-soluble chitosan derivative (hydroxypropyltrimethyl ammonium chloride chitosan, HACC) with a 26% degree of substitution (DS) of the quaternary ammonium [28] (referred to as 26% HACC), and a further in vitro study demonstrated that PMMA bone cements loaded with 26% HACC strongly inhibited the formation of biofilms caused by antibiotic-resistant staphylococci, whereas gentamicin-loaded PMMA had no effect [29]. However, the physical characterization and cytocompatibility of this new bone cement remain unknown. Therefore, the objectives of the present study are to investigate the influence of the inclusion of the 26% HACC on the physical properties of PMMA and also to investigate the in vitro cytocompatibility and osteogenic activity of the 26% HACC-loaded PMMA.

2. Materials and methods

2.1. Materials

26% HACC was prepared by combining chitosan and glycidyl trimethylammonium chloride (GTMAC), as previously reported [28]. Chitosan (CS) with a molecular weight of 2.0×10^5 and with an *N*-deacetylation rate of 91.83% was purchased from Zhejiang Yuhuan Ocean Biochemistry Co. Ltd., China. The CMW Endurance Bone Cement® containing PMMA/MMA with and without gentamicin (DePuy International Ltd, UK) was used as the control in this study. The PMMA with gentamicin contained 1 g gentamicin per 40 g PMMA powder. The other chemicals used were of analytical grade.

2.2. Preparation of cement specimens

Chitosan or 26% HACC powder (10 g) were added to the PMMA polymer powder (40 g) (referred to as PMMA-C and PMMA-H, respectively) and uniformly mixed. Then the powder was manually mixed with the liquid monomer at a ratio of 1.5 ml/2 g [30] in a bowl until the powder was fully wet. The mixture was subsequently poured into a PTFE mould and was pressed between two metal plates for 4 h. After the cement hardened, it was pulled out of the mould, and samples were stored in the dark at room temperature. The specimens were discs of 10.0 ± 0.1 mm in diameter and 3.0 ± 0.1 mm in height. PMMA and gentamicin-loaded PMMA (referred to as PMMA and PMMA-G, respectively) were also prepared according to the above procedure. Cements were sterilized by irradiation with 25 kGy of ^{60}Co before use.

2.3. Physical characterization of PMMA-based bone cements

The exothermic polymerization temperature–time profiles during solidification were measured using a thermocouple (M6, ShangHai FeiLong Instrument & Electronic Co. Ltd., China) connected to a digital temperature controller (XMT-D-3402, ShangHai FeiLong Instrument & Electronic Co. Ltd., China) and positioning its junction in the centre of the mould [31]. The temperatures were measured immediately after each bone cement was poured into the mould and recorded every 30 s for 20 min at 23 ± 1 °C. The maximum temperature (T_{max}) was obtained from the maximum of the exotherm, which corresponds to the maximum temperature attained during polymerization. The setting time was calculated according to the following formula: Setting time = $T_{amb} + T_{max}/2$, where T_{amb} is the ambient temperature (23 ± 1 °C), and T_{max} is the maximum temperature [32]. The measurements were repeated three times.

To determine the surface hydrophilicity and energy, static contact angle measurements were performed using distilled water and ethylene glycol as the media by the sessile drop method on a drop-shape analysis system (JC 2000D3, Shanghai Zhongcheng Digital Technology Co. Ltd., China) at ambient humidity and temperature. Five measurements were performed on different points of each specimen. The surface energy was calculated according to the surface tension and the contact angle. The surface roughness (R_a) of the cements was also determined with a stylus instrument (Japan Mitutoyo SJ-401).

2.4. Immersion of PMMA-based bone cements in simulated body fluid (SBF)

The SBF solution was prepared according to the literature (SBF: Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mol m^{-3}) [33]. Three samples of each bone cement were soaked in 40 ml of SBF solution at a temperature of 37 °C while shaking at 100 rpm. The solution was replaced with fresh SBF every 3 days. After 7, 14, and 28 days, the specimens were removed and were gently rinsed with distilled water and dried overnight at a temperature of 60 °C. The samples were then sputter-coated with gold, and the existence and morphology of the apatite formed on the material surface were observed using a scanning electron microscope (Joel JSM-6310LV, JEOL Ltd., Tokyo, Japan). In order to determine the apatite formation on the cement surface, wide-angle X-ray diffraction (XRD) was performed on an X-ray diffractometer (D/Max-2550 V, Rigaku, Japan) using fixed monochromatized Cu-Kα radiation (λ = 0.15406 nm) energized at 40 kV and 40 mA. The samples were measured at room temperature in the scan range of 10–80° (2θ) with scan rate of 0.02° min^{-1}.

2.5. Cell culture

The human mesenchymal stem cells (hMSCs) were cultured as described in a previous study [28]. In brief, cells were cultured in α-MEM culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 U ml^{-1}), and streptomycin sulphate (100 mg ml^{-1}) (GibcoBRL, Grand Island, NY). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, with the growth medium changed every 48 h. hMSCs at passage 3 were detached by 0.25% trypsin, resuspended in a different density using fresh culture medium, and used for the experiments described below.

2.6. Cell attachment

1 ml of the cell suspension with cell density of 2×10^4 viable cells was seeded in a 48-well plate (Costar3548, USA) that