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In Vivo Biodegradation and Biocompatibility of PEG/Sebacic Acid-Based Hydrogels using a Cage Implant System

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Abstract

Comprehensive in vivo biodegradability and biocompatibility of unmodified and Arg-Gly-Asp (RGD) peptide-modified PEG/Sebacic acid based hydrogels were evaluated and compared to the control material poly(lactide-co-glycolide) (PLGA) using a cage implantation system, as well as direct subcutaneous implantation for up to 12 weeks. The total weight loss after 12 weeks of implantation for unmodified PEGSDA and RGD-modified PEGSDA in the cage was approximately 42% and 52%, respectively, with no statistical difference ($p > 0.05$). The exudate analysis showed that PEGSDA hydrogels induced minimal inflammatory response up to 21 days following implantation, similar to the controls (empty cage and the cage containing PLGA discs). Histology analysis from direct subcutaneous implantation of the hydrogels and PLGA scaffold showed statistically similar resolution of the acute and chronic inflammatory responses with development of the fibrous capsule between the PEGSDA hydrogels and the control (PLGA). The cage system, as well as the histology analysis, demonstrated that the degradation products of both hydrogels, with or without RGD peptide modification, are biocompatible without statistically significant differences in the inflammatory responses, as compared to PLGA.

Keywords

In vivo biocompatibility; In vivo biodegradation; PEG sebacic acid diacrylate; Hydrogel; RGD-modified hydrogel; Cage implantation

Introduction

To determine the in vivo biocompatibility of a variety of potential biomaterials, researchers have sought numerous evaluation techniques. One of the most widely used methods for in vivo biocompatibility evaluation is based on histological or morphological analysis of adjacent tissues and/or cell responses to the implant.¹ Another approach is to determine cell function at the implant-tissue interface using enzyme histochemical techniques.² Over the past decades, Anderson's group has developed a novel technique towards in vivo evaluation of biocompatibility using stainless steel cage implantation.^{3,4} This cage implant system has been used to evaluate the dynamic nature of cell function at the site and proven to be sensitive,

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reliable, reproducible, and quantifiable without sacrificing animals at each time period.^{5,6} It also allows for the measurement of in vivo biodegradation characteristics of the implants.

Synthetic hydrogels have been widely used for biomedical applications, such as tissue engineering.^{7,8} However, the principle limitation to more extensive use of synthetic hydrogels is their lack of mechanical strength and/or biodegradability.⁸ To make synthetic hydrogels a more attractive candidate for tissue engineering applications, our laboratory has been developing relatively strong biodegradable hydrogel systems, compared to conventional hydrogels, such as PEG diacrylate (PEGDA).⁹ We have recently synthesized a novel biodegradable hydrogel system based on PEG and sebacic acid macromer terminated with acrylate groups (PEGSDA). It swells much less and is stronger compared to PEGDA, allowing it to maintain its structural integrity during tissue regeneration. Although this hydrogel system showed minimal cytotoxicity and excellent bioactivity in vitro, further evaluation of both in vivo biocompatibility and biodegradability is a prerequisite before extensive use of the hydrogel as a tissue engineering material as well as cell/drug delivery vehicle.⁹

The purposes of this study are 1) to determine the in vivo biodegradation characteristics and inflammatory responses of PEGSDA-based hydrogels and 2) to compare them with those of PLGA scaffold, which is one of most widely used biodegradable and biocompatible materials in biomedical application. The hypotheses are 1) PEGSDA-based hydrogels degrade faster in vivo than in vitro, 2) inflammatory response of the hydrogels is similar to that of PLGA control, and 3) RGD peptide modification of the hydrogel would not affect its in vivo biodegradation and biocompatibility characteristics. To test these hypotheses, we used the cage implantation system and histology to evaluate comprehensive in vivo biocompatibility and biodegradability of PEGSDA hydrogels and compared the results with a conventional synthetic polymer PLGA control. We investigated the extent and duration of inflammatory responses caused by implanted materials and also determined adjacent tissue responses and fibrous capsule formation by direct subcutaneous implantation into the back of rats.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Preparation of materials

PEGSDA was synthesized as previously described,⁹ and acrylated RGD peptide was synthesized by following a previous method.^{10,11} Their chemical structures are shown in Figure 1. The peptide with MW 3400 spacer arm was copolymerized with PEGSDA hydrogel. Briefly, 200 mg of PEGSDA prepolymer was dissolved in double-distilled water (DDW) (50% w/v) containing 0.05% photoinitiator (Irgacure D-2959, Ciba Geigy, Tarrytown, NY). One μ M of acrylated RGD peptide was added to the polymer solution. The final surface concentration of the peptides was one pmol/cm², estimated from the bulk concentration assuming that the peptide within 10 nm from the surface of a hydrogel was available.¹⁰ The solution was poured onto a glass plate and crosslinked under the UV light for 10 min. The hydrogel film was then lifted from the plate and immersed in DDW overnight to remove any remaining unreacted oligomers. After equilibration, the fully swollen hydrogel films were cut using a cork borer (diameter 10 mm). PLGA with a 85:15 lactic to glycolic acid ratio (Medisorb, Cincinnati, OH) was dissolved in methylene chloride, and the solution was cast into a mold to obtain discs of 10 mm diameter. The hydrogel and PLGA discs were disinfected by soaking in 70% ethanol for 1 day, and subsequently incubated in sterile PBS solution for 2 days.

Subcutaneous implantation of cages and hydrogels

Adult female Sprague Dawley rats (Charles River, MA), weighing 250–300 g were divided into four groups of six rats. All surgical procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic College of Medicine. Animals were anaesthetized with 80 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg/kg xylazine (Ben Venue Laboratories, Bedford, OH) by intraperitoneal (IP) injection. Cylindrical cages, with dimensions of 3.5 cm in length and 1 cm in diameter, were fabricated using 304 stainless-steel wire mesh with a mesh size of 24 and wire diameter of 0.3 mm (Cleveland Wire and Mesh Company, Cleveland, OH). The materials were inserted into the sterile stainless steel cages. The cages containing test materials and control empty cages were implanted subcutaneously into the lower back of the rats. Another 1 cm incision was made on the lower back of the rats, and the test material discs (10 mm diameter and 2 mm thickness) were directly implanted subcutaneously. Both incisions were closed using absorbable sutures. Postoperatively, animals were given buprenorphine 0.05 mg/kg subcutaneously for pain for the first 48 hours and Lactated Ringer's solution as needed.

Exudate analysis and biodegradation from cage explants

Inflammatory exudate analysis was conducted at 4, 7, 14, and 21 days post implantation, as previously described^{3,12}. Briefly, 0.2 mL of inflammatory exudate was aspirated from each cage using 1 mL tuberculin syringe. A drop from each exudate sample was cultured onto a brain-heart infusion plate to verify the absence of infection. The total leukocyte count was determined by hemocytometer using 10 μ L of exudate, mixed with 40 μ L of PBS-modified Wright's stain solution. The volume of each exudate sample containing 20,000 cells was calculated and mixed with RPMI to a total volume of 200 μ L. The cell mixture was spun at 700 rpm for 10 min onto microslides using Cytospin 2 (Shandon Inc., Pittsburgh, PA). The slides were stained with modified Wright's stain and differential cell counts of polymorphonuclear leukocytes (PMNs), macrophages, and lymphocytes were conducted. To measure the in vivo biodegradation rate, the remaining explants were removed after one or two months post-implantation. The explants were rinsed twice with distilled water, and subsequently with 1% Triton-X solution to remove the cells. All specimens were lyophilized and remaining mass was determined gravimetrically.

Tissue response evaluation

All animals were euthanized one or two months after implantation, with intramuscular (IM) injections of pentobarbital (Fort Dodge Animal Health, Fort Dodge, IA) and fixed by transcardial perfusion with 4% paraformaldehyde. The surrounding subcutaneous tissues with the implanted disks were removed. Samples were dehydrated in sequential ethanol and Hemo-De solutions, embedded in paraffin, and 8 μ m thick sections were cut with a tabletop microtome. Sections were stained with standard hematoxylin and eosin (H & E) and Masson's trichrome stains for histological examination. Tissue analysis was performed from all animal groups. Briefly, sections were washed and stained with hematoxylin for 10 min, rinsed, then stained with Biebrich scarlet for 5 min. Following rinsing and placing in phosphotungstic/phosphomolybdic acid for 10 min, the sections were transferred into Aniline blue, rinsed in 1% acetic acid, dehydrated, cleared, and cover-slipped. The collagen deposition for each specimen at two months after the surgery was determined by measuring the fibrous capsule thickness at least two readings per slide of three slides. Immunohistochemistry analysis was performed with biotin anti rat CD45 clone OX1 (BioLegend, San Diego, CA 92121) at a dilution of 1: 50 for lymphocytes and T cells and mouse anti-human CD68 clone PGM1 at a dilution of 1: 40 for macrophages, respectively (Dako Corp., Carpinteria, CA). Mouse anti-human CD68 clone PGM1 and tested on paraffin embedded sections for rat tissue and a negative control. The specificity was confirmed for CD68 which had reactivity for rat tissue

for both frozen and paraffin embedded tissue for IHC analysis. Stained sections were imaged on a Zeiss Axioplan II microscope using an AxioCam digital camera. Image analysis was performed using a digital image analyzing system (KS 400 Imaging System Release 3.0, Carl Zeiss Vision, Echting, Germany). Stained area measurements were calculated using the pixel calibration (both x and y) divided by the objective lens magnification.

Statistical analysis

All data are represented as means $\pm$ standard deviation. Statistical analysis was performed using either a student *t*-test for direct comparison between treatment groups and control or single factor analysis of variance (ANOVA) with Tukey-Kramer method for multiple comparison tests at a significance level of $p < 0.05$.

3. Results

PEGSDA macromer were successfully synthesized from initial monomers, e.g. PEG (MW: 1,000 Da) and sebacic acid. PLGA (85/15, lactide to glycolide ratio) was used as a control due to its well known biocompatibility and biodegradability.¹³

In vivo degradation of PEGSDA hydrogels

The structural integrity of the implants in the cages was well maintained during the experiment, and the implants were easily retrieved from the cage without any damage for further evaluation. The weight loss of PEGSDA-based hydrogels and PLGA in the cages over 12 weeks was measured to determine in vivo biodegradation rate, as well as the effect of RGD peptide incorporation on these properties. To evaluate in vivo degradation characteristics of PEGSDA hydrogels, the geometry of each material was monitored throughout the time course. The apparent in vivo degradation profiles of the hydrogels and PLGA were compared by measuring the weight loss of the materials over time. After 12 weeks of implantation in the cages, the weight losses of PEGSDA, RGD modified PEGSDA (PEGSDA+RGD) and PLGA were $41.6 \pm 2.1\%$, $52.1 \pm 19.7\%$, and $32.1 \pm 15.5\%$, respectively, without any significant differences among the test groups (Fig. 2). The degradation profiles of PEGSDA hydrogels showed that the weight loss of hydrogel during the first 4 weeks was minimal (4.3%), however, the hydrogel rapidly degraded thereafter, indicating that PEGSDA hydrogels degraded mostly by bulk degradation, similar to the degradation profile of PLGA.¹³

Acute inflammatory responses

Figure 3 shows typical distribution and morphology of various leukocytes in the exudates. It was observed that there were some degenerating neutrophils (PMNs, polymorphonuclear leukocytes) or reactive lymphocytes in the exudates, indicating that the concentration of neutrophils was decreasing and leukocytes were actively interacting with the materials.

Total and differential leukocyte concentrations in the cage implant exudates over time are shown in Fig. 4. No significant changes in total and differential leukocyte concentrations were observed during the tested time periods. The presence of PEGSDA hydrogels inside the cage did not cause any significant inflammatory change, as measured in total leukocyte concentrations throughout the time courses (Fig. 4A) compared to empty cage or PLGA-containing cage control, which are known to produce minimal inflammatory responses.¹⁴ Regarding differential leukocyte concentration, neutrophils were dominant during the first several days and rapidly decreased afterwards, indicating a typical acute inflammatory response to the implants. The rapid increase in lymphocyte concentration at later time periods in all treatment groups was seen, since the rats are lymphocyte predominant animals (Fig. 4B), as opposed to humans who are neutrophil predominant with regard to blood inflammatory response. However, the number of white cells in the exudates of all treatments was significantly

reduced during the time periods of 21 days, indicating that the acute inflammatory response subsided over time. RGDS peptide incorporation into the PEGSDA hydrogel did not significantly affect leukocyte concentrations in the exudates.

Direct subcutaneous implantation

Two months after the surgeries, tissue samples surrounding the implants were histologically analyzed. A comprehensive tissue response to the implants based on immunohistochemistry analysis was utilized in addition to H & E and Trichrome staining (Fig. 5). In general, no obvious adverse chronic inflammatory responses were found in the H & E staining of different implants and the staining images showed normal wound healing response to the materials. In this study, we also utilized CD45 and CD68 clones to detect the presence of lymphocytes or T cells and macrophages, respectively. Immunohistochemistry analysis showed that there were significantly less activated inflammatory cells around PEGSDA-based implants than PLGA implant (Fig. 6A). A thin layer of fibrous capsule stained with Trichrome was observed around the implants and the capsular thickness was measured (Fig. 6B). General trend was found that PEGSDA-based hydrogels tend to develop less fibrous capsule than PLGA. The fibrous capsule thickness in PEGSDA+RGD was significantly thinner ($p < 0.05$) than PLGA although there was no significant difference in fibrous thickness between PLGA and PEGSDA implants. Overall, these results demonstrated that PEGSDA-based hydrogels showed minimal inflammatory response when implanted in vivo, similar to the control (PLGA), which has been shown to be biocompatible in implant studies.¹⁵

4. Discussion

Synthetic polymeric materials to be used for biomedical applications should be biocompatible and should not be carcinogenic or immunogenic when implanted into the host.¹⁶ For tissue engineering applications, biodegradability of synthetic materials is also crucial, and the degradation rate need to be required to match the rate of tissue formation. Bioactivity is another requirement for synthetic materials to interact with surrounding cells towards wound healing or tissue regeneration.¹⁷ In the previous report, we have developed new exciting class of biodegradable hydrogels based on PEG and sebacic acid (e.g., PEGSDA) and have shown to be biocompatible and biodegradable in vitro and were equally bioactive with or without modification with cell adhesion peptides, such as RGDS. As mentioned previously, these hydrogels have two main building blocks, PEG and sebacic acid and PEG is supposed to render resulting macromers biocompatible due to its well known biocompatibility and sebacic acid makes the hydrogels absorb less water, thus increase mechanical properties due to its hydrophobic nature.^{9,18} We also evaluated the cytotoxicity of initial PEGSDA macromers before crosslinking and final degradation products by culturing rat-derived bone marrow stromal cells (MSCs) in the presence of these molecules and found no change in metabolic activity of the cells compared to those cultured on control tissue culture polystyrene.⁹ Although in vitro cellular responses to PEGSDA-based hydrogels appear to be promising, the evaluation of in vivo performance of the materials is a pre-requisite to eliminate the biocompatibility concern of the materials.

In current study, we further evaluated in vivo biodegradability and biocompatibility of PEGSDA hydrogels using a cage implantation system to assess inflammatory response of the materials and then we also evaluated tissue response using direct subcutaneous implantation of the materials. One of the advantages of the cage implantation system is the ability to evaluate the dynamic nature of inflammatory cell function at the implant site throughout the time course without sacrificing animals at each time period.¹⁹ Therefore, it is possible to determine the duration and extent of the inflammatory responses, which indicate in vivo biocompatibility of

the implanted materials.⁴ Another advantage of the system is that it provides additional in vivo biodegradation characteristics of the materials.

The stainless steel cages containing test articles were implanted into rats for up to 12 weeks to determine inflammatory response, as well as in vivo biodegradation characteristics. We expected that in vivo degradation rates of the PEGSDA hydrogel were faster than in vitro, as demonstrated by others with other materials due to enzymatic and/or cellular interaction at the interface of the material and tissue.¹³ As compared with our previously reported in vitro degradation profiles of PEGSDA hydrogels,⁹ there was no significant difference between in vitro and in vivo degradation rate of this specific hydrogel. We can speculate that non-specific protein adsorption such as albumin or immunoglobulin G (IgG), both of which are known to hinder the cellular interaction with the materials may prevent to accelerate in vivo degradation due to the hydrophobicity of the materials.²⁰ Regarding the effect of RGD peptide on the in vivo degradation rate, there was no significant difference in degradation profiles was detected between PEGSDA and PEGSDA+RGD hydrogels, although the trend showed that the weight loss of PEGSDA+RGD was greater than PEGSDA throughout the test periods. In general, RGD peptide is known to trigger significant interactions with surrounding cells which can trigger rapid degradation of a material.²¹ However, the peptide modification in this study appeared to show minimal interaction between surrounding cells and the materials likely due to the small amount of the peptide in the polymer network.²² Further, it is also possible to assume that since these materials are hydrophobic, non-specific protein adsorption may essentially 'hide' any cellular interaction with the materials.

The inflammatory cell analysis in the exudates from the cages revealed that there was no significant difference between each test group and the control (empty cage). Total leukocyte concentration data showed a typical acute and chronic inflammatory response over time regarding the number of leukocytes in the exudates (Fig. 4A), in which PMNs were predominant during the first 7 days (acute inflammatory response), and macrophages and lymphocytes are dominant thereafter, which represents typical chronic inflammatory response.²³ Further evaluation may be needed to determine the extracellular matrix protein concentration, such as alkaline phosphatase in the exudates, which may be helpful in understanding the interaction between the constituents of the exudates and the surface of the implants.¹⁹

In addition to the cage implant system, which provides a simple and effective means to examine in vivo biocompatibility of biomaterials by monitoring inflammatory cell responses of the exudates withdrawn from the animal, we further evaluated wound healing response associated with the implants by using histology and immunohistochemistry. Tissue response to the implanted materials showed a substantial amount of inflammatory cells in the tissues, regardless of the type of implants at two months after the surgeries (Fig. 5), likely due to an apparent active in vivo biodegradation process.²² Further, histology and immunohistochemistry revealed that PLGA implant showed normal wound healing response as evidenced by the significant presence of mononuclear cells (stained with CD45) such as lymphocytes which is considered chronic inflammation as well as macrophages (stained with CD68),²³ possibly foreign body giant cells (FBGCs) at the surface of the implant (Fig. 5). However, PEGSDA-based implants showed significantly less inflammatory cells, compared to PLGA implant. Fibrous capsule formation around PEGSDA and PEGSDA+RGD hydrogel implants was similar or significantly less as compared to the PLGA implant. In general, thick fibrous capsule formation should be avoided to facilitate mass transfer between the implants and surrounding tissues, which maintains the implant function.¹⁴ RGD peptide incorporation into the hydrogel appears to have minimal effect on the in vivo tissue responses, probably due to the relatively small amount of peptide incorporated into the hydrogel network (0.4% w/w), which is consistent to the previous study reported by others.¹ There was neither massive

inflammatory response nor thick fibrous capsule formation which should be avoided to facilitate mass transfer between the implants and surrounding tissues.¹⁴ Overall, the implant materials were found to be biocompatible in small animal model. These exciting novel biodegradable and biocompatible hydrogels may be useful in various tissue engineering applications. However, in vivo response studies in large animal models would be necessary before this exciting new class of biodegradable hydrogels can be realized in clinical settings.

5. Conclusions

Comprehensive in vivo biocompatibility and biodegradability of novel PEG-based hydrogels (PEGSDAs) were determined using a cage implant system and a direct subcutaneous implantation. Inflammatory cell response to the PEGSDA hydrogels was assessed by measuring total and differential leukocyte concentration in the exudate withdrawn from the cage containing the hydrogels and showed similar acute inflammatory responses compared to controls (empty cage and PLGA implant). Histology analysis and the thickness of fibrous capsule layer surrounding the hydrogel implants also showed minimal inflammatory and immune response of the surrounding tissues around hydrogels. The effect of RGDS peptide incorporation into the hydrogel on inflammatory response and biodegradability of the hydrogels was not significant. Overall, PEGSDA-based hydrogels showed normal wound healing response even with massive degradation and there was neither massive obvious inflammatory response nor thick fibrous capsule formation around PEGSDA hydrogel implants. This straightforward in vivo characterization method is useful to obtain comprehensive details of in vivo biodegradation and biocompatibility of biomaterials without sacrificing large number of animals.

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A

B

Figure 1. Chemical structures of (a) PEG sebacic acid diacrylate (PEGSDA), where molecular weight (MW) of PEG is 1,000 Da and (c) acrylated PEG-RGDS, where MW of PEG is 3,400 Da.

Figure 2.

In vivo degradation profiles of different scaffolds during 12 weeks of cage implantation. The percent weight loss after lyophilization was calculated as $(W_i - W_d)/W_i \times 100$, where W_i is dried initial weight and W_d is dried weight after incubation for a certain period. Error bars represent means $\pm$ standard deviation for $n=3$.

Figure 3. Representative photomicrographs of various leukocytes in exudates withdrawn from the implanted cages. The exudates were stained by Wright's modified staining: (A) lower magnification (200x) and (B) higher magnification (800x).

Figure 4.

(A) Total leukocyte concentrations and differential leukocyte concentrations of (B) PMN, (C) Lymphocyte, and (D) monocyte/macrophase in the exudates withdrawn from cage implants containing different scaffolds. Error bars represent means $\pm$ standard deviation for $n = 4-6$. * indicates significant ($p < 0.05$) difference from the control (empty).

Figure 5. Representative histological sections of different scaffolds after two months of subcutaneous implantation. H&E, Masson's Trichrome, CD45, and CD68 stain. "M" indicates the implantation sites. Scale bar = 100 μm. Arrows indicate the cells positively stained with CD45 and CD68.

Figure 6.

(A) The percentage of tissue area stained with CD45 and CD68 and (B) The thickness of fibrous capsule layers surrounding the different subcutaneous implants after two months of implantation. Error bars represent means $\pm$ standard deviation for $n=3-5$. * indicates significant ($p<0.05$) difference from PLGA sample.