Published in final edited form as:

J Biomed Mater Res A. 2011 March 15; 96(4): 621–631. doi:10.1002/jbm.a.33015.

Temporal progression of the host response to implanted poly(ethylene glycol)-based hydrogels

Aaron D. Lynn¹, Anna K. Blakney¹, Themis R. Kyriakides², and Stephanie J. Bryant¹

¹Department of Chemical and Biological Engineering, University of Colorado at Boulder, ECCH111, UCB 424, Boulder, Colorado 80309

²Department of Pathology, Yale University School of Medicine, New Haven, Ct 06536

Abstract

Poly(ethylene glycol) (PEG) hydrogels hold great promise as *in vivo* cell carriers for tissue engineering. To ensure appropriate performance of these materials when implanted, the host response must be well understood. The objectives for this study were to characterize the temporal evolution of the foreign body reaction (FBR) to acellular PEG-based hydrogels prepared from PEG diacrylate precursors when implanted subcutaneously in immunocompetent c57bl/6 mice by (immuno)histochemical analysis and gene expression. Compared to a normal FBR elicited by silicone (SIL), PEG hydrogels without or with a cell adhesion ligand RGD elicited a strong early inflammatory response evidenced by a thick band of macrophages as early as day 2, persisting through 2 weeks, and by increased interleukin-1 β expression. PEG-only hydrogels showed a slower, but more sustained progression of inflammation over PEG-RGD. Temporal changes in gene expression were observed in response to PEG-based materials and in general exhibited, elevated expression of inflammatory and wound healing genes in the tissues surrounding the implants, while the expression patterns were more stable in response to SIL. While a stabilized FBR was achieved with SIL and to a lesser degree with PEG-RGD, the PEG-only hydrogels had not yet stabilized after 4 weeks. In summary, PEG-only hydrogels elicit a strong early inflammatory reaction, which persists throughout the course of the implantation even as a collagenous capsule begins to form. However, the incorporation of RGD tethers partially attenuates this response within 2 weeks leading to an improved FBR to PEG-based hydrogels.

1 Introduction

When foreign materials are implanted into a wound space, the normal series of events that lead to resolution of inflammation and wound healing are interrupted, resulting in what is commonly referred to as the foreign body response (FBR).¹ Initially, neutrophils and macrophages are recruited to the implant site and subsequently interrogate it by adhering to its surface via non-specific protein absorption. This adhesion leads into activation of the host inflammatory cells resulting in the secretion of a number of cytokines and reactive oxygen and nitrogen species aimed at degrading the material. This reaction leads into persistent inflammation characterized by fusion of macrophages into foreign body giant cells (FBGCs) that mount a continued attack against the material. Eventually, fibroblast cells are recruited, which are responsible for secreting and laying down a collagenous, fibrous matrix that essentially walls off the implant, leading to the stabilization of the response. Macrophages and FBGCs may persist for years at the material surface, continuing to attack the material and causing long-term damage via oxidative mechanisms.²⁻⁴ From a tissue engineering

perspective where synthetic scaffolds are employed, the FBR may have deleterious consequences.⁵

While numerous studies have probed the final stages of the FBR through capsule formation, only a few studies have examined the early stages of the *in vivo* response. The initial response will involve the secretion of a variety of cytokines and other signaling molecules that give rise to inflammation and progression of the FBR. For example, Brodbeck *et al.*⁶ described the mRNA expression for pro-inflammatory and pro-wound healing cytokines in cells that were adhered to different biomaterials *in vivo* to gain insights into the activation state of cells at the material surface. Most notably, the authors observed that hydrophobic, cationic, and anionic materials led to pro-inflammatory and wound healing responses after 7 days, but which were followed by decreases only in inflammation after 14 days. Contrarily, hydrophilic surfaces showed decreased pro-inflammatory and wound healing profiles after 7 days and generally remained low through 21 days. In a separate study, Rodriguez *et al.*⁷ showed material dependent differences in protein concentration for different cytokines. For example, the *in vivo* response to polyether urethane, using a cage implant system, led to high levels of interleukin-6 (IL-6), a pro-inflammatory cytokine, and interleukin-10 (IL-10), an anti-inflammatory cytokine, after 4 days post-implantation when compared to polyethylene terephthalate and silicone rubber. By day 14, IL-6 remained elevated, while IL-10 continued to increase. The authors proposed that the prolonged elevation of pro-inflammatory cytokines, even with increased IL-10, may negatively affect the normal inflammatory and wound healing processes particularly for PEU materials and hence may impact their long-term success *in vivo*. Gretzer *et al.*⁸ described cytokine concentrations in the exudate surrounding several biomaterials *in vivo*. Their findings indicated an overall acute inflammatory response after 1 day with high levels of TNF- α and low levels of IL-10, which was followed by a steady increase in IL-10 over 3 weeks and low levels of TNF- α at 3 weeks suggesting a transition from an early inflammatory to a later wound healing response. Together, these studies and others illustrate that there is indeed a temporal response in cytokine gene expression and production, which generally follows the progression of the FBR with a strong early inflammatory response, followed by resolution of inflammation with the transition to the formation of a fibrous capsule and stabilization of the response. However, the severity of the FBR is highly dependent on the type of material.

Poly(ethylene glycol) (PEG) based hydrogels are promising for cell encapsulation strategies in tissue engineering neural,⁹ cartilaginous,^{10,11} bone,¹² pancreatic,^{13,14} and cardiovascular^{15,16} tissues. However, their *in vivo* success will largely depend on the severity of the FBR. Recently, we explored the *in vivo* response to PEG hydrogels by probing for capsule formation, which typically occurs within 3-4 weeks after implantation.¹⁷ Surprisingly, stabilization of the response had not yet occurred after 4 weeks. Rather, a strong inflammatory reaction was present as evidenced by a thick band of macrophages at the implant surface. Interestingly, the incorporation of RGD, a cell adhesion oligopeptide, into PEG hydrogels was sufficient to partially attenuate this reaction resulting in a reduced presence of macrophages with some evidence of capsule formation. *In vitro* studies confirmed that macrophages indeed adhere to PEG hydrogels, even in the absence of any cell adhesion moieties, and that PEG hydrogels elicit a stronger macrophage activation than PEG hydrogels containing RGD. Kao and co-workers have reported similar *in vitro* findings with PEG-based hydrogels.¹⁸

Based on findings from our prior work, this study aimed to better characterize the *in vivo* reactions to PEG and PEG-RGD by investigating the progression of the FBR over the course of four weeks. Specifically, (immuno)histochemical analysis was performed to monitor the influx of inflammatory cells, namely macrophages, and the deposition of new matrix and the formation of a fibrous capsule. The 'state' of the FBR was also monitored by evaluating the

cells and tissues adjacent to the implant through gene expression analysis for genes indicative of inflammation (i.e., interleukin-1 β , interleukin-12 β , and inducible nitric oxide synthase), wound healing (i.e., arginase I and vascular endothelial growth factor A) and immune regulation (i.e. interleukin-10). By better understanding the temporal progression of the FBR, this study also aimed to provide some insights into the disparate responses observed between PEG and PEG containing RGD. In particular, we hypothesize that differences occurring early in the FBR due to the presence of RGD lead to a reduction in recruitment of inflammatory cells and initiation of resolution at late time points. The FBR was evaluated at 2, 4, 7, 14 and 28 days and medical grade silicone was employed as a positive control to represent a typical progression of the FBR.

2 Materials and Methods

2.1 Material preparation

Poly(ethylene glycol) diacrylate (PEG-dA) was synthesized by reacting acryloyl chloride (AC, Sigma) and triethylamine (TEA, Sigma) with poly(ethylene glycol) (3000 Da, PEG, Fluka) in dry toluene. Briefly, acryloyl chloride was added dropwise into a solution of PEG and triethylamine in excess toluene to a final molar ratio of 1 mol PEG to 8 mol TEA to 8.8 mol AC and reacted for 24 hours at room temperature. PEG-dA product was purified by repeated precipitation in diethyl ether and acrylation percentage was verified by $^1\text{H-NMR}$ to be above 94% and of high purity.

Monoacrylated poly(ethylene glycol)-YRGDS was synthesized by reacting 3400 DA monoacrylated poly(ethylene glycol) N-hydroxysuccinimide (Laysan Bio) with tyrosine-arginine-glycine-aspartic acid-serine (YRGDS) (Genscript) oligopeptide in a 50mM sodium bicarbonate buffer at pH of 8.4 for 2 hours. The monoacrylated PEG-YRGDS product was purified by dialysis, lyophilized for storage and characterized with $^1\text{H NMR}$.

Silicone disks were punched from medical grade silicone sheets (Invotec) of thickness 0.8mm into 5mm diameter disks. PEG-dA hydrogels were formed by photopolymerization of a 20% (w/w) PEG-dA solution with 0.0125% (w/w) photoinitiator (I2959, Irgacure, Ciba Specialty Chemical) with or without 5mM monoacrylated PEG-YRGDS between two glass slides with 0.8mm spacers under 365nm ultraviolet light (5-10mW/cm²) for 10 minutes. All hydrogels were prepared under sterile conditions. Hydrogel sheets were punched into 5mm disks. All materials were rinsed 3-4 times in 70% ethanol overnight to sterilize followed by 4-5 rinses in endotoxin free and sterile phosphate buffered saline. Prior to implantation, all materials were verified to be free of endotoxins.

2.2 Implantation Study

Implants were placed into dorsal subcutaneous pockets of 6-8 week old, male c57bl/6 mice. One pocket was formed over each shoulder and hip for a total of four pockets per animal. One material of PEG-only, PEG-RGD or SIL was placed into a single pocket with the remaining fourth pocket left empty. The incisions were closed with surgical staples. Animals were monitored daily for any abnormalities at the wound site. Mice were sacrificed at 2, 4, 7, 14, and 28 days post implantation by CO₂ asphyxiation and cervical dislocation. Implants were excised with surrounding tissue and processed for either (immuno)histological analysis (n=2) or quantitative reverse transcription polymerase chain reaction (q RT-PCR, n=4).

2.3 (Immuno)histological Analysis

Explants were fixed in 4% paraformaldehyde immediately for 24 hours after explanting. Paraformaldehyde was replaced with 15% sucrose to store the explants until staining.

Dehydration and paraffin embedding were carried out with standard protocols. Sections (10µm) were stained with Masson's Trichrome following standard protocols.

Immunohistochemical staining was carried out for detection of the mac3 antigen as a marker for macrophages. Briefly tissue sections were deparaffinized, blocked and incubated with rat anti-mouse mac3 primary antibodies (BD Biosciences) at a 1:30 dilution followed by a biotinylated anti rat secondary antibody (BD Biosciences) at a 1:30 dilution. The samples were subsequently treated with Streptavidin-HRP (BD Biosciences) followed by 3,3'-diaminobenzidine (BD Biosciences) and counterstained with methyl green to visualize the presence of Mac3.

All tissues were imaged using light microscopy (Zeiss, Axioskop 40) with 10 and 40× objectives with a digital camera (Diagnostic Instruments, MN 14.2 Color Mosaic) using SPOT Software v. 4.6. Cell and capsule thickness were evaluated semi-quantitatively using NIH ImageJ software by measuring the thickness of the inflammatory cell layer at the implant surface (days 14 and 28) and the overall thickness of the capsule surrounding the implant (day 28 only).

2.4 RNA isolation and q RT-PCR

Tissue immediately surrounding the implant was excised and placed into ependorf tubes along with the implanted material and 300µL of TRK Lysis Buffer (Omega). Adjacent skin was discarded. Explanted tissue and disks were snap frozen in liquid nitrogen and stored at -80 °C until further processing. Samples were homogenized with mechanical mortar and pestles and RNA was isolated using homogenization columns and E.Z.N.A. microelute columns (Omega) according to manufacturers instructions. The purity and quantity of RNA was determined using a Nanodrop instrument (ND-1000, Thermo Scientific) with A260/280 greater than 1.90. Pure RNA was transcribed into complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). CDNA was stored at -80 °C until analysis. Primers were custom designed to span across an intron-exon boundary to prevent amplification of any contaminating genomic DNA. All primers were validated for each gene and melt curves were performed to ensure that there was only one amplicon product. Their sequences are listed in Table 1. The quantitative polymerase chain reaction was performed using FAST Sybr Green reagents (Applied Biosystems) and expression was measured using the 7500 Fast system (Applied Biosystems). The ribosomal RNA *L32* was used as a housekeeping gene,¹⁹ which was verified to be stable across the *in vivo* samples. All results are reported as relative expression (RE):²⁰

$$\text{relative expression} = \frac{E^{C_{t(\text{HKG})}}}{E^{C_{t(\text{GOI})}}}$$

where E is the primer efficiency, HKG is the housekeeping gene and GOI is the gene of interest.

2.5 Statistical Analysis

One and two-way analysis of variance was performed using the general linear model within Minitab 15 statistical analysis software for semi-quantitative histological measurements (material type and implantation time) and relative gene expression (implantation time only). Where appropriate, pair-wise comparisons were performed using Tukey's post-hoc testing. Statistical significance was confirmed when p values were less than 0.05 unless otherwise indicated. All data are represented as the mean value ± standard deviation.

2.6 Animal Use

Animal protocols were approved by the University of Colorado at Boulder animal use committee.

3 Results

3.1 Histological analysis of subcutaneous implantation

The foreign body response to subcutaneously implanted biomaterial disks made of PEG-only, PEG with RGD (PEG-RGD) and medical grade silicone (SIL) were excised with surrounding tissue, sectioned and stained for (immuno)histological analysis by Masson's trichrome (Fig. 1,2), and anti-Mac-3, a macrophage specific antigen (Fig. 3).

SIL was employed to represent a typical FBR. The early host response (day 2-4) to SIL is characterized by mild early inflammation with one to two layers of inflammatory cells and by the deposition of a loose collagen matrix. By day 7 non-inflammatory cells have been recruited to the ventral capsule with only a thin layer of inflammatory cells remaining. Fourteen days after implantation, capsule thickness has increased and is composed of cells and loosely organized collagen with inflammatory cells adjacent to the material surface. At days 21 (data not shown) and days 28, the capsule has become well organized with a thin layer of inflammatory cells, staining positive for mac-3, remaining immediately at the implant surface. The dense collagenous capsule is surrounded by a disorganized matrix. Mac3 staining is present throughout the entire course of the study, but is restricted to a thin layer of cells exclusive to the material tissue interface.

PEG-only materials elicited an acute inflammatory reaction characterized by fibrin deposition at the dorsal implant surface with strong Mac3 staining within the cell layer immediately adjacent to the hydrogel surface. This response continues to progress through days 4 and 7 as more macrophages accumulate at the material surface. A loose collagenous matrix was observed encapsulating the layer of inflammatory cells adjacent to the hydrogel by day 7. Additionally, blood vessels could be observed adjacent to the material. Although a thick layer of inflammatory cells remained present at the material surface, an ill-defined collagenous capsule continued to develop over the second and third week primarily composed of a loosely formed collagen based matrix with associated cells. Mac3 staining remained strong at the material surface throughout weeks 2 and 4. No stabilization of the FBR to PEG-only hydrogels was observed by 4 weeks.

The overall FBR to PEG-RGD materials resulted in a thick cellular capsule composed of macrophages and a non-collagenous matrix at early timepoints. After 4 and 7 days, the capsule was more defined as collagen was deposited on the ventral surface of the implant. Macrophages continued to accumulate at the hydrogel surface as evidenced by an increase in the thickness of inflammatory cell layer present at the material surface on the ventral side. By day 14, the ventral surface of the implant was covered with three distinct layers of inflammatory cells at the material surface followed by a dense collagenous layer and a loose collection of cells. By day 28, the ventral portion of the capsule was reduced in overall thickness and was composed of a layer of inflammatory cells and a dense collagenous capsule with apparent angiogenesis. Positive Mac3 staining was observed throughout the entire study and was present on both ventral and dorsal surfaces of the implant.

3.2 Semi-quantitative analysis of capsule

To characterize the thickness of the capsule surrounding each implant, measurements were made of both the overall thickness of the capsule surrounding implants (day 28 only, Fig. 4a,b) and the layer of inflammatory cells immediately at the material interface (days 14 and

28, Fig. 4c,d). At the ventral surface of the implant (Fig. 4a), the overall capsule thickness was largest with PEG-only implants (~430 μm). PEG-RGD implants had a thinner capsule (~200 μm) with the thinnest capsule found surrounding SIL implants (~110 μm). On the dorsal surface of the implant (Fig. 4b), the capsule was approximately the same thickness with both PEG-only and PEG-RGD implants while the capsule size surrounding SIL implants was approximately half that size.

Additionally the recruitment of inflammatory cells was measured as a function of implantation time. On the dorsal surface (Fig. 4d), both material type and implantation time were significant factors ($p < 0.01$ and 0.05 respectively). On this side, frontier layer of cells decreased in size from day 14 to day 28 on all implants. At the ventral surface of the implant (Fig. 4c) material type was a significant factor ($p < 0.05$). Here, the frontier layer of cells decreased in thickness for both PEG-RGD and SIL implants, however on PEG-only implants there was a trend toward increased thickness of inflammatory cells.

3.3 qRT-PCR analysis of subcutaneous implantation

Genes associated with inflammation (*IL-1 β* , *IL-12 β* , *iNOS*), immunoregulation (*IL-10*), and wound healing (*arginase 1* and *VEGF-A*) were evaluated to assess the overall cellular responses during the FBR. Relative expression data appear in Fig. 5 and the corresponding statistical analysis is given in Table 2.

Genes associated with inflammation were evaluated where implantation time was a significant factor for *IL-1 β* (Fig. 5a) and *IL-12 β* (Fig. 5b). Early after implantation from day 2 to 4, PEG-only implants elicited increases in *IL-1 β* expression, decreases in *IL-12 β* expression, but no change in *iNOS* (Fig. 5c) expression. PEG-RGD elicited similar responses for *IL-1 β* and *iNOS*, but *IL-12 β* expression did not change. SIL elicited similar responses for *IL-1 β* , while *iNOS* expression increased and *IL-12 β* expression did not change. From day 7 to 14, expression of all three inflammatory genes tended to increase in tissues surrounding PEG-only and PEG-RGD implants reaching their highest levels observed in the study, while in tissues surrounding SIL implants *IL-12 β* expression increased, but *IL-1 β* and *iNOS* expressions were relatively unchanged. It is notable that increases were highest in tissues surrounding PEG-only implants when compared to the other two materials. After 28 days, cells and tissue surrounding PEG-only, PEG-RGD and SIL materials had similar levels of inflammatory gene expression with one notable exception where *iNOS* expression was lowest in tissue surrounding SIL.

Overall temporal patterns of expression for the gene associated with immune regulation, *IL-10* (Fig. 5d) was only significant with implantation time for PEG-RGD implants, but significant pairwise comparisons and general trends were noted. At early time points, from day 2 to 4, *IL-10* expression tended to decrease by approximately an order of magnitude in cells and tissues surrounding PEG-only but remained relatively constant for PEG-RGD and SIL. At intermediate times from days 7 to 14, *IL-10* expression increased for PEG-only and PEG-RGD, with the largest increases observed for PEG-RGD by ~ 30-fold. By 28 days, *IL-10* expression levels were similar to day 14 levels for all materials and remained similarly higher in the PEG-only and PEG-RGD when compared to the SIL materials.

Genes associated with wound healing were evaluated and implantation time was only significant with SIL materials for *arginase 1* expression (Fig. 5e) and PEG-only and PEG-RGD materials for *VEGF-A* expression (Fig. 5f), but significant pairwise comparisons and general trends were noted. From day 2 to 4 *arginase* and *VEGF-A* expression tended to decrease in PEG-only and PEG-RGD implants while expression in tissue surrounding SIL implants remained relatively constant. At intermediate implantation times, day 7 to 14, *arginase* and *VEGF-A* expression increased, significantly in PEG-RGD samples, while

remaining relatively unchanged in tissues surrounding SIL implants. In general, there was a trend of decreasing *arginase* and *VEGF-A* expressions at 28 days for all materials when compared to day 14. It is noteworthy that after 28 days, *arginase* expression in cells and tissue surrounding PEG-only was approximately an order of magnitude higher than its expression associated with PEG-RGD and SIL.

4 Discussion

The results presented in this study demonstrate very different responses to PEG-only, PEG-RGD, and SIL. While all materials elicited a foreign body reaction, SIL implants appear to have a stable response by day 28 characterized by minimal presence of inflammatory cells and a thin, dense collagenous capsule. The hydrogel materials implanted however have much different responses throughout the course of the implantation study. PEG-only implants displayed prolonged inflammation at the tissue-material interface characterized by increasing numbers of macrophages at the material surface throughout the implantation period, which did not stabilize within 4 weeks. PEG-RGD materials elicited a strong initial inflammatory response followed by a reduction in inflammation, which was delayed when compared to SIL implants. Nonetheless, PEG-RGD hydrogels still exhibited a significant presence of Mac3 positive cells at the material-tissue interface.

In an effort to gain insights into the 'state' of the cells at the material-tissue interface, gene expression for several different cytokines, enzymes, and signaling molecules were investigated. Since macrophages are thought to be the cells primarily responsible for orchestrating the FBR, we chose to explore genes that capture the three main activation states of macrophages associated with inflammation, wound healing, and regulation.²¹ Although, it is important to note that other cells involved in the FBR may also express these genes. More importantly, as seen by the immunohistochemical analysis, the cellular composition of the FBR to SIL and PEG-based hydrogels differs. Nonetheless, the spectrum of genes evaluated along side (immuno)histological analyses provides new insights into the temporal progression of the FBR to PEG-based hydrogels.

The initial inflammatory response to SIL was quickly stabilized by fibrous capsule formation; however, stabilization of the FBR did not occur for the PEG-based hydrogels. Of the pro-inflammatory genes studied, *IL-1 β* expression was most highly up-regulated in response to the PEG-based hydrogels when compared to SIL. *IL-1 β* up-regulation was highest in PEG-only, which correlated to a greater number of macrophages present on the PEG-only compared to PEG-RGD materials. Interestingly, *IL-12 β* expression levels were not up-regulated in the PEG-based materials and at certain times were lower when compared to SIL. While the role for *IL-12 β* is not well established in the FBR, it has been shown to increase macrophage phagocytic activity and production of interferon gamma from stimulated T-cells, which may aid in macrophage recruitment and activation at the implantation site.²² However, it appears that in the presence of high levels of *IL-1 β* , *IL-12 β* may not be a significant regulator of the FBR to PEG-based materials, at least based on gene expression. It should be noted that the relative expression levels of *IL-12 β* were 1 to 2 orders of magnitude lower than the relative expression levels of *IL-1 β* .

Inducible nitric oxide synthase (*iNOS*) catalyzes the reaction to form nitric oxide, which when produced by macrophages can serve multiple roles in the course of wound healing and the foreign body reaction. For example, nitric oxide is expressed in human macrophages present at the interface of implanted orthopedic devices,²³ which is thought to contribute to a persistent inflammatory response leading to premature failure in hip implants.²⁴ However nitric oxide is also required in wound healing, playing important roles in angiogenesis²⁵ and matrix synthesis.²⁶ In this study, there were temporal changes in *iNOS* expression levels in

response to all materials. A few interesting trends are worth noting. Early after implantation, *iNOS* expression was highest, by nearly an order of magnitude, in cells and tissue surrounding PEG-only materials compared to PEG-RGD and SIL materials at 2 days. By day 28, the expression remained highest in the PEG-based materials, for which the FBR had not stabilized compared to SIL. While the role of NO in the FBR is not well-understood, our findings suggest that NO release might be important in the FBR, warranting further investigation.

IL-10 is a potent regulator of many pathways involved in the innate and adaptive immune system.²⁷ In response to a biomaterial, *IL-10* has been found within the exudate surrounding materials,^{7,8} and in macrophages and foreign body giant cells that are present at the tissue-material interface in the later stages of the FBR suggesting that it has a role in macrophage fusion and stabilization of the FBR.²⁸ Here, *IL-10* gene expression did not change significantly in cells surrounding SIL implants, correlating with a quickly stabilized FBR. Contrarily, *IL-10* expression in response to the PEG-based hydrogels dropped significantly at early time points, but was followed by a large upregulation by day 14. This temporal response could be partly attributed to the fact that a strong inflammatory reaction ensues, but which is also combined with evidence of some collagen deposition distal to the implant site.

Arginase type I metabolizes arginine into ornithine, which is used by fibroblasts to synthesize proline and ultimately hydroxyproline, a collagen precursor. While the role of arginase type I in the foreign body reaction is not well known, it has been shown that IL-4, an important cytokine in wound healing, upregulates arginase expression in macrophages.²⁹ In this study, there was an increase in *arginase* expression in response to SIL materials from day 2 through 14, which dropped by day 28. This temporal expression pattern correlates with the deposition of collagen during the FBR resulting in a stabilized dense fibrous capsule by day 28. Interestingly, *arginase* expression also increased in response to PEG-based hydrogels resulting in the highest observed expression at day 14. While stabilization and capsule formation was not observed, the increased expression of *arginase* may still be indicative of its role in facilitating collagen deposition, which was present surrounding the PEG-based hydrogels. Early upregulations of arginase (leading to development of collagen precursors from arginine) in tissues surrounding implanted materials may facilitate fibroblast collagen deposition but may not be indicative of stabilization of the FBR.

VEGF-A plays an important part in stimulating angiogenesis in inflammation and wound healing, but its role in mediating the FBR has not been defined.³⁰⁻³² *VEGF-A* expression was unchanged over time in response to SIL implants, but was temporally regulated in response to PEG-based hydrogel materials. Interestingly, there was a lack of vessel formation proximal to SIL implants, which correlates with reduced inflammation and the lack of temporal changes in *VEGF-A* expression. Although there was an initial drop in *VEGF-A* expression in response to PEG-based materials, a strong upregulation followed 2 weeks post implantation, which correlated with the persistence of a strong inflammatory and neovascularization proximal to the implants. These observations suggest that the PEG-based hydrogel materials can induce more neovascularization compared to the SIL materials, but the implications of this observation on the long-term *in vivo* reaction remain to be elucidated.

Overall, the PEG-based hydrogels elicited a very different temporal progression of the FBR exhibiting a strong inflammatory reaction when implanted into subcutaneous pockets compared to SIL materials. From a material perspective there are several key differences. While neutral and hydrophilic surfaces tend to lead to decreased protein absorption, cell adhesion, giant cell formation and inflammation when compared to hydrophobic surfaces,^{29,33-36} hydrogels are highly hydrophilic absorbing many times their dry weight in water.

These materials form loosely cross-linked networks that result in facile transport of water and other molecules through the gel. It is possible that when the hydrogel materials are implanted they are able to 'soak up' many of the pro-inflammatory cytokines, which are normally present at the early stages of any wound, leading to their prolonged presence and subsequent release. A second potential explanation for the differences observed from a "normal" FBR with SIL and that observed by the hydrogel materials may involve differential levels and/or types of protein adsorption on hydrogel versus SIL materials. It is generally accepted that materials modified with PEG do not adsorb significant quantities of protein on their surface,^{37,38} but these observations may not directly translate into a *crosslinked* PEG hydrogel consisting of polyacrylate chains crosslinked with PEG. In fact we have observed that macrophages do adhere to PEG-only hydrogels indicating that there is a sufficient level of protein adsorption.¹⁷ However, it is likely that protein adsorption is still considerably lower than that observed on SIL systems,³⁹ suggesting that the type of protein adsorbed may be playing a more significant role in the abnormal progression of the FBR. Interestingly, the FBR to bioinert and highly water swollen hydrogels, such as poly(vinyl alcohol) crosslinked by γ -ray irradiation, led to fibrous capsule formation within three weeks post-implantation,⁴⁰ which is more characteristic of the response to SIL observed in this study. One key difference between SIL and hydrogels, such as poly(vinyl alcohol), is that the PEG-based hydrogels are likely more susceptible to degradation. Namely, degradation may occur through hydrolysis or enzymatic degradation of the ester bond linking the PEG crosslink with the polyacrylate chains or through oxidative degradation.¹⁷ Degradation of a material can contribute to inflammation by releasing potentially inflammatory degradation products such as acrylic acid or by leading to a persistent production of radical species from oxidative degradation.

Differences observed between the PEG-only implants and PEG-RGD materials were less dramatic, but still significant. RGD is a polypeptide highly conserved throughout many matricellular and extracellular matrix proteins for which macrophages have many integrin pairing partners.⁴¹ While macrophage interactions with RGD and RGD containing proteins have been shown to both decrease⁴² and increase⁴³ inflammatory cytokine production in macrophages, their effects when tethered to a PEG hydrogel materials are less studied. We have shown the presence of RGD tends to attenuate expression of inflammatory cytokines from murine macrophages *in vitro* when compared to PEG-only hydrogel materials. However, others have shown that the presence of RGD and/or PHSRN (representing the synergistic cell adhesion sites in fibronectin) can increase cytokine production from human macrophages *in vitro* compared to PEG only surfaces¹⁸. Macrophage will interact with the PEG-RGD hydrogels likely through their integrins either by binding directly to RGD or to proteins that may have adsorbed to the surface, but which is mediated in part by the presence of RGD. Interestingly, Hennessy *et al.*⁴⁴ showed that incorporating RGD onto the surface of hydroxyapatite decreased protein absorption and reduced mesenchymal stem cell (MSC) spreading *in vitro*. However, *in vivo* the milieu of proteins in the exudate will likely lead to enhanced protein adsorption over what would be observed *in vitro*. Therefore, we suspect that RGD is likely playing a role, in part, by modulating the type of proteins adsorbed to the surface and hence affecting the progression of the FBR. However, additional studies are necessary to elucidate the exact role of RGD in the *in vivo* environment.

While the findings in this study clearly indicate elevated inflammatory responses are occurring in response to PEG-based hydrogel implants compared to SIL, it is important to note limitations of the current study. The materials were implanted directly adjacent to the tissue in an effort to replicate the type of implantation that would occur for a tissue engineered construct. However, due to differences in mechanical properties between the tissue and the SIL and differences in the degree of (de)swelling between the tissue and the hydrogel materials, processing and sectioning inevitably resulted in the loss of the

biomaterial in many instances. The immediate layer of cells adjacent to the implants was not captured and subsequently we were not able to easily detect the presence of FBGCs. Additionally, gene expression while providing insights into the 'state' of the cells and tissue during the progression of the FBR, does not capture the levels of protein production, which are ultimately the mechanisms by which the FBR is orchestrated. Finally, it is important to recognize that gene expression, while likely mostly derived from macrophages (notably for the PEG-based hydrogels) may also include other types of cells such as fibroblasts and endothelial cells and that the relative cell contents change with time and differ by material. Despite these limitations, our findings provide new insights into the temporal progression of the FBR to PEG-based hydrogels.

5 Conclusions

The current study further corroborates the data from our previous work that PEG-based hydrogels elicit an abnormal FBR when implanted into subcutaneous pockets in mice. The temporal progression of the FBR to PEG-based hydrogels begins with a strong infiltration of macrophages, which persists throughout the FBR indicating a prolonged inflammatory reaction along with what appears to be a classically activated macrophage phenotype. The level of inflammation was dependent on the presence of RGD cell adhesion moieties. Based on macrophage presence at the tissue-material interface, inflammation was highest initially in response to PEG-RGD. However, the inflammatory response to PEG-only implants becomes much more severe within 1-2 weeks. The 'state' of the cells in the tissue surrounding the PEG-based hydrogels, characterized by gene expression, indicated a similar or slightly reduced inflammatory state with RGD throughout the FBR. Together, these findings indicate that the presence of RGD, while initially may contribute to an increased recruitment/adhesion of macrophages, attenuates the inflammatory response initiated by PEG hydrogels. Interestingly, this inflammatory reaction was accompanied by the progression of collagen deposition distal to the implant surface. While capsule formation and stabilization typically occurs within 3 to 4 weeks post-implantation, stabilization was not evident in response to the PEG-based hydrogels indicating that perhaps longer times are required to reach this point. Future studies are planned to extend the implantation time to assess whether stabilization indeed does occur and its just delayed or if stabilization never occurs which may indicate that degradation of the gel is occurring and hence prolonging the inflammatory reaction. Overall, our findings indicate that further study and manipulation of the *in vivo* response to PEG-based hydrogels are needed before their success *in vivo* can be realized.

Acknowledgments

This project was supported by the National Institutes of Health grant number 1R03DE019505 in addition to the Department of Education Graduate Assistance in Areas of National Need for a fellowship to ADL and a HHMI/UROP fellowship to AKB.

References

1. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol.* 2008; 20(2):86–100. [PubMed: 18162407]
2. Zhao Q, Topham N, Anderson JM, Hiltner A, Lodoen G, Payet CR. Foreign-Body Giant-Cells and Polyurethane Biostability - Invivo Correlation of Cell-Adhesion and Surface Cracking. *Journal of Biomedical Materials Research.* 1991; 25(2):177–183. [PubMed: 2055915]
3. Dadsetan M, Christenson EM, Unger F, Ausborn M, Kissel T, Hiltner A, Anderson JM. In vivo biocompatibility and biodegradation of poly(ethylene carbonate). *Journal of Controlled Release.* 2003; 93(3):259–270. [PubMed: 14644576]

4. Bergsma JE, Debruijn WC, Rozema FR, Bos RRM, Boering G. Late Degradation Tissue-Response to Poly(L-Lactide) Bone Plates and Screws. *Biomaterials*. 1995; 16(1):25–31. [PubMed: 7718688]
5. Babensee JE, Anderson JM, McIntire LV, Mikos AG. Host response to tissue engineered devices. *Advanced Drug Delivery Reviews*. 1998; 33(1-2):111–139. [PubMed: 10837656]
6. Brodbeck WG, Voskerician G, Ziats NP, Nakayama Y, Matsuda T, Anderson JM. In vivo leukocyte cytokine mRNA responses to biomaterials are dependent on surface chemistry. *Journal of Biomedical Materials Research Part A*. 2003; 64A(2):320–329. [PubMed: 12522819]
7. Rodriguez A, Meyerson H, Anderson JM. Quantitative in vivo cytokine analysis at synthetic biomaterial implant sites. *Journal of Biomedical Materials Research Part A*. 2009; 89A(1):152–159. [PubMed: 18431759]
8. Gretzer C, Emanuelsson L, Liljensten E, Thomsen P. The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. *J Biomater Sci Polym Ed*. 2006; 17(6):669–87. [PubMed: 16892728]
9. Namba RM, Cole AA, Bjugstad KB, Mahoney MJ. Development of porous PEG hydrogels that enable efficient, uniform cell-seeding and permit early neural process extension. *Acta Biomaterialia*. 2009; 5(6):1884–1897. [PubMed: 19250891]
10. Elisseeff J, McIntosh W, Fu K, Blunk BT, Langer R. Controlled-release of IGF-I and TGF-beta1 in a photopolymerizing hydrogel for cartilage tissue engineering. *J Orthop Res*. 2001; 19(6):1098–104. [PubMed: 11781011]
11. Nicodemus GD, Bryant SJ. The role of hydrogel structure and dynamic loading on chondrocyte gene expression and matrix formation. *J Biomech*. 2008; 41(7):1528–36. [PubMed: 18417139]
12. Yang F, Williams CG, Wang DA, Lee H, Manson PN, Elisseeff J. The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells. *Biomaterials*. 2005; 26(30):5991–5998. [PubMed: 15878198]
13. Cruise GM, Hegre OD, Lamberti FV, Hager SR, Hill R, Scharp DS, Hubbell JA. In vitro and in vivo performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes. *Cell Transplantation*. 1999; 8(3):293–306. [PubMed: 10442742]
14. Mason MN, Arnold CA, Mahoney MJ. Entrapped Collagen Type 1 Promotes Differentiation of Embryonic Pancreatic Precursor Cells into Glucose-Responsive beta-Cells When Cultured in Three-Dimensional PEG Hydrogels. *Tissue Engineering Part A*. 2009; 15(12):3799–3808. [PubMed: 19537960]
15. Mann BK, Gobin AS, Tsai AT, Schmedlen RH, West JL. Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering. *Biomaterials*. 2001; 22(22):3045–3051. [PubMed: 11575479]
16. Iyer RK, Chiu LL, Radisic M. Microfabricated poly(ethylene glycol) templates enable rapid screening of triculture conditions for cardiac tissue engineering. *J Biomed Mater Res A*. 2008
17. Lynn AD, Kyriakides TR, Bryant SJ. Characterization of the in vitro macrophage response and in vivo host response to poly(ethylene glycol)-based hydrogels. *J Biomed Mater Res A*. 2010; 93(3): 941–53. [PubMed: 19708075]
18. Schmidt DR, Kao WJ. Monocyte activation in response to polyethylene glycol hydrogels grafted with RGD and PHSRN separated by interpositional spacers of various lengths. *Journal of Biomedical Materials Research Part A*. 2007; 83A(3):617–625. [PubMed: 17503491]
19. Ishii T, Wallace AM, Zhang X, Gosselink J, Abboud RT, English JC, Pare PD, Sandford AJ. Stability of housekeeping genes in alveolar macrophages from COPD patients. *European Respiratory Journal*. 2006; 27(2):300–306. [PubMed: 16452584]
20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. 2001; 29(9)
21. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*. 2008; 8(12):958–969.
22. Luttkhuizen DT, Harmsen MC, Van Luyn MJA. Cellular and molecular dynamics in the foreign body reaction. *Tissue Engineering*. 2006; 12(7):1955–1970. [PubMed: 16889525]
23. Moilanen E, Moilanen T, Knowles R, Charles I, Kadoya Y, AlSaffar N, Revell PA, Moncada S. Nitric oxide synthase is expressed in human macrophages during foreign body inflammation. *American Journal of Pathology*. 1997; 150(3):881–887. [PubMed: 9060826]

24. Hukkanen M, Corbett SA, Batten J, Kontinen YT, McCarthy ID, Macloof J, Santavirta S, Hughes SPF, Polak JM. Aseptic loosening of total hip replacement - Macrophase expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. *Journal of Bone and Joint Surgery-British Volume*. 1997; 79B(3):467–474. [PubMed: 9180331]
25. Shi HP, Efron DT, Most D, Barbul A. The role of iNOS in wound healing. *Surgery*. 2001; 130(2): 225–229. [PubMed: 11490353]
26. Shi HP, Efron DT, Most D, Tantry US, Barbul A. Supplemental dietary arginine enhances wound healing in normal but not inducible nitric oxide synthase knockout mice. *Surgery*. 2000; 128(2): 374–378. [PubMed: 10923019]
27. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001; 19:683–765. [PubMed: 11244051]
28. Higgins DM, Basaraba RJ, Hohnbaum AC, Lee EJ, Grainger DW, Gonzalez-Juarrero M. Localized Immunosuppressive Environment in the Foreign Body Response to Implanted Biomaterials. *American Journal of Pathology*. 2009; 175(1):161–170. [PubMed: 19528351]
29. Anderson JM, Jones JA. Phenotypic dichotomies in the foreign body reaction. *Biomaterials*. 2007; 28(34):5114–5120. [PubMed: 17706278]
30. Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol*. 1998; 152(6):1445–52. [PubMed: 9626049]
31. Chen SL, Jones JA, Xu YG, Low HY, Anderson JM, Leong KW. Characterization of topographical effects on macrophage behavior in a foreign body response model. *Biomaterials*. 2010; 31(13):3479–3491. [PubMed: 20138663]
32. Schutte RJ, Parisi-Amon A, Reichert WM. Cytokine profiling using monocytes/macrophages cultured on common biomaterials with a range of surface chemistries. *Journal of Biomedical Materials Research Part A*. 2009; 88A(1):128–139. [PubMed: 18260130]
33. Chuang HYK, King WF, Mason RG. Interaction of Plasma-Proteins with Artificial Surfaces - Protein Adsorption-Isotherms. *Journal of Laboratory and Clinical Medicine*. 1978; 92(3):483–496. [PubMed: 681830]
34. Shen MC, Garcia I, Maier RV, Horbett TA. Effects of adsorbed proteins and surface chemistry on foreign body giant cell formation, tumor necrosis factor alpha release and procoagulant activity of monocytes. *Journal of Biomedical Materials Research Part A*. 2004; 70A(4):533–541. [PubMed: 15307157]
35. Jenney CR, Anderson JM. Effects of surface-coupled polyethylene oxide on human macrophage adhesion and foreign body giant cell formation in vitro. *Journal of Biomedical Materials Research*. 1999; 44(2):206–216. [PubMed: 10397922]
36. Jenney CR, Anderson JM. Alkylsilane-modified surfaces: inhibition of human macrophage adhesion and foreign body giant cell formation. *J Biomed Mater Res*. 1999; 46(1):11–21. [PubMed: 10357131]
37. Cao L, Chang M, Lee CY, Castnet' DG, Sukavaneshvar S, Ratner BD, Horbett TA. Plasma-deposited tetraglyme surfaces greatly reduce total blood protein adsorption, contact activation, platelet adhesion, platelet procoagulant activity, and in vitro thrombus deposition. *Journal of Biomedical Materials Research Part A*. 2007; 81A(4):827–837. [PubMed: 17236214]
38. Jeon SI, Lee JH, Andrade JD, Degennes PG. Protein Surface Interactions in the Presence of Polyethylene Oxide .1. Simplified Theory. *Journal of Colloid and Interface Science*. 1991; 142(1): 149–158.
39. Backovic A, Huang HL, Del Frari B, Piza H, Huber LA, Wick G. Identification and dynamics of proteins adhering to the surface of medical silicones in vivo and in vitro. *Journal of Proteome Research*. 2007; 6(1):376–381. [PubMed: 17203981]
40. Nho YC, Moon SW, Lee KH, Park CW, Suh TS, Jung YA, Ahn WS, Chun HJ. Evaluations of poly(vinyl alcohol) hydrogels cross-linked under gamma-ray irradiation. *Journal of Industrial and Engineering Chemistry*. 2005; 11(1):159–164.
41. Erickson, C.; Albrecht, R. Monocytes and Macrophages. In: Graziano, FM.; Lemanske, RF., editors. *Clinical immunology*. Baltimore: Williams & Wilkins; 1989. p. 27-51.

42. Bonfield TL, Colton E, Marchant RE, Anderson JM. Cytokine and growth factor production by monocytes/macrophages on protein preadsorbed polymers. *J Biomed Mater Res.* 1992; 26(7):837–50. [PubMed: 1607370]
43. Beezhold DH, Lause DB. Stimulation of Rat Macrophage Interleukin-1 Secretion by Plasma Fibronectin. *Immunological Investigations.* 1987; 16(5):437–449. [PubMed: 3500916]
44. Hennessy KM, Clem WC, Phipps MC, Sawyer AA, Shaikh FM, Bellis SL. The effect of RGD peptides on osseointegration of hydroxyapatite biomaterials. *Biomaterials.* 2008; 29(21):3075–3083. [PubMed: 18440064]

Figure 1.

Host response to subcutaneously implanted PEG-only (a,d,g), PEG-RGD (b,e,h) and SIL (c,f,i) materials in immunocompetent c57bl/6 mice over 28 days. Tissue was stained with Masson's Trichrome, which stains connective tissue blue, nuclei dark red/purple and cytoplasm red/pink. The sections were imaged with a 10× objective at day 2 (a,b,c), 14 (d,e,f) or 28 (g,h,i). * denotes location of implant. Scale bar is 500µm.

Figure 2.

Ventral side capsule formed in response to subcutaneously implanted PEG-only (a,d,g), PEG-RGD (b,e,h) and SIL (c,f,i) disks in immunocompetent c57bl/6 mice over 28 days. Tissue was stained with Masson's Trichrome, which stains connective tissue blue, nuclei dark red/purple and cytoplasm red/pink. The sections were imaged with a 40 $\times$ objective at day 2 (a,b,c), 14 (d,e,f) or 28 (g,h,i). * denotes location of implant. Scale bar is 100 μ m.

Figure 3. Ventral side capsule, stained for macrophages, in response to subcutaneously implanted PEG-only (a,d,g), PEG-RGD (b,e,h) and SIL (c,f,i) disks in immunocompetent c57bl/6 mice over a 28 day study. Tissues were stained with an antibody for mac3 (brown), counter stained with methyl green, which stains the cytoplasm of cells green, and imaged with a 40× objective at day 2 (a,b,c), 14 (d,e,f) or 28 (g,h,i). * denotes location of implant. Scale bar is 100µm.

Figure 4.

Semi-quantitative analysis of subcutaneously implanted PEG-only, PEG-RGD and SIL. Capsule thickness was measured at day 28 on both the ventral (a) and dorsal (b) side of the implanted material. The frontier layer of inflammatory cells was measured on the ventral (c) and dorsal (d) side of the implant at day 14 and 28. Symbols represent significant difference, $p < 0.05$, from PEG-only (*) and PEG-RGD (#) implants.

Figure 5.

Gene expression, as measured by real-time RT-PCR, in tissues surrounding subcutaneously implanted PEG-only, PEG-RGD and SIL over 28 days. Genes were selected to represent markers of inflammation: a) *Interleukin 1 β* , b) *Interleukin 12 β* and c) *inducible nitric oxide synthase*, immunoregulation: d) *Interleukin 10*, and wound healing: e) *arginase type I* and f) *vascular endothelial growth factor A*. Bars represent statistical significance between time points within the same material. * - $p < 0.05$.

Table 1
Primers used for quantitative RT-PCR

Gene of Interest	Primer Sequence	Efficiency	Accession #
<i>Interleukin 1 beta</i>	F: 5'-CAGGTCGCTCAGGGTCACA-3' R: 5'-TCAGAGGCAAGGAGGAAAACA-3'	1.91	NM_008361
<i>Inducible nitric oxide synthase</i>	F: 5'-AAGGCCACATCGGATTAC-3' R: 5'-GTTGATGAACTCAATGGCATG-3'	1.92	NM_010927
<i>Interleukin 12 beta</i>	F: 5'-CATCAGGGACATCATCAAACC-3' R: 5'-CAAAGAACTTGAGGGAGAAAGT-3'	1.85	NM_008352
<i>Arginase type 1</i>	F: 5'-TGTGTCATTGGGTGGATGCT-3' R: 5'-TGGTACATCTGGGAACCTTCC-3'	2.01	NM_007482
<i>Vascular endothelial growth factor A</i>	F: 5'-CC TGT GTG CCG CTG ATG-3' R: 5'- CGC ATG ATC TGC ATG GTG AT-3'	2.05	NM_001025250
<i>Interleukin 10</i>	F: 5'-CAGAGAAGCATGGGCCAGAA-3' R: 5'-CCACTGCCTTGCTCTTATTTTC-3'	1.98	NM_010548
<i>L32</i>	F: 5'-CCATCTGTTTTACGGCATCATG-3' R: 5'-TGAACCTCTTGGTCCTCTTTTGA-3'	2.07	NM_172086

Table 2
One-way ANOVA for quantitative RT-PCR

Gene of Interest	ANOVA Factor – Implantation Time		
	PEG-Only	PEG-RGD	SIL
<i>Interleukin 1 Beta</i>	p = 0.001	p = 0.005	p = 0.102
<i>Interleukin 12 Beta</i>	0.001	0.222	0.004
<i>Inducible Nitric Oxide Synthase</i>	0.081	0.054	0.358
<i>Interleukin 10</i>	0.317	0.015	0.324
<i>Arginase Type 1</i>	0.064	0.065	0.023
<i>Vascular Endothelial Growth Factor A</i>	0.006	0.034	0.544