Biocompatibility of Polysebacic Anhydride Microparticles with Chondrocytes in Engineered Cartilage

Sathish Ponnuram^{1†}, Grace D. O'Connell², Clark T. Hung³, and Ponisseril Somasundaran¹

¹Earth and Environmental Engineering, 500 W, 120th street, 918 Mudd Columbia University, New York, NY, 10027,

²Mechanical Engineering, 5122 Etcheverry Hall, University of California, Berkeley, CA 94720

³Biomedical engineering, 351 Engineering Terrace, 1210 Amsterdam Avenue, Columbia University, New York, NY, 10027

† Corresponding author, email: sp2522@columbia.edu, Fax: (212) 854-8362

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.

Keywords: Articular cartilage, polysebacic anhydride, microparticles, delivery, lipotoxicity, hydrogel

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].

1 Cartilage tissue-engineering techniques that encapsulate chondrocytes within a three-dimensional
2 scaffold have been successful in promoting extracellular matrix deposition *in vitro* [3-5]. However, nutrient
3 diffusion into the engineered tissue decreases over time due to the elaborated extracellular matrix on
4 scaffold periphery. As a result, compressive mechanical and biochemical properties are lower in the
5 center of the scaffold than the periphery [6]. One approach to overcome limited nutrient diffusion is to
6 decrease the diffusion path length through macro-channels [6] or localized delivery of nutrients
7 throughout the scaffold. Improved nutrient diffusion from macro-channels can be limited due to cells on
8 the channel periphery depositing extracellular matrix into the channel and occluding the channel within a
9 few weeks in culture [6]. In this study, we use nutrients-encapsulated microparticles to deliver nutrients
10 throughout the scaffold.

11 There are several current and emerging applications that have used biocompatible polymers to
12 fabricate delivery vehicles for drugs, genes, and vaccines [7, 8]. One challenging factor of using delivery
13 vehicles for nutrient distribution is the ability to accurately control the release profile and to provide
14 uniform distribution of delivered nutrients. For example, transforming growth factor- β 3 (TGF β 3), which is
15 crucial for cartilage growth and development, must be delivered uniformly during the first two weeks in
16 culture [9, 10]. Therefore, an ideal polymeric delivery device should be biocompatible and exhibit a
17 surface erosion profile with fast release kinetics (i.e. order of weeks).

18 Polyanhydrides are a class of biodegradable and biocompatible polymers that are used for
19 sustained delivery of bioactive agents [11-13]. They have several advantages, including surface erosion
20 properties and tunable degradation rates suitable for short-term release profiles [14, 15]. Previous studies
21 have used polyanhydrides to deliver vaccines, adjuvants, and proteins, and demonstrated that the
22 immunogenicity of these actives is preserved [16-19]. Moreover, polyanhydride-based wafers are
23 approved by the Federal Drug Administration (FDA) for drug delivery to treat brain tumors [20].

24 Polymer degradation rate is highly dependent on molecular weight, hydrophobicity, pH,
25 crystallinity, porosity, and surface area of the polymeric device [21-27]. Biocompatible polyanhydrides
26 such as poly(sebacic anhydride) (PSA) prepared from sebacic acid can degrade at a faster rate than
27 other biocompatible polymers (eg., poly(esters)) [14, 15, 28]. Copolymerization of PSA with an aromatic

1 anhydride (e.g., poly(carboxyphenoxy propane-co-sebacic anhydride), P(CPP-SA)) decreases
2 degradation rate, compared to PSA-alone [14]. Polyanhydrides and their degradation products are
3 considered non-cytotoxic [13, 29-35], non-mutagenic [36], non-carcinogenic [13], and are extensively
4 metabolized by rat brain tissue [37, 38]. In particular, 80% of the sebacic acid was metabolized and
5 eliminated as CO₂ [37, 38]. Other cells, such as aorta epithelial osteoblast-like and smooth muscle cells,
6 also did not show cytotoxic effects due to polyanhydrides [13, 39]. However, recent work suggests that
7 polyanhydrides are biocompatible for J774 macrophages (0.5 - 1.25 X 10⁶ cells/mL) for polymer
8 concentrations up to 2.8 mg/mL, above which cytotoxicity was observed [40].

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

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

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).

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 minutes 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 hours. 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).

Fabrication of PSA microparticles and encapsulation of bioactive molecules

Fabrication of PSA microparticles with encapsulated nutrients into 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 seconds. 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 μ L 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 seconds. The obtained double emulsion was then diluted with 20 mL aqueous solution of 0.5 % PVA and stirred vigorously for 4 hours 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 $^{\circ}$ 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 amitriptyline. Amitriptyline (15 mg) was dissolved in a phosphate buffered saline solution (PBS; 150 μ L) and was encapsulated in PSA (200 mg) using double emulsion, as described above. Amitriptyline encapsulated PSA microparticles (175 mg) were resuspended in 10 mL of

PBS. To measure the release concentration of amitriptyline from PSA microparticles, the suspension was centrifuged (7000 rpm for 5 minutes) and 5 mL of the supernatant was extracted once a week for three weeks. Fresh PBS (5 mL) was added at each time point to maintain the total volume. Amitriptyline standards were made in PBS (range 0 - 0.025 mg/mL) and used to determine the amount of amitriptyline released to the supernatant.

Tissue-engineering studies

Knee joints from bovine calves were acquired from a local abattoir. Articular cartilage was harvested aseptically and digested overnight in collagenase media (Type 4, Worthington Biochemical Corp. Lakewood, NJ). Chondrocytes were passaged in DMEM with 10% serum and a growth factor cocktail (0.5 ng/mL bFGF, 0.5 ng/mL PDGF, 1.0 ng/mL TGF β 1) [4]. Once cells reached confluence, they were removed from the culture dish by trypsinization.

Dosage-dependent biocompatibility was evaluated by encapsulating L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (stabilized-vitamin C) in PSA microparticles. PSA microparticles were incorporated into a 4% weight/volume (w/v) agarose solution (40°C, Sigma) at varying weight/volume percentages (0.2%, 2%, 3%, and 10% by w/v; 2, 20, and 100 mg/mL, respectively). Passaged cells (concentration of 60×10^6 cells/mL) were encapsulated in the agarose mixture for a final concentration of 30×10^6 cell/mL in 2% w/v agarose with PSA-microparticles. Each experimental group concentration was cast and studied in separate batches and thus had its own control without PSA microparticles.

Cylindrical constructs were cored from the agarose slab (diameter = 4 mm, thickness = 2.34 mm) and cultured in chemically defined medium (DMEM with 0.1 μ M dexamethasone, 40 mg/mL L-proline, 50 μ g/mL ascorbate 2-phosphate, 100 mg/mL sodium pyruvate, 1X ITS+premix, 100 U/mL penicillin, and 100 mg/mL streptomycin and amphotericin B (Invitrogen Co., Carlsbad, CA)). The medium was changed every three days and supplemented with 10 ng/mL of TGF β 3 during the first 14 days in culture. Cell viability was assessed using a live/dead cytotoxicity kit (Molecular Probes, Invitrogen), where living cells are stained with calcein-AM and dead cells are stained with ethidium bromide.

The efficacy of bovine serum albumin (BSA) as mitigation agent for cytotoxicity of PSA polymer (not microparticles) and its degradation products was evaluated. BSA protein was added during the casting process (at 1.1% w/v BSA and 3% w/v PSA polymer) and in the growth medium at 1 mg/mL. BSA was evaluated at 1:2 ratio of BSA to PSA (i.e. 1.1% w/v BSA and 3% w/v PSA). Experimental groups without BSA but with 3% w/v PSA or no PSA polymer served as controls.

Mechanical properties

The equilibrium Young's modulus (E_Y) and dynamic modulus (G^* , at 1 Hz) were measured under unconfined compression ($n = 3 - 7$ per group) on a custom-built mechanical tester. A 10% strain was applied at a rate of 0.05 %/s and held for 30 minutes. The equilibrium stress was used to calculate the E_Y . Following stress-relaxation, the dynamic modulus was measured by imposing 2% sinusoidal strain at frequency 1 Hz.

Biochemical properties

Following mechanical testing, the sample's wet weight was obtained and prepared for biochemical analyses. Constructs were lyophilized for 48 h to obtain the dry weight and digested overnight using Proteinase K at 56^o C. The agarose was separated by centrifugation as undigested mass and the supernatant was collected for biochemical analysis. Glycosaminoglycan (GAG) content was assessed using 1,9-dimethylmethylene blue (DMMB) dye-binding assay for measuring sulfated-GAGs. The GAG content was quantified using chondroitin-6-sulfate standards and reported as a ratio of constructs' dry weight. The amount of DNA present in the samples was measured using PicoGreen assay.

Statistical Analysis

All reported data are presented as averages with standard deviations. A one-way ANOVA was performed on mechanical and biochemical properties. Once significance was found, a post hoc t-test was performed to compare experimental groups with the control.

Results and Discussion

Characterization and release kinetics of polysebacic anhydride microparticles.

The PSA microparticles were spherical with sizes ranging from several hundreds of nanometers to few tens of microns (Figure 1). A 50% efficiency in amitriptyline encapsulation was observed during the fabrication process. The release profile of amitriptyline from the PSA microspheres was linear during the first two weeks (Figure 2). Within 2 weeks, approximately 60% of the encapsulated amitriptyline was released from the PSA microparticles, after which the concentration of the released drug was too low for detection (Figure 2). The fast release (< two weeks) and linear profile show that PSA-based polymeric microparticles can be utilized as carriers to provide sustained uniform delivery of nutrients.

Figure 1 A. Representative scanning electron microscopy (SEM) images of PSA-microparticles. B. High resolution image of a single PSA-microparticle from the same batch.

Figure 2 Amitriptyline release profile from PSA-microparticles (maximum standard deviation, SD = 9%, for n=4).

Dose dependent cellular viability in the presence of PSA

The dose-dependence biocompatibility of PSA microparticles with chondrocytes was determined using a 3D agarose hydrogels. Initial cell viability observed within 24 hours of hydrogel fabrication demonstrated little to no cell death for the 0.2% (2 mg/mL) PSA microparticles group (Figure 3A).

However, adding the PSA microparticles at a concentration of 2% (20 mg/mL) and 10% (100 mg/mL) resulted in widespread cell death throughout the construct (Figure 3B & C). As expected, the drop in cell viability subsequently affected tissue growth and mechanical properties of engineered cartilage (Figure 4; $p < 0.01$ versus control). Equilibrium and dynamic moduli of the 0.2% w/v group were not significantly different from the control ($p = 0.43$). Taken together, these results indicate that the non-cytotoxicity of PSA polymeric microparticles is limited to concentrations below 0.2 % w/v (2 mg/mL).

In contrast to the earlier studies on biocompatibility of polyanhydrides [13, 29-35], our results indicate cytotoxicity at higher concentrations of PSA microparticles. This agrees with recent work reported by Adler and coworkers [44], who found cytotoxic effects of polyanhydride copolymer (P(CPP-SA)) at concentrations at or above 2.8 mg/mL. However, the mechanism of sebacic acid polymer cytotoxicity at higher concentrations is not well understood. Moreover, no methods have been proposed to mitigate cytotoxicity of PSA polymer.

Figure 3 Cell viability of chondrocytes encapsulated in agarose hydrogel with PSA polymer at concentrations of (A) 0% w/v (control group), (B) 0.2% w/v (2 mg/mL) (C) 2% w/v (20 mg/mL) and (D) 10% w/v (100 mg/mL). Green color indicates living cells and red color indicates dead cells. Bar represents 50 μ m.

Figure 4 Compressive Young's modulus of engineered cartilage containing PSA-microparticles encapsulated with L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate. Separate tissue engineering studies were run to evaluate each concentration group; therefore, the values were normalized to the respective control group (i.e. constructs without PSA; represented by the solid black line). The E_Y of 0.2, 2, and 10 w/v% are reported for days 46, 52, and 14, respectively. The 10 w/v%

experimental group was discontinued after 14 days, due to lack of metabolic activity. SD=2.5-10%, n=3 per group. *Represents significant differences from the control group ($p < 0.01$).

Mechanisms of the cytotoxicity of PSA microparticles

Several factors contribute to inflammatory, cytotoxic, or genotoxic effect on cells, including changes in local pH, osmolality, disruption of cytoplasmic membrane, uptake of biomaterials beyond biocompatible limits, Ca^{2+} levels, lipotoxicity, and induction of reactive oxygen or nitrogen species [46-48].

It is already known that a change in local pH from the physiological value of 7.2 significantly decreases matrix synthesis from chondrocytes [49]. In particular, the pH of our growth medium dropped from 7.5 to 6.3 when PSA-microparticles were suspended at 30 mg/mL for 5 hours. However, increasing the buffer capacity of the culture medium (5mM HEPES buffer) demonstrated that a drop in local pH was not the primary cause for cell death. HEPES buffer stabilized pH of the culture medium at 7.2, but did not improve cell viability in the presence of PSA microparticles (Supplementary Information, Figure S3).

The cytotoxicity of PSA may be due to cellular uptake of degradation products beyond biocompatible limits. The main degradation product of PSA is polysebacic acid, which can be considered a fatty acid-like molecule due to the presence of carboxylic acid groups and long hydrophobic chains. It is well known that excessive intracellular concentrations of alkyl fatty acids can cause abnormal levels of reactive oxygen species ROS and even result in cellular death [48, 50], which is called lipotoxicity. If the chondrocytes ingest the PSA-microparticles or its degradation products, then the alkyl fatty acid-like concentrations inside cells can be expected to increase, causing lipotoxicity. The natural pathway by which cells cope with lipotoxicity is through the intracellular regulation of lipids by serum albumin proteins [51, 52]. That is, by maintaining a ratio of serum albumin/lipids of 0.7 - 2, cells are able to combat the adverse effects caused by increased concentrations of fatty acids [52].

Thus, we hypothesized that if cytotoxicity was caused by lipotoxicity, the addition of bovine serum albumin (BSA) can mitigate cytotoxicity of PSA degradation products. To test this, BSA was added to the

agarose hydrogel during casting and the culture medium was supplemented with 1 mg/mL of BSA throughout the culture period. BSA significantly decreased cytotoxicity from PSA microparticles at a concentration of 3% w/v (Figure 5). At day 30, equilibrium compressive Young's and dynamic moduli of BSA-protected constructs were comparable to the PSA-free control (Figure 5A, and 5B). The biochemical composition, GAG and DNA content (Figure 5C, and 5D day 60), were also similar for these two groups.

These findings demonstrate that BSA can mitigate cytotoxic effects of PSA polymer, which presents a potential to use PSA microparticles as a delivery vehicle in engineered cartilage constructs. This finding of cytotoxic effects of hydrophobic anhydride polymers is important, since it has been earlier suggested that these polymers, when hydrolyzed to carboxylic acids, can act as nutrients for cells [53, 54].

Figure 5 Compressive mechanical and biochemical properties of engineered cartilage constructs with BSA protection from PSA degradation byproducts. (A.) Equilibrium Young's modulus (E_v), (B.) Dynamic modulus (G^*), and (C) GAG content, (D) DNA content. Groups include BSA- and PSA-free control (solid), PSA-polymer without BSA (dots), and PSA-polymer with BSA (slant). The amount of GAG and DNA are reported in grams per gram of wet construct. SD=2-16%, n=4 per group. *Represents: No significant differences were observed between the control and BSA protected constructs ($p > 0.13$), .

Conclusions

Polysebacic anhydride (PSA) polymer and PSA-based microparticles were evaluated for biocompatibility with chondrocytes and were tested for short-term (2 weeks) and sustained release of encapsulated nutrients within an agarose hydrogel for articular cartilage tissue engineering. PSA microparticles were biocompatible with chondrocytes at concentrations below 2 mg/mL and were found to be cytotoxic at a loading concentration of 20 mg/mL. The mechanism of cytotoxicity is suggested to be due to the intracellular accumulation of PSA or its fatty acid-like hydrolysis products and the resulting lipotoxicity. Bovine serum albumin (BSA) was found to offer the best protection from this adverse effect. Incorporating BSA into the hydrogel and culture system increased the usable PSA-microparticles towards 3% w/v (30 mg/mL) within the agarose hydrogel. In this study, we only evaluated one concentration of BSA with PSA polymer. Future work will evaluate whether BSA protection is maintained for higher doses of PSA polymer (e.g. 50-200 mg/mL). In conclusion, the findings reported here show the importance of determining dose dependent effects of PSA on biocompatibility and the potential use of PSA microparticles as a delivery vehicle for cartilage tissue engineering applications.

Acknowledgements

This material is based upon work partially supported by NIH grant AR46568, NSF under Grant 0749461, as well as by NSF and the Environmental Protection Agency under Cooperative Agreement EF 0830117. We acknowledge partial support from Center for Particular and Surfactant Systems (CPaSS) at Columbia

University and from the Tata Research Development and Design Center, Pune, India. We also thank Dr. Irina Chernyshova for her corrections and suggestions.

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