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Fabrication and characterization of carboxymethyl cellulose novel microparticles for bone tissue engineering

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ABSTRACT

In this study we developed carboxymethyl cellulose (CMC) microparticles through ionic crosslinking with the aqueous ion complex of zirconium (Zr) and further complexing with chitosan (CS) and determined the physio-chemical and biological properties of these novel microparticles. In order to assess the role of Zr, microparticles were prepared in 5% and 10% (w/v) zirconium tetrachloride solution. Scanning electron microscopy (SEM) with energy dispersive X-ray spectrometer (EDS) results showed that Zr was uniformly distributed on the surface of the microparticles as a result of which uniform groovy surface was obtained. We found that Zr enhances the surface roughness of the microparticles and stability studies showed that it also increases the stability of microparticles in phosphate buffered saline. The crosslinking of anionic CMC with cationic Zr and CS was confirmed by Fourier transform infrared spectroscopy (FTIR) results. The response of murine pre-osteoblasts (OB-6) when cultured with microparticles was investigated. Live/dead cell assay showed that microparticles did not induce any cytotoxic effects as cells were attaching and proliferating on the well plate as well as along the surface of microparticles. In addition, SEM images showed that microparticles support the attachment of cells and they appeared to be directly interacting with the surface of microparticle. Within 10 days of culture most of the top surface of microparticles was covered with a layer of cells indicating that they were proliferating well throughout the surface of microparticles. We observed that Zr enhances the cell attachment and proliferation as more cells were present on microparticles with 10% Zr. These promising results show the potential applications of CMC-Zr microparticles in bone tissue engineering.

1. Introduction

Carboxymethyl cellulose (CMC) is a hydrophilic biocompatible polymer obtained through the chemical modification of cellulose. It consists of carboxymethyl group attached to the polysaccharide backbone making it a polyelectrolyte. It has been identified as a smart cellulose material as its properties such as shape, mechanical rigidity and porosity can be altered in a controlled manner [1,2].

The presence of carboxymethyl group in CMC makes it soluble in water and negatively charged polymer. This enables it to undergo complexation with oppositely charged materials thereby forming a crosslinked matrix structure (Fig. 1) with improved physico-chemical and biological properties. CMC has been studied as an injectable gels, composites and films for potential bone regeneration applications [3–5]. *In vivo* study done with CMC showed that when used as a hybrid injectable material with calcium phosphate and bone morphogenetic protein can induce greater bone formation in rat tibial defect site [6].

Injectable scaffolds have been extensively studied in bone tissue engineering in the recent years mainly because of their potential to minimize the surgical interventions. Scaffolds in the form of injectable gels offer advantages such as easy handling properties and adaptability to the defect site [7–9]. In order to make these injectable gels promising for bone tissue engineering, they should be able to incorporate drugs and bioactive agents and release them in a controlled manner. Studies have shown that growth factors incorporated directly into gels showed the large initial burst release [10,11]. Particle (micro- and nano-) based injectable scaffolds can offer advantages in this aspect because of their ability to encapsulate the bioactive agents and release them in controlled manner. In addition, microparticle based scaffolds provide a temporary support for cells to attach and proliferate [12,13].

For microparticles to be successfully used in bone regeneration, they need to be biocompatible, biodegradable and should enhance the osteoblast adhesion and proliferation without inducing any harmful effects to them [14,15]. CMC beads prepared by crosslinking with multivalent metals have been used in many drug delivery applications which showed good encapsulation of drugs and pH sensitive release [16,17]. These crosslinked 3-D CMC matrices are restricted to be used in bone tissue engineering applications due to the potential harmful effects of

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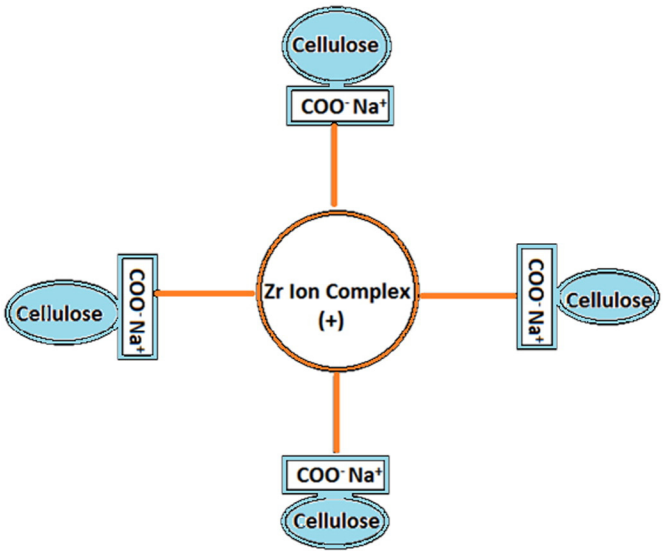

Fig. 1. Ionic crosslinking interaction model for anionic CMC with cationic Zr ion complex.

metal ions to osteoblasts [18,19]. Zr is a tetravalent cationic metal with an interesting aqueous chemistry. In its aqueous solution, it rarely exists as a pure Zr^{4+} ion but rather undergoes complexation with hydroxide ion thereby forming complex cation among which $Zr_4(OH)_4^{8+}$ (Zr-ion) is the predominant one in low pH solution [20]. Zr has been extensively used in the development of prosthetic devices in the form of biologically inert zirconia due to their good mechanical properties and corrosion resistivity. It has also been used as an alloy with other metals as a bearing surface for bone implants because of its wear resistivity [21–23]. The occupational health guideline from center for disease control and prevention (CDC) has classified Zr as a metal with very low systemic toxicity and studies have shown that it has no adverse effects on osteoblasts [24]. It was recently found that Zr when added to cell culture medium with controlled concentration improves the differentiation and proliferation of human osteoblasts [25]. Due to its good mechanical properties and improved cell responses, Zr has also been incorporated into different scaffolds recently and has shown excellent biological response with osteoblasts and mesenchymal stem cells [26,27].

After performing literature reviews, we found that although CMC has been extensively used as composites and injectable gel scaffolds for bone cells, the use of injectable microparticles has not been reported yet [3–5,28–30]. In this study we aim to develop CMC microparticles that show a favorable biological response to osteoblasts and promote their adhesion and proliferation. These microparticles were fabricated by crosslinking anionic CMC with complex cationic species of Zr. The fabricated microparticles were further stabilized by introducing them to the cationic CS solution. In order to see the effects of Zr, microparticles were prepared with two different concentration of Zr and the effects on stability, surface structure, osteoblasts response were assessed. In order to study the drug release kinetics, we used cefazolin as a model drug which has been used in the treatment of bone diseases associated with the bacterial invasion [31].

2. Materials and methods

2.1. Materials

Sodium carboxymethyl cellulose (average molecular weight 250,000 kDa, DS 0.7), zirconium (IV) chloride ($ZrCl_4$) (≥99.5% trace metal basis), chitosan (medium molecular weight) were purchased from Sigma-Aldrich (USA). Cefazolin sodium salt was purchased from MP Biomedicals (USA). Phosphate buffered saline (PBS), minimum essential media (MEM), penicillin/streptomycin and fetal bovine serum (FBS) were all purchased from Gibco (USA). LIVE/DEAD viability/Cytotoxicity kit was purchased from Invitrogen (USA).

2.2. Fabrication of microparticles

Microparticles were fabricated by crosslinking CMC with ionic species of zirconium present in hydrolyzed solution of $ZrCl_4$. Aqueous solution of CMC (3% w/v) was prepared by slowly adding CMC into deionized (DI) water under stirring at room temperature. The viscous CMC solution thus obtained was then added dropwise into hydrolyzed solution of $ZrCl_4$ through a syringe fitted with 30 gauge needle and left under stirring at 300 rpm for 3 h for crosslinking. Two different concentration of $ZrCl_4$ was used (5% w/v-P1 and 10% w/v-P2) for the preparation of microparticles. The particles thus formed were washed with DI water to eliminate the $ZrCl_4$ solution on their surface and were suspended in 2% CS solution for 24 h for complexation with CS. The particles looked more rigid and somewhat reduced in size after soaking in CS for 24 h. This might be due to the complexation of anionic CMC with cationic CS. They were again washed with DI water and were dried at room temperature for 48 h before further analysis.

2.3. Characterization of microparticles

- SEM observation
  The surface morphology of microparticles was analyzed using scanning electron microscope (SEM) (FEI quanta 3D FEG, FEI Company, USA) after sputter coating with gold for 30 s. Energy dispersive X-ray detector (EDS) with SEM was used to map the elemental Zr along the surface of microparticle and quantify the elements. Due to the spherical morphology of the microparticles it was only possible to collect the mapping results from top half of microparticle and bottom half was shaded.
- IR spectroscopy
  The intramolecular interaction between the components in microparticle was determined using Fourier transform infrared (FTIR) spectrometer (Excalibur Series FTIR, Varian). Dried samples were mixed with potassium bromide (KBr) and pressed into pellets using KBr kit. The pellets were loaded into the instrument and analyzed at a resolution of 8 cm^{-1}, average 100 scans from 4000 to 400 cm^{-1}.
- X-ray diffraction (XRD) study
  The phase analysis of zirconium present on microparticle was done with powder X-ray diffractometer (pXRD) (X'Pert Pro, PANalytical) with the crushed microparticles presented in powder form.

2.4. Stability of microparticles

The stability of microparticles was studied in PBS medium at pH 7.4. 10 mg of both microparticles (P1 and P2) were immersed in PBS at 37 °C under continuous shaking at 25 rpm up to 35 days. PBS was changed every 24 h for first 2 days and every five days afterwards in order to mimic *in vivo* conditions. In order to monitor the hydrolysis of microparticles, pH of collected PBS was measured and the change in pH was plotted for 25 days. The microparticles after 35 days were imaged with SEM to see the integrity and intactness in PBS.

2.5. Osteoblast culture and live/dead cell assay

Murine osteoblast cells (OB-6) were cultured in α-MEM medium supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. To assess the *in vitro* cytotoxicity of microparticles, they were first soaked in culture medium for 24 h and UV sterilized for