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In vitro cytocompatibility evaluation of poly(octamethylene citrate) monomers toward their use in orthopedic regenerative engineering

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ABSTRACT

Citrate based polymer poly(octamethylene citrate) (POC) has shown promise when formulated into composite material containing up to 65 wt% hydroxylapatite (HA) for orthopedic applications. Despite significant research into POC, insufficient information about the biocompatibility of the monomers 1,8-Octanediol and Citrate used in its synthesis is available. Herein, we investigated the acute cytotoxicity, immune response, and long-term functionality of both monomers. Our results showed a cell-type dependent cytotoxicity of the two monomers: 1,8-Octanediol induced less acute toxicity to 3T3 fibroblasts than Citrate while presenting comparable cytotoxicity to MG63 osteoblast-like cells; however, Citrate demonstrated enhanced compatibility with hMSCs compared to 1,8-Octanediol. The critical cytotoxic concentration values EC30 and EC50, standard for comparing cytotoxicity of chemicals, were also provided. Additionally, Citrate showed slower and less inhibitory effects on long-term hMSC cell proliferation compared with 1,8-Octanediol. Furthermore, osteogenic differentiation of hMSCs exposure to Citrate resulted in less inhibitory effect on alkaline phosphatase (ALP) production. Neither monomer triggered undesired pro-inflammatory responses. In combination with diffusion model analysis of monomer release from cylindrical implants, based on which the maximum concentration of monomers in contact with bone tissue was estimated to be 2.2×10^{-4} mmol/L, far lower than the critical cytotoxic concentrations as well as the 1,8-Octanediol concentration (0.4 mg/mL or 2.7 mmol/L) affecting hMSCs differentiation, we provide strong evidence for the cytocompatibility of the two monomers degraded from citrate-based composites in the orthopedic setting.

1. Introduction

Despite the innate regenerative capacity of human bone, healing of nonunion defects, defined as incomplete defect closure, remains a challenge clinically, creating a substantial need for the development of bone grafts to bridge defects and guide tissue regeneration [1]. The development of orthopedic biomaterials that are totally synthetic, readily available, capable of fully degrading *in vivo*, and mimic natural bone has been strongly encouraged to replace the limited supply of autografts [2–4]. Notably, citrate based materials with rich -COOH groups capable of incorporating up to 65 wt% of hydroxylapatite (HA), simulating the inorganic composition of natural bone, have shown great promise in bone regeneration compared to traditional degradable polymers such as polylactide (PLA) capable of compositing a maximum of 25–30 wt% of HA before becoming excessively brittle [5]. In contrast to PLA's bulk degradation, the degradation of citrate-based material proceeds in a form of surface erosion, which could avoid the accumulation of massive acidic degradation products [6]. Moreover, the strongest citrate based polymer/HA composites possessed a compressive strength of ~250 MPa, falling within the range of human cortical bone (100–230 MPa) [7]. Therefore, citrate-based polymers could serve as ideal base materials to prepare bone-like composite

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orthopedic biomaterials [7,8].

The first citrate-based polymer composited with HA for orthopedic applications was poly(octamethylene citrate) (POC) synthesized by reacting Citric Acid and 1,8-Octanediol through a convenient one-pot polycondensation reaction [5,8–10]. In addition to POC, both Citrate and 1,8-Octanediol have been widely used for the synthesis of a family of citrate-derived polymers with various functionalities for diversified applications such as soft and hard tissue engineering, drug delivery, bioimaging, and biosensing in the last decade [5,11–21]. Although both *in vitro* and *in vivo* biocompatibility of POC and POC/HA composites have been well tested in previous studies [5,8,10], there is surprisingly limited information about the safety of the two monomers given the cytocompatibility of individual components of materials should be considered at the onset of materials design [22–24]. Moreover, *in vivo* degradability of POC/HA is a highly desired material property that allows gradual replacement of the bulk implant with functional tissue. The main degradation mechanism is cleavage of the ester bond formed by Citrate and 1,8-Octanediol [5]. It means that Citrate and 1,8-Octanediol would comprise the majority of the degradation products of POC/HA, and would readily contact with host tissue, largely affecting the long-term tissue response. Citrate has historically been regarded as a biocompatible monomer, since it is a well-known naturally occurring metabolite in the TCA cycle, and its application in certain medical situations has been approved by the FDA: for example, the citrate containing drug “PREPOPIK” has been approved for cleansing of the colon as a preparation for colonoscopy. However, almost no biocompatibility information is available for its orthopedic applications. In comparison, the safety of 1,8-Octanediol in biomedical applications remains much more underexplored, although 1,8-Octandiol has been reported to be used in cosmetics as a plasticizer [25].

In the present study, we filled the blanks by investigating the biocompatibility of the two POC monomers to different cells with the objective of answering the following questions: 1) Is toxicity of Citrate or 1,8-Octanediol cell-type dependent? 2) What are the tolerant concentrations of the two monomers to different cells? 3) What are the critical concentrations of the two monomers for bone forming cells to maintain their functionality towards bone formation? 4) How do we estimate the *in vivo* release and diffusion of monomers from implants, particularly from cylindrical implants often tested in animal studies, after material degradation? Answering the above questions is critical for the future translation of the biomaterials made of Citrate and 1,8-Octanediol.

2. Material and methods

2.1. Solution preparation

1,8-Octanediol (Alfa Aesar), which is sparingly soluble in water, was dissolved using complete medium to prepare fresh solutions at the concentration of 15 mg/mL and was subjected to further dilution using complete medium prior to testing. Citrate (Alfa Aesar) stock solution at the concentration of 150 mg/mL was prepared with DI water and buffered to pH 7.2–7.4 with 1N NaOH solution, followed by aliquoting and storage at −20 °C. Sodium dodecylsulfate salt (SDS) stock solution was prepared with DI water at a concentration of 2 mg/mL followed by aliquoting and storage at −20 °C.

2.2. Cell culture

Mouse fibroblast cells 3T3 and human osteoblast-like cells MG63 were purchased from ATCC and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS). Human mesenchymal stem cells (hMSCs) were obtained from Lonza and cultured with low glucose DMEM with 10% FBS and GlutaMAX (Gibico). hMSCs with passage ≤7 were used in the present study. All the cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Human acute monocytic leukemia cells THP-1 obtained from ATCC were cultured in suspension using RPMI-1640 with 20% FBS and 0.05 mM 2-Mercaptoethanol, with the culture flask placed upright for better cell recovery. After passage cells one time, the culture flask was lied down and cells were maintained in complete RPMI-1640 medium with 10% FBS and 0.05 mM 2-Mercaptoethanol.

2.3. Cytotoxicity evaluation

All *in vitro* cytotoxicity tests were conducted according to the international standard ISO 10993-5:2009(E). Dilutions of SDS producing a reproducible cytotoxic response served as positive control while blank wells without the test sample served as negative control to reflect the background response. Both positive and negative controls were included in each assay. Briefly, cells were seeded to 96 well plates at desired density (Seeding density: 3T3, 20,000 cells/cm^2; MG63, 20,000 cells/cm^2; hMSCs, 10,000 cells/cm^2). After the cultured cells reached subconfluency (approximately 80% confluency), the culture medium was removed and 100 μL of 1,8-Octanediol, Citrate or SDS solution at various concentrations were added to cells. After incubation for 24 h, the medium was removed followed by the addition of Cell Counting Kit-8 (CCK-8; Dojindo) solution diluted 1:10 with complete medium. Finally, the absorbance was measured at 450 nm with a Microplate reader.

2.4. Cell proliferation assay

In the cell proliferation assay, hMSCs were seeded to 96 well plates at a density of 5000 cells/cm^2. After 24 h, culture medium was removed and test samples at different concentrations were added and cultured with cells for 1, 3, and 5 days. Finally, CCK-8 assay was performed according to manufacture instructions. Proliferation rate was identified as the increasing in cell viability/day from day 1-day 3 and day 3-day 5, respectively.

2.5. Osteogenic differentiation of hMSCs

To test the effect on osteogenic differentiation, hMSCs at passage≤7 were used. Cells were seeded to 48 well plates at a density of 10,000 cells/cm^2, and cultured to reach subconfluency. Then, differentiation was initiated by adding osteogenic medium supplemented with 10^{-7} M Dexamethasome, 0.05 mM ascorbate-2-phosphate, and 0.01M β-glycerophosphate. Dilutions of 1,8-Octanediol and Citrate were added in osteogenic medium to test their effect on differentiation.

2.6. ALP assay and ALP staining

After differentiation for 14 days, part of the cell sample was collected for alkaline phosphatase (ALP) assay while the other part was fixed for ALP staining. In the ALP assay, cells were first washed twice with PBS and lysed with RIPA lysis buffer. Then, the cell lysate was transferred to microcentrifuge tubes and centrifuged at 14,000 g for 15 min at 4 °C. The supernatant was subsequently transferred to a new tube and a PicoGreen DNA quantification assay (Molecular Probes) was performed to determine total DNA concentration in lysate according to the manufacturer's instructions. Meanwhile, the cell lysate was diluted with Assay buffer to a final volume of 50 μL per sample and added to 96 well plates. Stock