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Fabrication of pluronic and methylcellulose for etidronate delivery and their application for osteogenesis

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ABSTRACT

Novel hydrogels were prepared by blending 4% (w/w) methylcellulose (MC) with various concentrations of 12, 14, 16, 18 and 20% (w/w) pluronic F127 (PF) to form injectable implant drug delivery systems. The blends formed gels using lower concentrations of PF compared to when using PF alone. Etidronate sodium (EDS) at a concentration of 4×10^{-3} M was loaded into these blends for producing an osteogenesis effect. The pure gels or EDS loaded gels exhibited cytocompatibility to both the osteoblast (MC3T3-E1) and myoblast (C2C12) cell lines whereas the gels of 16PF, 18PF and 20PF were very cytotoxic to the cells. The EDS loaded gels demonstrated significantly greater alkaline phosphatase (ALP) activities compared to the pure gels. The longer exposure time periods of the samples to the cells, the greater was the ALP activity. These EDS loaded gels significantly increased proliferation of both cell lines thus indicating a bone regeneration effect. The PF/MC blends prolonged the *in vitro* release of EDS for more than 28 days. Based on the *in vitro* degradation test, the MC extensively improved the gel strength of the PF and delayed the degradation of the gels thus making them more functional for a sustained drug delivery for osteogenesis.

1. Introduction

Hydrogels are hydrophilic polymeric networks that have been widely used for pharmaceutical and biomedical applications. They have created a great deal of interest owing to their numerous advantages such as their biodegradability and cell encapsulation. Exceptionally, thermoreversible hydrogels with an ability to undergo a phase transition from a sol to a gel form following an external stimulus, such as temperature, have been used for several applications in drug delivery (Fundueanu et al., 2009; Klouda et al., 2011; Misra et al., 2009), tissue engineering (Tang et al., 2010b), biosensors (Gant et al., 2010) and myocardial injection therapy (Nelson et al., 2011). Injectable hydrogels, in particular, can be used as encapsulation devices that is they are in a sol state at room temperature but form a gel at a physiological temperature. As the material is injected in the liquid state, it can maintain a high local concentration of a drug in the surrounding tissues. One of the examples of sol–gel reversible hydrogels includes pluronics [poloxamers or poly(ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) (PEO–PPO–PEO) triblock copolymers] that exhibit thermoreversibility based on micellization or micelle aggregation (Kohori et al., 2002; Neradovic et al., 2001). The thermoreversible nature of pluronic materials permit them to be used as a carrier for oral, topical, intranasal, vaginal, ocular and parentral administration (Escobar-Chávez et al., 2006). In more recent years pluronics have been explored for applications in tissue engineering and biomedical applications. Although pluronic has been used for the *in-situ* drug delivery of various drugs, there are certain issues of concern such as possible cytotoxicity, mechanical strength as well as their stability due to its delicate network. Pluronic F127 (Escobar-Chávez et al., 2006) (PF, PEO_{106}–PPO_{70}–PEO_{106}) has been used in this study. PF above 10% was found to be cytotoxic towards HepG2, HMEC-1 (endothelial) and L6 (muscle) cell lines (Khattak et al., 2005). For PF to form a thermoreversible gel, its concentration should be higher than 15% (Sun and Raghavan, 2010) which causes cytotoxicity. Nevertheless this cytotoxicity of PF can be reduced by using membrane stabilizing agents such as hydrocortisone, glucose and glycerol (Khattak et al., 2005).

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Methylcellulose (MC) is a polymer derived from cellulose that is widely used as a drug excipient. It undergoes thermoreversible gelation in the region of 50–70 °C, hence MC alone cannot be used as an injectable material because that would require it to be in the gel form at body temperature (37 °C). A blend of MC and chitosan in the presence of salts has been reported to be useful for applications in tissue engineering (Tang et al., 2010a). Furthermore, a blend of MC and PF can form a gel at body temperature in the presence of ammonium sulfate and this blend prolonged the release of docetaxel (Kim et al., 2012). MC can protect the cells from a physical stress (Kuchler et al., 1960) and has a stimulatory effect on cells (Mizrahi and Moore, 1970). Therefore, it was of interest to blend MC and PF and to investigate the possibility that perhaps the blend was a gel at body temperature. In addition, based on the known effects of MC on the cells, MC may be able to reduce the cytotoxicity of PF.

Bisphosphonates are pyrophosphate analogues that are resistant to hydrolysis and have exceptional affinity for bone tissue. Bisphosphonates have been known to inhibit the activity of osteoclasts (Czuryszkiewicz et al., 2005). The anti-resorption activities of bisphosphonates make them the drug of choice for several bone diseases such as paget's disease, multiple myeloma, bone metastases, hypercalcemia, osteoporosis and breast cancer (Czuryszkiewicz et al., 2005; Nancollas et al., 2006). However they have poor bioavailability (oral absorption less than 2%) that has resulted in investigating other administration routes such as intravenous, subcutaneous and intramuscular therapy (Salzano et al., 2011). However intravenous (IV) administration of bisphosphonates suffers another drawback as 30–80% of drug can be excreted through the urine and this may cause renal failure due to the formation of calcium complexes in the blood (Grainger, 2012). Thus localized delivery of bisphosphonates has, in recent years, focused on the ability to enhance their therapeutic efficiencies and minimize their adverse effects. At the present time liposomes, a microsphere based delivery system, have been explored for the local delivery of bisphosphonates (Czuryszkiewicz et al., 2005). Nitrogen containing bisposphonates are many times more potent than the non-nitrogen containing bisphosphonates such as etidronate sodium (EDS). In this work, EDS was the drug of choice since the latter may cause necrosis (Tanaka et al., 2013). Moreover nitrogen containing bisphosphonates were cytotoxic to periodontal tissues.

The aim of this present study was to probe the feasibility of incorporating EDS into the gels and check the blends of PF and MC for use as injectable thermoresponsive gels for local delivery of drugs. The interactions between MC and PF were determined using attenuated total reflection Fourier transform spectroscopy (ATR-FTIR). The viscosity of the blends was determined. The morphologies of the freeze dried gels were examined by environmental scanning electron microscopy (ESEM). The *in vitro* release of EDS and the degradation of the gels, PF and PF/MC, were also investigated. The *in vitro* cytotoxicity of pure PF, PF/MC gels towards MC3T3-E1, a murine osteoblast cell line and also C2C12, a murine myoblast cell line, was investigated. The alkaline phosphatase (ALP) activity, a routine method used to determine cell differentiation, of these gels with and without EDS was evaluated using both cell lines.

2. Materials and methods

2.1. Materials

Pluronic F127, PBS (phosphated buffer saline pH 7.4), MC (powder form; viscosity 10–25 mPa s for a 2% solution in H_2O at 20 °C; methoxyl content: 27.5–32%), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent, Triton X-100, p-nitrophenylphosphate, zinc acetate and EDS were from Sigma Co., St. Louis, MO, USA. Mouse osteoblastic cells (MC3T3-E1) and C2C12 cells were obtained from the ATCC, Manassas, VA, USA. All other chemicals (magnesium chloride, NaOH, carbonate buffer *etc.*) were all reagent grades obtained from RCI chemicals.

2.2. Preparation of samples

The PF solutions were prepared at various concentrations (16, 18 and 20% w/w) using a cold method as previously described (Lin et al., 2004). Briefly, required amount of PF was dissolved in MillQ water at 4 °C and refrigerated overnight to ensure that the polymer was thoroughly dissolved. For the PF/MC blends, MC was separately dispersed in distilled water with continued stirring at a cold temperature to produce 4% w/w solution. The appropriate amount of PF was then dispersed in the MC solution to obtain 12, 14, 16, 18 and 20% w/w of PF in MC (4% w/w) and these blends were referred to as 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC, respectively. The resulting combination was mixed thoroughly and refrigerated until the blend was completely dissolved.

This sample preparation method was also employed to prepare the samples for the determination of cytotoxicity. However, the PF and MC powder were UV sterilized before use and sterilized water was employed in these sample preparations as previously described (Li et al., 2014). The sample solutions, 0.2 g equivalence of PF or PF/MC, were added to a 24-well plate. After the samples formed a gel, PBS (1 mL) was added to the well, thus the concentration of the PF or PF/MC blend was 0.2 g/mL. The 24-well plate was incubated for 24 h and the PBS extract was filter sterilized. These extract solutions (100 μl) were further used for the cytotoxicity study (Jamuna-Thevi et al., 2011).

EDS has been previously found to be nontoxic at concentrations that ranged from 10^{-4} to 10^{-5} M to the various cell lines (Itoh et al., 2003; Tanaka et al., 2013). In this study, the pure drug solution at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M of EDS were used to determine the concentration-dependent cytotoxicity of EDS on MC3T3-E1 and C2C12 cell lines. These solutions were prepared by dissolving appropriate amounts of EDS in water. For the gel samples containing EDS, the required amount of the drug to obtain the final concentration of 4×10^{-3} M EDS, was dispersed to the 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC solutions, the mixtures were thoroughly mixed until the drug was completely dissolved and the mixtures were referred to as 12PF/MC/EDS, 14PF/MC/EDS, 16PF/MC/EDS, 18PF/MC/EDS and 20PF/MC/EDS, respectively.

2.3. ATR-FTIR measurement

ATR-FTIR spectra were recorded using a PerkinElmer Spectrum One spectrometer, equipped with an attenuated total reflectance (ATR) sampling device containing a ZnSe crystal. The spectra were scanned over the range of 4000–650 cm^{-1} using 64 scans at a resolution of 2 cm^{-1}. Prior to each scan, a background spectrum of water was scanned using the same parameters. The samples in the sol state were measured immediately after taking the samples from the refrigerator. The samples in the gel state were collected after incubating the sample at 37 °C. All samples remained in the same states before during and after measurements. Deconvolution of the FTIR spectra over the range from 1390 to 1366 cm^{-1} was performed using the GRAMS/AI (7.01) software (Thermo Galactic, Salem, NH, USA) by fitting the spectra with a Gaussian function.

2.4. Cell cultures

MC3T3-E1 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal