Colloids and Surfaces B: Biointerfaces 136 (2015) 207–213

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Biocompatibility of polysebacic anhydride microparticles with chondrocytes in engineered cartilage

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ARTICLE INFO

Article history:
Received 23 April 2015
Received in revised form 30 June 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Articular cartilage
Polysebacic anhydride
Microparticles
Delivery
Lipotoxicity
Hydrogel

ABSTRACT

One of main challenges in developing clinically relevant engineered cartilage is overcoming limited nutrient diffusion due to progressive elaboration of extracellular matrix at the periphery of the construct. Macro-channels have been used to decrease the nutrient path-length; however, the channels become occluded with matrix within weeks in culture, reducing nutrient diffusion. Alternatively, microparticles can be imbedded throughout the scaffold to provide localized nutrient delivery. In this study, we evaluated biocompatibility of polysebacic anhydride (PSA) polymers and the effectiveness of PSA-based microparticles for short-term delivery of nutrients in engineered cartilage. PSA-based microparticles were biocompatible with juvenile bovine chondrocytes for concentrations up to 2 mg/mL; however, cytotoxicity was observed at 20 mg/mL. Cytotoxicity at high concentrations is likely due to intracellular accumulation of PSA degradation products and resulting lipotoxicity. Cytotoxicity of PSA was partially reversed in the presence of bovine serum albumin. In conclusion, the findings from this study demonstrate concentration-dependent biocompatibility of PSA-based microparticles and potential application as a nutrient delivery vehicle that can be imbedded in scaffolds for tissue engineering.

1. Introduction

Articular cartilage, the connective tissue of diarthrodial joints, absorbs and distributes large compressive stresses placed on the joint, and enables low friction sliding. Treatment of diseased or damaged articular cartilage is challenging due to its avascular nature and poor self-repair capability [1,2]. Cartilage tissue-engineering techniques that encapsulate chondrocytes within a three-dimensional scaffold have been successful in promoting extracellular matrix deposition *in vitro* [3–5]. However, nutrient diffusion into the engineered tissue decreases over time due to the elaborated extracellular matrix on scaffold periphery. As a result, compressive mechanical and biochemical properties are lower in the center of the scaffold than the periphery [6]. One approach to overcome limited nutrient diffusion is to decrease the diffusion path length through macro-channels [6] or localized delivery of nutrients throughout the scaffold. Improved nutrient diffusion from macro-channels can be limited due to cells on the channel periphery depositing extracellular matrix into the channel and occluding the channel within a few weeks in culture [6]. In this study, we use nutrients-encapsulated microparticles to deliver nutrients throughout the scaffold.

There are several current and emerging applications that have used biocompatible polymers to fabricate delivery vehicles for drugs, genes, and vaccines [7,8]. One challenging factor of using delivery vehicles for nutrient distribution is the ability to accurately control the release profile and to provide uniform distribution of delivered nutrients. For example, transforming growth factor-β3 (TGFβ3), which is crucial for cartilage growth and development, must be delivered uniformly during the first two weeks in culture [9,10]. Therefore, an ideal polymeric delivery device should be biocompatible and exhibit a surface erosion profile with fast release kinetics (i.e., order of weeks).

Polyanhydrides are a class of biodegradable and biocompatible polymers that are used for sustained delivery of bioactive agents [11–13]. They have several advantages, including surface erosion properties and tunable degradation rates suitable for short-term release profiles [14,15]. Previous studies have used polyanhydrides to deliver vaccines, adjuvants, and proteins, and demonstrated that the immunogenicity of these actives is preserved [16–19]. Moreover, polyanhydride-based wafers are approved by the Federal

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http://dx.doi.org/10.1016/j.colsurfb.2015.08.040
0927-7765/

Drug Administration (FDA) for drug delivery to treat brain tumors [20].

Polymer degradation rate is highly dependent on molecular weight, hydrophobicity, pH, crystallinity, porosity, and surface area of the polymeric device [21–27]. Biocompatible polyanhydrides such as poly(sebacic anhydride) (PSA) prepared from sebacic acid can degrade at a faster rate than other biocompatible polymers (e.g., poly(esters)) [14,15,28]. Copolymerization of PSA with an aromatic anhydride (e.g., poly(carboxyphenoxy propane-co-sebacic anhydride), P(CPP-SA)) decreases degradation rate, compared to PSA-alone [14]. Polyanhydrides and their degradation products are considered non-cytotoxic [13,29–35], non-mutagenic [36], non-carcinogenic [13], and are extensively metabolized by rat brain tissue [37,38]. In particular, 80% of the sebacic acid was metabolized and eliminated as CO_2 [37,38]. Other cells, such as aorta epithelial osteoblast-like and smooth muscle cells, also did not show cytotoxic effects due to polyanhydrides [13,39]. However, recent work suggests that polyanhydrides are biocompatible for J774 macrophages ($0.5 - 1.25 \times 10^6$ cells/mL) for polymer concentrations up to 2.8 mg/mL, above which cytotoxicity was observed [40].

For nutrient delivery applications in cartilage tissue engineering, in situ release of sufficient bioactive molecules such as vitamin C would require a PSA microparticle concentration in excess of 50 mg/mL (see supplementary information). This necessary concentration (i.e., >50 mg/mL) for delivery of an appropriate concentration of nutrients throughout engineered cartilage may affect biocompatibility of polymeric microparticles due to accumulation of degradation by-products. Currently, there is no data in the literature that evaluates the biocompatibility and cytotoxicity of PSA with chondrocytes. Furthermore, there are no studies exploring the potential mechanisms of cytotoxicity or mitigation strategies. Without answering these questions, the utility of polyanhydrides (in particular PSA) as a short-term delivery device in articular cartilage tissue engineering will be very limited.

Therefore, the objectives of this study are to evaluate biocompatibility of PSA polymer with chondrocytes and to evaluate PSA-based microparticles as nutrients delivery device when embedded in agarose hydrogels for cartilage tissue engineering. Specifically, we found PSA polymer to be nontoxic to chondrocytes at or below 2 mg/mL PSA, above which cytotoxicity was observed. The mechanism of cytotoxicity at higher concentrations is deduced to be due to lipotoxicity because of structural similarities of PSA degradation products with fatty acids. Several mitigation strategies for PSA were tested and bovine serum albumin (BSA) treatment was found to be effective. The results from this study demonstrate potential use of PSA-microparticles with BSA protection as a nutrient or drug delivery vehicle in engineered cartilage.

2. Materials and methods

Chemicals required for synthesis of polymeric micro-particles were of analysis or higher grade: Sebacic acid (Alfa Aesar, Ward Hill, MA), methylene chloride (Acros Organics, New Jersey—US), poly(vinyl) alcohol (MP Biomedicals, Santa Ana, CA) petroleum ether (Fisher Scientific, New Jersey—US), acetic anhydride, ethyl ether, and amitriptyline hydrochloride from (Sigma Aldrich, St. Louis, MO). Aqueous solutions were made from Millipore™ water (with resistivity of ~18.1 MΩ cm).

2.1. Synthesis and characterization of PSA polymer

The poly(sebacic anhydride) (PSA) was synthesized using sebacic acid as a monomer by melt condensation method [12]. Recrystallized sebacic acid (5 mg) was mixed with 50 mL of acetic anhydride and stirred for 20 min at 40 °C until a clear solution was obtained. The excess acetic anhydride, after the de-hydration and acetylation of the diacid groups of sebacic acid monomer, was removed by increasing the temperature to 70–120 °C under vacuum. The precursor sebacic anhydride molecule was polymerized by increasing and maintaining temperature at 150 °C with constant stirring for 2 h. The final PSA product was dissolved in a minimum amount of methylene chloride and recrystallized using a large excess of ethyl ether and petroleum ether. The resulting suspension was centrifuged, dried at room temperature under vacuum, and stored at −20 °C. The chemical identity of the polymer was characterized using nuclear magnetic resonance (NMR) and vibrational spectroscopies which confirmed successful synthesis of the PSA polymer (see Supplementary information).

2.2. Fabrication of PSA microparticles and encapsulation of bioactive molecules

Fabrication of PSA microparticles with encapsulated nutrients was performed using a double emulsion method (water/oil/water) [41]. Methylene chloride was used as an oil phase and poly(vinyl) alcohol (PVA) as an emulsifier at the interface between the external water and oil phases. An evaporative process was used to eliminate methylene chloride leaving behind the polymer and encapsulated bioactives in the internal water phase. Alternatively, bioactive solids were encapsulated by using them as the internal phase (solid/oil/water) instead of their aqueous solution. In particular, 100 mg of PSA was dissolved in the 1 mL of methylene chloride and the bioactive internal phase, either as aqueous solution or solid phase, was emulsified/dispersed by ultra-sonication and homogenization for 60 s. A typical internal phase composition is 5–50% by weight of the encapsulating polymer [41–43]. Therefore, we set the internal phase at 50% by weight of PSA (i.e. 50 μL of aqueous bioactive to 100 mg of PSA) to maximize encapsulation. This water/oil emulsion was added drop-wise to 2 mL of the external phase consisting of aqueous solution of emulsifier (PVA) at 1 wt.% under continuous sonication and homogenization for 120 s. The obtained double emulsion was then diluted with 20 mL aqueous solution of 0.5% PVA and stirred vigorously for 4 h to evaporate the oil-phase (methylene chloride). A dense layer of polymer thus encapsulates the internal bioactive-phase forming microparticles. The encapsulated-microparticles suspension was centrifuged and washed with water three times. The precipitates were lyophilized and stored at −20 °C until use. The bioactive internal phases that are encapsulated include an aqueous solution of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate. The microparticles intended for the delivery in the tissue constructs were fabricated under sterile conditions. Both the internal and external water phases in the double emulsion, as well as water in all stages of fabrication procedure, were replaced with Dulbecco's modified essential medium (DMEM) solution.

Scanning electron microscopy (SEM) was used to characterize size and morphology of PSA microparticles. The microparticles were deposited on a carbon substrate and coated with a 2 nm coating of Au-Pd and SEM images were obtained with a voltage setting of 0.8 kV and current at 20 mA (4700-Hitachi SEM, Roslyn Heights, NY).

Previous work on release kinetics from nanogel particles demonstrated that the release profile of amitriptyline, a tricyclic anti-depressant drug, was easily monitored by ultraviolet-light absorption (UV-light; wavelength = 239 nm) [44,45]. While amitriptyline has limited applications for cartilage tissue engineering applications, ascorbic acid oxidizes easily in the presence of water and dissolved oxygen, making it difficult to evaluate its release kinetics. Therefore, we evaluated the release kinetics of PSA microparticles by encapsulating microparticles with amitripty-