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In vivo acute and humoral response to three-dimensional porous soy protein scaffolds

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ABSTRACT

Increasing interest in using soy biomaterials for tissue engineering applications has prompted investigation into the in vivo biocompatibility of soy implants. In this study, the biocompatibility of soy protein scaffolds fabricated using freeze-drying and 3-D printing was assessed using a subcutaneous implant model in BALB/c mice. The main objectives of this study were: (1) to compare soy protein with bovine collagen, a well-characterized natural protein implant, by implanting scaffolds of the same protein weight, and (2) to observe the effects of soy scaffold microstructure and amount of protein loading, which also alters the degradation properties, on the acute and humoral immune responses towards soy. Results showed that freeze-dried soy scaffolds fully degraded after 14 days, whereas collagen scaffolds (of the same protein weight) remained intact for 56 days. Furthermore, Masson's trichrome staining showed little evidence of damage or fibrosis at the soy implant site. Scaffolds of higher soy protein content, however, were still present after 56 days. H&E staining revealed that macrophage infiltration was hindered in the denser bioplotted soy scaffolds, causing slower degradation. Analysis of soy-specific antibodies in mouse serum after implantation revealed levels of IgG_1 that correlated with higher scaffold weight and protein density. However, no soy-specific IgE was detected, indicating the absence of an allergic response to the soy implants. These results demonstrate that soy protein could be an acceptable biocompatible implant for tissue regeneration, and that scaffold porosity, soy protein density and scaffold degradation rate significantly affect the acute and humoral immune response.

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1. Introduction

Soy protein is a natural, renewable and abundant resource readily extracted and isolated from whole soybeans. Versatility in soy protein processing has fueled interest in using this material for tissue engineering [1,2] and wound healing [3,4] applications. Initial evaluations of soy as a biocompatible biomaterial *in vitro* have established that cells remain viable when seeded on soy structures [5–7] or when soy is dissolved in cell culture media [8]. Soy protein membranes prepared by casting solutions of soy protein in water and air-drying passed both direct contact (scanning electron microscopy (SEM) observation) and indirect contact (Dulbecco's modified Eagle medium extraction and MTT viability) biocompatibility assays using L929 mouse fibroblasts [5]. Human mesenchymal stem cells have been shown to adhere and proliferate *in vitro* on porous soy protein scaffolds fabricated through freeze-drying and three-dimensional (3-D) printing [6,7]. Soy granules made from defatted soy (containing soy protein) have been shown to stimulate the synthesis of collagen [9] and mineralized bone noduli [8] *in vitro*, as well as promote the formation of trabecular bone in a femoral critical sized defect *in vivo* [10]. The inherent bioactivity of soy and the achievable range of structural and mechanical properties with soy structures [6,7,11] demonstrate that soy can be a promising biomaterial for a variety of tissue engineering targets.

Soy protein contains all essential and non-essential amino acids including cysteine [12]. Cysteines allow for disulfide bonding during heat treatment, which can affect protein solubility and mechanical properties of casted products [12]. Soy has unique intermolecular and intramolecular forces, allowing for a wide range of gelation properties [13]. Hydrogen bonds, disulfide bonds and hydrophobic interactions resulting from the molecular structure of soy are necessary for the formation and maintenance of 2-D and 3-D stable structures [6,13]. Studies from the food and

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biomedical industries have demonstrated that soy protein materials can be fabricated into a variety of structures with varying properties, including films [14–16], hydrogels [17] and scaffolds [6,7]. Therefore, soy protein serves as an ideal material for studying the effects of scaffold microstructure on the biological response *in vitro* and *in vivo*.

As with any novel material being developed as an implant, there is a demand for further biocompatibility characterization of soy protein structures *in vivo*. This is particularly relevant in the case of soy, since spontaneous allergy to soy affects 0.4% of children; the levels of soy-specific IgE are predictive of disease progression [18]. To date, the *in vivo* biocompatibility of soy has mostly been explored with soy as a composite material mixed with other natural materials such as chitosan [19] and cellulose [20] in the form of membranes and sponges. In this study, the safety of implanting soy protein scaffolds fabricated by freeze-drying and 3-D printing was assessed with two main objectives. The first objective was to compare soy protein with collagen, a commonly used FDA-approved natural protein implant material. The second objective was to observe the effects of scaffold microstructure and amount of protein loading, which also alters the degradation properties, on the immune response towards soy. These 3-D porous soy protein scaffolds were hypothesized to be degradable with resolvable inflammation levels by tuning the open porosity of the scaffolds, which can also promote cell infiltration and better integration of the scaffold with surrounding tissue.

2. Materials and methods

2.1. Fabrication of freeze-dried collagen and soy scaffolds

Soy protein isolate containing 87.6% protein (Solae LLC, St Louis, MO) as determined by the combustion method [21] was used for this study. Both 1 and 3 wt.% soy protein slurries were formed by mixing with glycerol (1:1 weight ratio to soy protein) in MQ water. Glycerol was added as a biocompatible plasticizer specifically required for the soy scaffold fabrication process to achieve porous structures [7]. Solutions were homogenized for 5 min, and the 1% and 3% solutions were casted into 7 cm aluminum dishes in volumes of 13 ml and 30 ml, respectively to achieve scaffolds of similar weight when punched with a 6 mm punch. Collagen slurries were fabricated by dissolving 1 wt.% bovine type I collagen (Sigma–Aldrich, St Louis, MO) in 0.05 M acetic acid (Sigma–Aldrich) and homogenizing for at least 30 min until all protein was dissolved. Slurries were vacuumed until all air pockets were removed, and 25 ml of the slurry was casted into 7 cm aluminum dishes. Upon casting, all slurries were immediately lyophilized in an AdVantage BenchTop lyophilizer (VirTis, Gardiner, NY) using a procedure described previously [7]. Briefly, slurries were frozen at a rate of $-0.5\ ^{\circ}C\ min^{-1}$ down to $-15\ ^{\circ}C$ and held at temperature for 5 h, then dried at a pressure of 100 mtorr for at least 2 days. All freeze-dried scaffolds were punched using a 6 mm biopsy punch. The weights of the scaffolds were measured prior to surgery preparations. Soy scaffolds of 1 and 3 wt.% with weights of 1.65 ± 0.1 and 14 ± 0.5 mg were chosen for surgeries. The heights of 1% collagen scaffolds were trimmed using micro-scissors to achieve equivalent weight for comparison with 1% soy scaffolds.

2.2. Fabrication of bioplotted soy scaffolds

Slurries consisting of 20 wt.% soy protein and 4 wt.% glycerol in MQ water were fabricated and printed into 3-D scaffolds with controlled pore size and geometry using the Bioplotter (EnvisionTEC GmbH, Germany) employing a previously described method [6]. Briefly, the mixed slurries were sieved sequentially through autoclaved 297 μm and 105 μm pore size sieves and printed through a 200 μm needle with inter-strut spacing of 400 μm. Every other layer was rotated 90°, with a lateral shift of 0.5 mm every four layers to achieve an overlapping conformation to minimize through pores and enhance cell seeding efficiency. Scaffolds were fabricated to the given height of 12 layers with a layer spacing of 250 μm to achieve equivalent weight for comparison with 3% soy protein freeze-dried scaffolds (14 ± 0.5 mg).

2.3. Characterization of scaffold structure using SEM and porosimetry

SEM was performed using a LEO Gemini 1525 FEG SEM with an acceleration voltage of 15 kV (Oberkochen, Germany) to observe and compare the microstructure of the various scaffolds. Scaffolds were coated with 10 nm of osmium using an osmium plasma coater (Filgen, Structure Probe, West Chester, PA) prior to imaging. A mercury intrusion porosimeter (Micromeritics, Norcross, GA) was used to determine the volume percent porosity and pore size diameter distributions for all groups ($N = 3$) using a previously described method [22].

2.4. Implantation of 3-D scaffolds

Scaffolds were immersed in 70% ethanol for at least 12 h and soaked in phosphate buffered saline (PBS, HyClone, Logan, UT) overnight prior to surgery. Female BALB/c mice age 6–8 weeks were used for this study ($N = 6$ per group per time point, 96 mice total). Mice were anesthetized using isoflurane, and a 5–6 mm incision was made on the shaved dorsal side. Forceps were used to create a subcutaneous pocket in which one scaffold was placed per mouse. Non-absorbable nylon sutures were used to close the incision site. Mice were killed at days 1, 14, 28 and 56 after scaffold implantation. The surgical protocol followed NIH guidelines for the care and use of laboratory animals and was approved by Northwestern University's Animal Care and Use Committee (Chicago, IL, USA).

2.5. Assessment of implant degradation

The size of the implants in the mice was measured using digital calipers every 3 days after surgery. The implant volume was assumed to be the cylindrical area underneath the skin. The thickness of the scaffold (h) was measured, and the diameter of the implant under the skin (d) was measured three times and averaged. The implant volume was calculated using the formula $\pi \cdot (d/2)^2 \cdot h$ and observed for all mice with a palpable implant.

2.6. Histology of implanted scaffolds

Subcutaneous implants were excised and fixed in 10% neutral buffered formalin. Control tissue was obtained by excising an isolated area of the lower dorsal side of a mouse that had no contact with the implant. Tissues were dehydrated using graded ethanol solutions and embedded in paraffin wax. The samples were sectioned into 4 μm thick sections. Sections were deparaffinized, rehydrated and stained using routine hematoxylin and eosin (H&E) stain and Masson's trichrome stain.

2.7. Evaluation of serum immunoglobulin levels using ELISA

Naive mice ($N = 5$) were immunized with 100 μl soy protein (10 μg) with aluminum hydroxide adjuvant (3 mg) on days 0 and 7 and sacrificed at day 14, to establish a reference sera pool for comparing serum from implanted mice for all assays performed. Sera of scaffold implanted mice were collected at each time point, and the soy-specific antibody levels were detected using enzyme-

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