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# SCAFFOLD DESIGN FOR THE REDUCTION OF HOST TISSUE RESPONSE

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## ABSTRACT

In scaffold materials, the family of poly(*α*-hydroxy acid)s, such as polyglycolide (PGA) and polylactide (PLA) and its copolymers such as poly(lactide-*co*-glycolide) (PLGA), has been extensively used in tissue-engineered organs due to its biocompatibility, controllable biodegradabilitiy, and relatively good processability. However, it is more desirable to endow the PLA, PGA, and PLGA scaffold with new functionality for tissue-engineered bioorgans. This review introduces the focus of synthetic/natural hybrid biomaterials as PLGA/2-methacryloyloxyethyl phosphorylcholine (MPC) polymers, in order to approach to a more natural environment and support biological signals for tissue growth and reorganization. The reduction of inflammatory reaction of PLGA through the hybridization of demineralized bone particles (DBPs) and small intestine submucosa (SIS) is also being reviewed.

## Introduction

At present, numerous biodegradable polymeric biomaterials have been employed in devices for orthopedic surgery, scaffolds for tissue engineering and as a vehicle for drug delivery system. Unfortunately some implanted biomaterials and drug delivery vehicles have been reported to induce sequential events of immunologic reactions in response to injury caused by implantation procedures resulting in acute inflammation marked by a dense infiltration of inflammation-mediating cells at the materials-tissue interface.[1~5] Prolonged irritations provoked by implanted biomaterials further advance the acute inflammation into chronic adverse tissue response characterized by the accumulation of dense fibrotic tissue encapsulating the implants.[3]

PLGA is a member of a group of poly(α-hydroxy acid) that is among the few synthetic polymers approved for human clinical use by FDA. Consequently, it has been extensively used and tested for scaffold materials due to its good biocompatibility, relatively good mechanical property, lower toxicity and controllable biodegradability. It has been clinically utilized for three decades as sutures, bone plates, screws and drug delivery vehicles and its safety has been proved in many medical applications.[1] PLGA degrades by nonspecific hydrolytic scission of their ester bonds into their original monomer, lactic acid and glycolic acid. During these processes, there is minimal systemic toxicity, however, in some cases, their acidic degradation products can decrease the pH in the surrounding tissue resulting in local inflammatory reaction and poor tissue development as shown in Figure 1.[6]

Furthermore its poor mechanical strength, small pore size and hydrophobic surface properties for cell seeding have limited its usage.

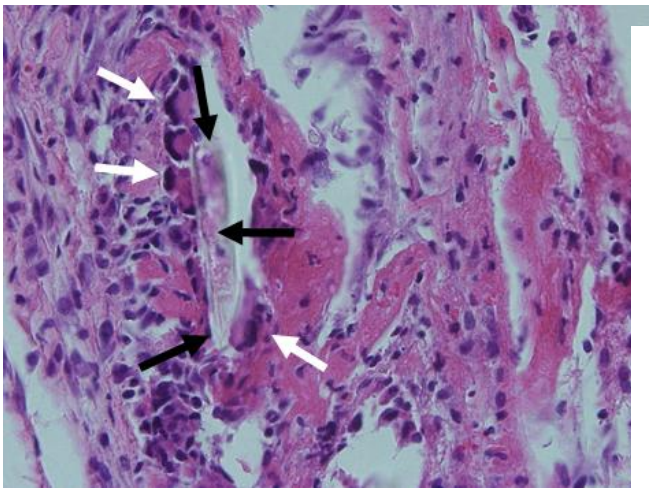

**Fig. 1** Foreign body granuloma. Small PLGA debris (black arrows) broken off from the PLGA film and is surrounded by macrophages and multinucleated giant cells (white arrows). These induced macrophages and multinucleated giant cells were remaining over 2 months.

We have reported that 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers (PMEH, Figure 2) are

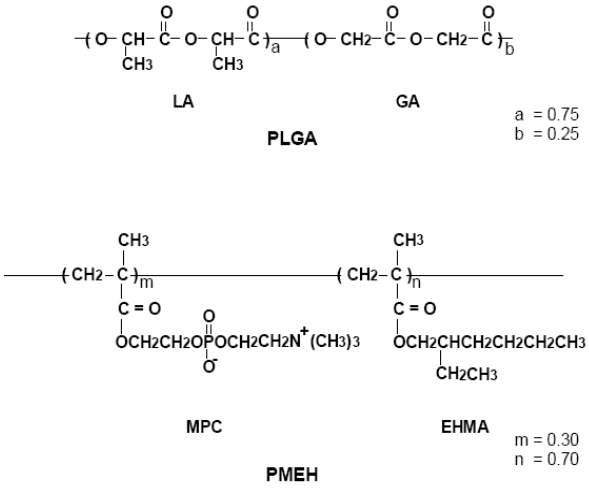


**Fig. 2** Chemical structures of PLGA and PMEH

Currently, biomaterials are endowed with biocompatibility through three different methods which are: coating with hydrophilic molecules, modifying surface characteristics using physiochemical methods and impregnating bioactive substances. Previous reports showed that application of mineral layer or localized delivery of anti-inflammatory agent such as corticosteroid with cytokine could be effectively suppressed inflammation and fibrosis of implant.[7]Although the methods of such studies are experimentally available, it is usually complicated to prepare the implants, and adverse effects of a specific growth factor have not been clearly defined. In addition, the mechanisms by which PLGA induces local inflammatory responses have not been discussed sufficiently.[8]

In our laboratory, the natural/synthetic hybrid scaffolds have been investigated during the last 15 years such as small intestine submucosa (SIS)[9,10], demineralized bone particles (DBP)[8,11], DBP gel[12], fibrin[13], keratin[14], hyaluronic acid[15], collagen gel[4], silk[4] and a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer (PMEH)[16] with PLGA to reduce cellular inflammatory response. In this chapter, we introduced MPC/PLGA, DBP/PLGA and SIS/PLGA hybrid scaffold in terms of scaffold design for the reduction of host response and the augmentation of tissue formation.

**1. Reduction of inflammatory reaction on MPC/PLGA polymer blend**

synthesized as biomimetics to the biomembrane structure.[16] The MPC polymers are useful for surface modification of conventional materials to improve their biocompatibility even when the random copolymers composed of MPC and alkyl methacrylates were applied as coating polymers. They effectively reduce protein adsorption and denaturation and inhibited cell adhesion even when the polymer is in contact with whole blood in the absence of any anticoagulants.

We hypothesized that the inflammatory reaction of adherent cells on PLGA might occur and could be reduced by blending a PMEH with the PLGA. PLGA/PMEH blend membranes were prepared by a solvent evaporation technique. The thermal properties of the PLGA/PMEH membrane were determined using a differential scanning calorimeter. The glass transition temperature of the PLGA/PMEH membranes was slightly decreased compared to that of a PLGA membrane. X-ray photoelectron spectrum analysis revealed that the MPC unit was exposed on the PLGA/PMEH membrane and that the surface concentration of the MPC unit on the membrane was increased with an increase in the concentration of the PMEH in the blended membrane.[16]

NIH-3T3 mouse fibroblast cells were cultured on the PLGA/PMEH membrane for 2 days. The number of adherent cells on the PLGA/PMEH membrane was decreased with an increase in the concentration of the PMEH. Using the RT-PCR method, the amount of an inflammatory cytokine, IL-1β; mRNA expressed from adherent human premyelocytic

leukemia (HL-60) cells on PLGA and PLGA/PMEH membranes were determined. On a PLGA/PMEH membrane containing 0.2 wt% of PMEH, the expression of IL-1β mRNA was significantly lower than that on PLGA, but no difference in the number of adherent cells was found as shown in Figures 3 and 4. The properties that resist protein adsorption on PMEH would be one of the reasons to reduce expression of IL-1β from HL-60 cells adhered on a PLGA/PMEH membrane. Cell adhesion and proliferation are quite necessary for tissue engineering materials, which is one of the most attractive applications of the PLGA.[17] By controlling the concentration of PMEH in the PLGA/PMEH membrane, the inflammatory reaction of adherent cells could be effectively reduced with no decrease in the adherent cell number and proliferation as shown on PLGA/0.2PMEH.

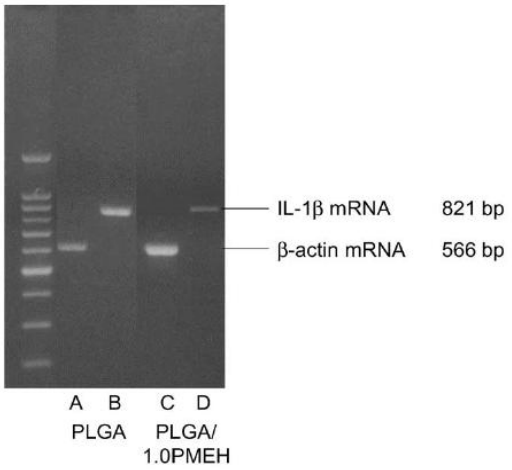


**Fig. 3** Expression of IL-1β and β-actin mRNA from HL-60 cells adhered on PLGA and PLGA/1.0PMEH membranes

## 2. Reduction of inflammatory reaction of PLGA using DBP

DBP have long been recognized as a powerful inducer of new bone growth. Many authors reported that this osteoinductive property is mainly due to bone morphogenetic proteins (BMPs).[3~5]After demineralization process using an acid solution such as HCl, an acid-insoluble matrix of collagen and growth factors, including BMP, is left behind. In the bone defect site as well as in non-skeletal areas DBP induces osteogenesis without a fibrous reaction.[18] In a more recent study, we demonstrated that DBP enhanced hydrophilicity of PLGA scaffold with an increase of content and reduced adverse cellular response associated with inflammation.[4] For this, we hypothesized that the inflammatory response of cells neighboring PLGA implant may occur and can be reduced by impregnating DBP into PLGA. We focused our attention on the early stages of inflammatory reaction and used histological and molecular analyses to assess how cells and tissue responded to DBP-PLGA hybrid materials *in vivo* and *in vitro*. We evaluated the effect of five different ratios DBP/PLGA hybrid materials on cellular inflammatory response and tissue reaction induced by PLGA.[8]

### 3.1 Cell Viability

In order to evaluate the influence of DBP content in PLGA materials on cells, we analyzed viability of mouse fibroblasts on PLGA and the five different ratios DBP/PLGA scaffold during the *in vitro* culture and found that DBP enhanced initial attachment of fibroblast on the scaffold. At day 1, the number of vital cells was significantly higher in culture of the scaffold containing DBP, than that of the scaffold without

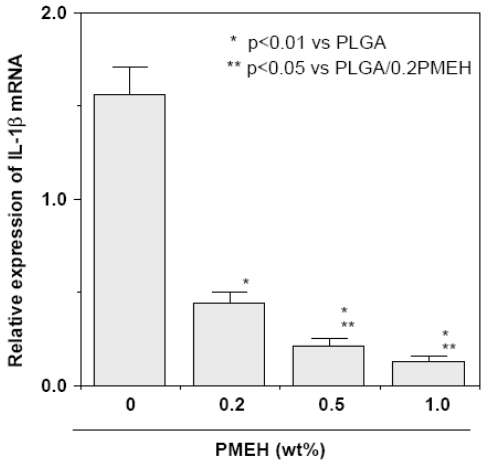


**Fig. 4** Relative expression of IL-1b expressed from HL-60 cell adhered on PLGA and PLGA/PMEH membrane

DBP (Figure 5).

Particularly, 20% and 40% DBP/PLGA scaffold maintained the number of viable cells highly compared to PLGA scaffold through 3 days. The range of DBP contents of PLGA scaffolds showed no adverse effects on fibroblasts cell attachment, proliferation and viability compared as PLGA scaffold did. No significant differences were found between the PLGA and 10% DBP/PLGA scaffold at day 2 and 3. Next we evaluated viability of HL-60 cells on samples and did not found statistical significances between PLGA and DBP hybrid scaffold at day 1 and 2. By culture day 5, proliferation of HL-60 cells with PLGA increased slightly more than with DBP/PLGA scaffold (Figure 6).

### 3.2 Inflammatory Cytokine Expression

To elucidate the cellular responses associated with

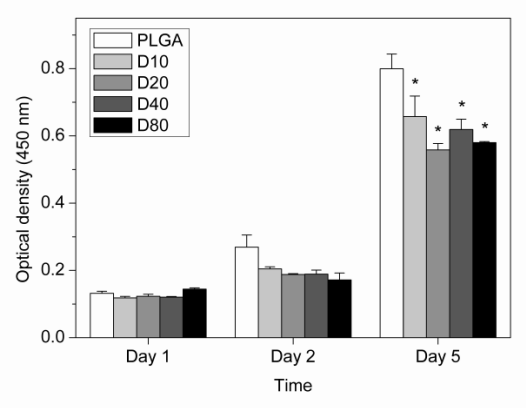


**Fig. 5** The number of viable fibroblasts on PLGA and DBP/PLGA scaffolds at 1, 2 and 3 days as determined by MTT colorimetric assay. *Corresponds to $P < 0.05$ in comparison with PLGA scaffold for each day

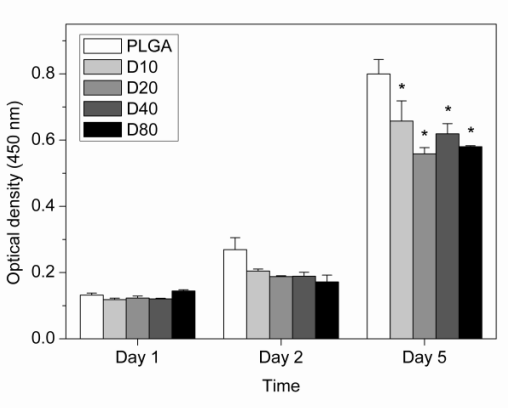


**Fig. 6** The number of viable HL-60 cells on PLGA and DBP/PLGA scaffold at Day 1, 2 and 5 as determined by MTT colorimetric assay. *Corresponds to $P < 0.05$ in comparison with PLGA scaffold for each day

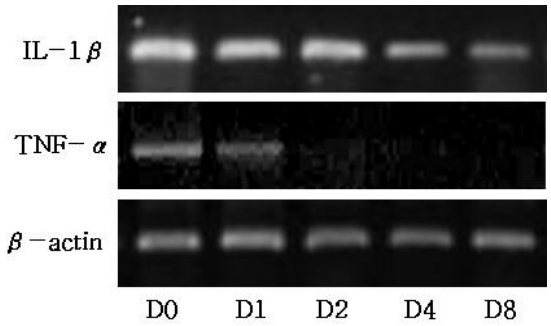


**Fig. 7** Representative gene expression band as IL-1β, TNF-α and β-actin. These bands were normalized and showed in Figures 16-8 and 16-9.

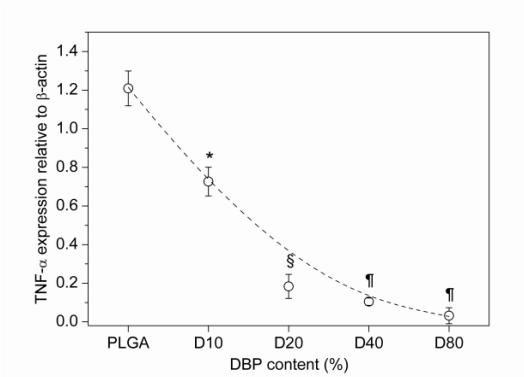


**Fig. 8** The expression of TNF-α mRNA on HL-60 cells fell continuously with an increase in content of DBP in PLGA. The significant decrease of a TNF-α expression on HL-60 cells in each scaffold as DBP contents increases from 0 to 80%.

inflammation on sample films, we measured the level of mRNA expression of TNF-α and IL-1β from HL-60 cell in 48 hours after culture with PLGA or DBP/PLGA films as shown in Figure 7. TNF-α mRNA in HL-60 highly expressed following PLGA film, as compared to DBP/PLGA films; it was significantly lower following DBP/PLGA films than PLGA film with increases in contents of DBP, 10, 20, 40 and 80% of DBP ($p < 0.005$, $p < 0.0001$, $p < 0.00005$ and $p < 0.00005$, respectively) (Figure 8). The intensity of TNF-α expression of PLGA film was significantly ten times higher or more than that of 40% DBP/PLGA films. HL-60 cell with 80% DBP/PLGA film rarely expressed TNF-α mRNA. Similarly, IL-1β mRNA expression decreased markedly with 40% and 80% DBP/PLGA film compared to PLGA film. No significant differences of IL-1β mRNA expression were observed between PLGA, 10% DBP/PLGA and 20% DBP/PLGA (Figure 9).

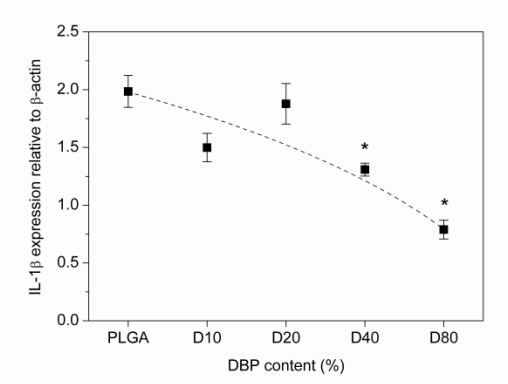


**Fig. 9** The expression of IL-1β mRNA on HL-60 cells fell continuously with an increase in content of DBP in PLGA. IL-1β mRNA expression decreased markedly on 40% and 80% DBP/PLGA film. *Corresponds to $P<0.05$ in comparison with PLGA film

### 3.3 *In Vivo* Tissue Response

To further characterize *in vivo* inflammatory response surrounding the implants, histological examination was performed at Day 5 after implantation. Remarkable inflammation was observed in tissue surrounding the PLGA film; however this inflammatory reaction was progressively diminished with an increase in contents of DBP in PLGA film. We observed numerous recruited neutrophils infiltrates with a large number of multinucleated giant cells (MNGCs) adjacent tissue after PLGA film implantation. However this inflammatory cellular response decreased as content of DBP continuously increased in PLGA film. The density of inflammatory cell following PLGA film implantation was approximately two times higher than that following 40% DBP/PLGA film. The DBP film had fewer inflammatory cells relative to the PLGA film (Figure 10). PLGA, DBP/PLGA and DBP film had a noticeable difference of fibrotic band encapsulation. The fibrotic thickness was significantly decreased in DBP/PLGA hybrid and DBP film. PLGA film had five times or more broad fibrotic band than the other samples (Figure 11). In 40% or 80% DBP/PLGA hybrid film, macrophages or foreign body giant cells were rarely observed in immediate contact with DBP fragment surface and a thin collagenous fibrous band surrounded the samples. The DBP film seldom recruited MNGCs compared to PLGA or DBP/PLGA hybrid film (Figure 12).

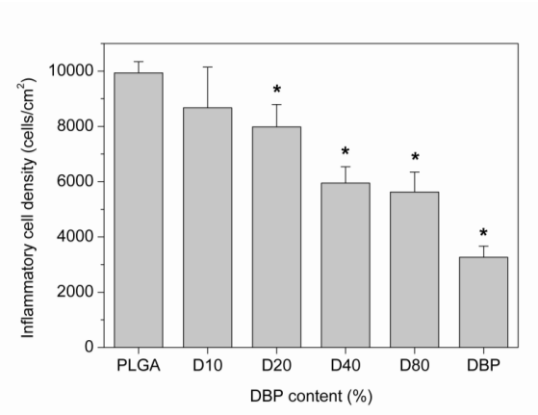


**Fig. 10** The inflammatory response to PLGA film, DBP hybrid PLGA films and DBP. The density of inflammatory cells. *Corresponds to P<0.05 in comparison with PLGA film

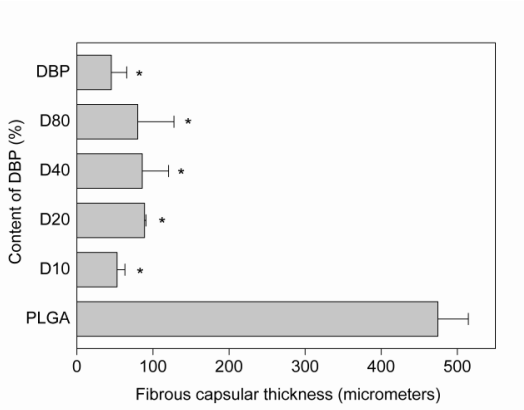


**Fig. 11** The inflammatory response to PLGA film, DBP hybrid PLGA films and DBP. Fibrous wall thickness elicited by PLGA film was nearly five times that measured for DBP impregnated PLGA films. *Corresponds to P<0.00001 in comparison with PLGA film.

These results indicate that DBP hybrid PLGA film elicits decreased tissue reactivity that can result from biocompatibility of DBP. TNF-α plays a role of inflammatory response that activates leukocytes, enhances adherence of neutrophils and monocytes, promotes the migration of inflammatory cells into the intercellular space and triggers local production of other pro-inflammatory cytokines such as IL-1β. TNF-α and IL-1β which are potent stimulator of fibroblast growth.[19] Therefore, temporospatial expression of these proinflammatory mediators allows fine tuning of the inflammatory response.[8] DBP has been reported as a biocompatible inducer of bone formation which is mainly responsible to bone morphogenetic proteins and several growth factors including insulin-like growth factor (IGF) and transforming growth factor β1 (TGF-β1) in DBP has been described.[20]

In this study, we observed that DBP hybrid PLGA materials did not significantly affect the viability of HL-60 cells during 3D *in vitro* culture however; their inflammatory response to DBP hybrid polymeric materials was obviously reduced. The probable cause for these results is that TNF-α expression may be strongly suppressed by bioactive substances released from DBP; this pattern was enhanced with an increase in content of DBP in PLGA film, which can result in suppression of the IL-1β expression that reduces proliferation and fibrous capsular formation of fibroblasts.

DBP/PLGA materials may have more mechanical stability compared to PLGA materials without DBP, that can led to decrease production of implant debris and reduction of tissue response. Whether this reduction of foreign body reaction occurs because the bioactive molecules released from DBP induced suppression of local inflammation or DBP impregnated PLGA materials provided a favorable surface or rigid structural support to cell remains to be determined.

In this study, we have shown that by impregnating DBP in the PLGA materials, the inflammatory reaction could be effectively reduced *in vivo* and *in vitro*. This result suggests that hybridization of natural materials such as DBP is suitable

for control of an adverse tissue reaction of polymeric materials shown *in vivo* application.

**4. Effect of DBP/PLGA scaffold on angiognesis during the repair of calvarial bone**

As we discussed Section 3, DBP have long been recognized as a powerful inducer of new bone growth due to BMPs.[8,18~20] BMPs is one of specific members of transforming growth factor-β (TGF-β) superfamily with a known potential as promoting the various stages of intramembranous and endochondral bone ossification during fracture healing. BMPs may also stimulate the synthesis and secretion of other angiogenic growth factors as vascular endothelial growth factors (VEGF). [21]

Development of microcirculation via microvascularization is a crucial factor in the regeneration of bone. For these reasons, DBP is commonly used to facilitate bone grafting after skeletal injury or disease, however, the biologic mechanisms for its osteogenic potential still remain obscure, and DBP does not provide structural support to an injured bony structure without another artificial fixation device. Additionally, DBP cannot contain alone live progenitor cells to enhance the osteogenesity. To resolve these problems, we manufactured a highly porous DBP/PLGA hybrid scaffold which can be adapted to the shape of defect and load the lots of live progenitor cells. The purpose of our study was to evaluate the maintaining capacity of osteogenic and angiogenic properties of DBP itself in the DBP/PLGA hybrid scaffold *in vivo*

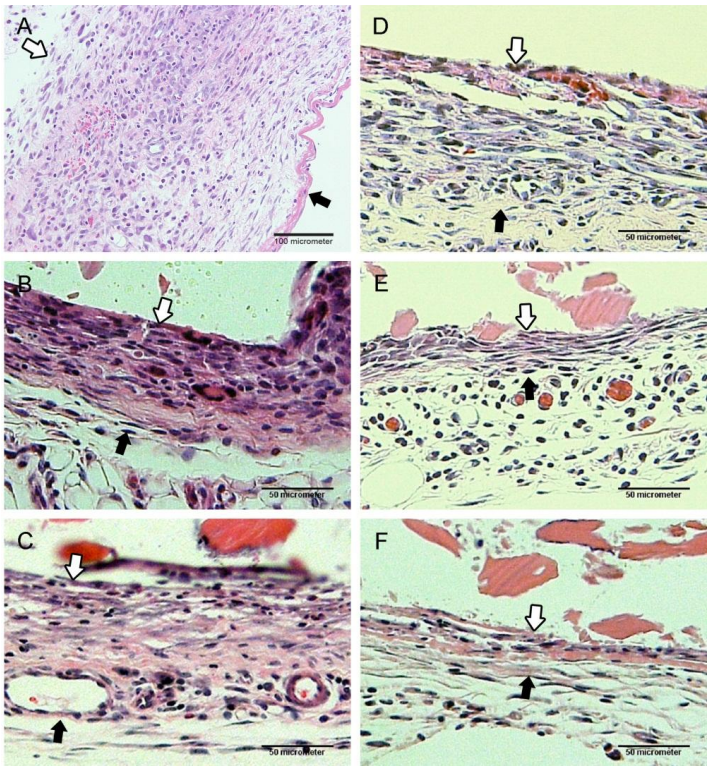


**Fig. 12** Photomicrographs of HE stain sections of the directly bordering tissue after PLGA, DBP hybrid PLGA films and DBP. (A) shows the tissue implanted with PLGA, bar length = 100 μm (x100), (B)-(F) show the tissue implanted with 10% DBP/PLGA, 20% DBP/PLGA, 40% DBP/PLGA, 80% DBP/PLGA and DBP, respectively, bar length = 50 μm (x400). Note that the number of inflammatory cell and fibrous band thickness in vicinity to tissue implanted samples was decreased as DBP content in PLGA film was increased. Polymer-tissue interface surfaces are indicated by white arrow. The fibrous wall thickness was represented by black and white arrow.

**4.1 Effect of DBP on VEGF mRNA Expression.**

In order to evaluate the effect of DBP on angiogenesis during cortical bone healing process, we studied the molecular biologic events by analyzing the activation of VEGF that has known as an endothelial specific mitogen.

By postsurgical days 7, we observed an increase of VEGF mRNA expression in those scaffolds contained with DBP, and these upregulations lasted by days 28 continuously (Figure 13). On the other hand, only PLGA group did not exhibit the expression of VEGF at 7 days postoperatively, and it had a quiet restricted expression around days 14 transiently and

decreased by days 28. DBP induced VEGF activation in 20 and 40% DBP groups were higher than that in other groups. Two spliced variants of VEGF were detected at 492 and 564 bp. These results indicated that DBP in the PLGA scaffold accelerated VEGF expression at the early phase of bone repair.

**4.2 Osteocalcin mRNA Expression during DBP Induced Bone Formation.**

Next we analyzed the osteocalcin expression for differentiation; its presence has been considered to establish the differentiated state of the osteoblast.[22] We compared the time frame in which relative osteocalcin expression to GAPDH in the different ratios DBP/PLGA scaffolds compared to PLGA scaffold and found that DBP induced the osteocalcin expression during the early phase of osteoinduction (Figure 14). Conversely, throughout the course of investigation, beginning at days 3 and extending until days 28, we did not observe a marked increase of osteocalcin expression in only PLGA group. These RT-PCR observations indicated that DBP/PLGA hybrid scaffolds may not only activate endothelial cell proliferation, but also induce of osteoblast differentiation.

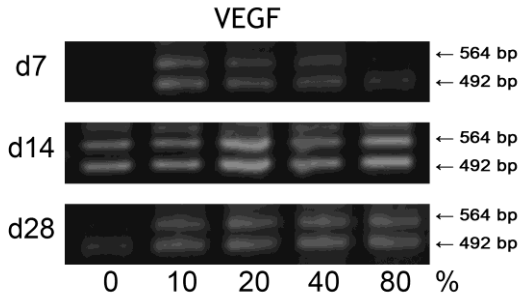


**Fig. 13** VEGF mRNA recovery using RT-PCR. Zero, 10, 20, 40 and 80% means DBP content in PLGA and same with PLGA, D10, D20, D40 and D80 respectively in Figure 16-11.

**4.3 Histology**

After 3 days, we observed that a marginal gap and inside implant were filled with blood coagulum in all groups. In the defect filled with DBP/PLGA implants, a small amount of new osteoid matrix is detected around the some active fragments of DBP nearby defect margin (Figure 15A). By postsurgical days 7, the fibro-osseous like tissue was found at the area between defected bone and implant (Figure 15B). After days 7, first vascular structures were visible on every groups depending contents of DBP within the scaffolds, implant without DBP stained positive weakly for CD31/PECAM-1, but especially 20% and 40% DBP/PLGA implants resulted in an enhanced expression until postsurgical days 28 (Figure 15C). On days 28, we could see various stages of differentiation and maturation of bone, starting from cortex to center of the implant. The area of new bone inside of scaffold was found higher in 20% and 40% DBP groups than other groups (Figure 16).

The DBP in defect degraded into smaller pieces progressively. In contrast, 80% DBP groups exhibited only small amount of newly formed osteoid tissue compared to even only PLGA groups, suggesting that the increased DBP contents compromised the porosity and interconnectivity of composite implant, which may enable successful invasion and ingrowth of osteoprogenitor cell to it.[4,5]

In conclusions, DBP inside of PLGA scaffold is not only attracting osteoblasts into the bony defect site and stimulating their differentiation, but also leading more intensive angiogenesis by means of angiogenic activation, which plays an important role in bone formation and maintenance, and bone tissue differentiation.

**4.4 The host tissue response to PLGA/small intestine submucosa (sis) hybrid scaffolds**

SIS consists of more than 90% types I and III collagen, plus a wide variety of cytokines, including basic fibroblast growth factor (bFGF), transforming growth factor-b (TGF-b), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1), as well as glycosaminoglycans, fibronectins, chondroitin sulfates, heparins, heparin sulfates, and hyaluronic acids.[23] These constituents are expected to facilitate the function of SIS as a tissue engineering scaffold by supporting cell attachment, proliferation, differentiation, and migration. We tried to compare the host tissue response to representative synthetic and natural biomaterials by assessing inflammation at the implanted area. We prepared and characterized PGLA/SIS films with five different ratios of SIS as 0, 10, 20, 40 and 80% to PLGA, performed subcutaneous implantation of these scaffolds into rats, and then compared the host tissue response by RT-PCR analysis of TNF-α, IL-1β and IL-6 mRNA expression as shown in Figure 16 and then normalized in Figure 17.

As shown in Figure 17, TNF-α mRNA in *in vivo* highly expressed following PLGA film, as compared to SIS/PLGA films; it was significantly lower following SIS/PLGA films than PLGA film with increases in contents of SIS, 10, 20, 40 and 80% of SIS ($p < 0.05$). The intensity of TNF-α expression of PLGA film was significantly ten times higher or more than that of 40% SIS/PLGA films. Similarly, IL-1β mRNA expression decreased markedly with 40% and 80% SIS/PLGA film compared to PLGA film.

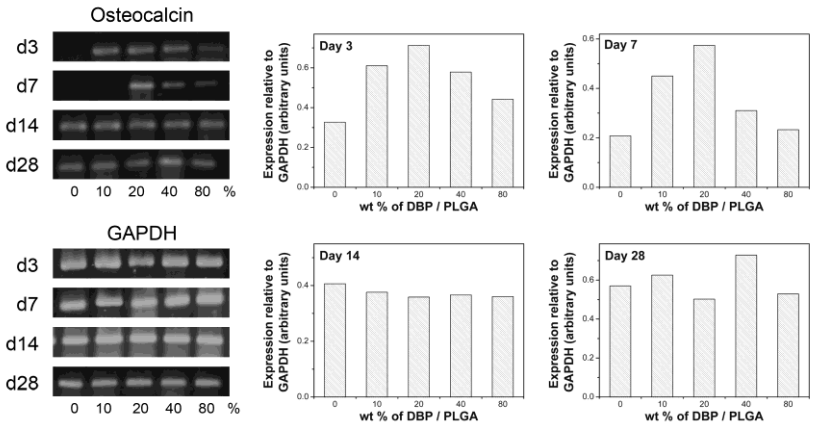


**Fig. 14** Representative patterns and densitometric comparisons of osteocalcin expression relative GAPDH

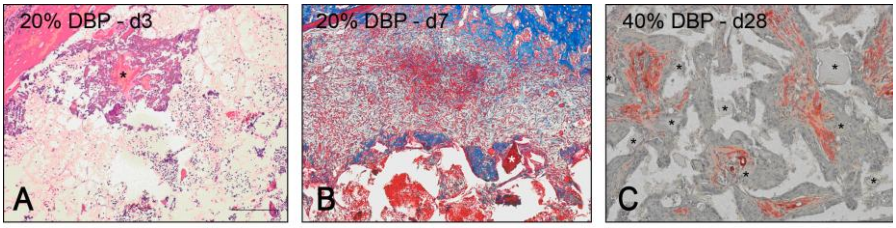


**Fig. 15** Horizontal sections. A. 20% DBP after 3 days; HE stain, B. 20% DBP after 7 days; TC stain, C. 40% DBP after 28 days; immunohistological staining using CD31. (Asterisks are the fragments of DBP, Original magnification; A and B = 40x, and C = 100x)

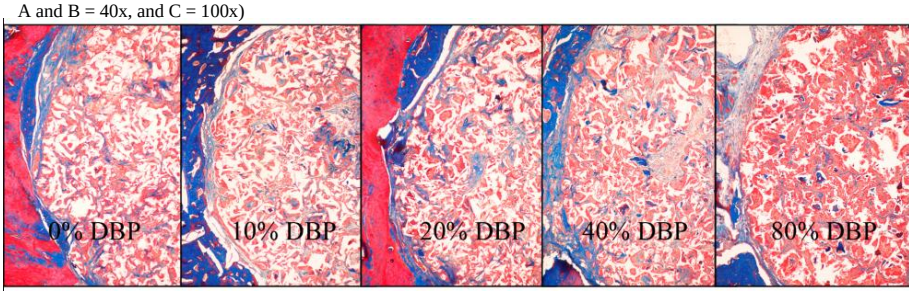


**Fig. 16** Horizontal sections after 28 days; TC stain (Original magnification x40

TNF-α plays a role of inflammatory response that activates leukocytes, enhances adherence of neutrophils and monocytes, promotes the migration of inflammatory cells into the intercellular space and triggers local production of other pro-inflammatory cytokines such as IL-1β.[19] TNF-α, IL-6 and IL-1β which are potent stimulator of fibroblast growth.[18] Therefore, temporospatial expression of these proinflammatory mediators allows fine tuning of the inflammatory response.[24]SIS has been reported as a biocompatible inducer of cell growth which is mainly responsible to several growth factors as we discussed earlier in SIS has been described.[23]In this study, we observed that their inflammatory response to SIS hybrid polymeric materials was obviously reduced. The probable cause for these results is that TNF-α expression may be strongly suppressed by bioactive substances released from SIS; this pattern was enhanced with an increase in content of SIS in PLGA film, which can result in suppression of the IL-1β and IL-6 expression that reduces proliferation and fibrous capsular formation of fibroblasts (data not shown).

## Discussion and Conclusion

Tissue engineering including regenerative medicine shows tremendous potential as a revolutionary research. Successful results have been reported for regenerating tissues and organs such as skin, bone, cartilage, nerve, tendon, muscle, corneal, bladder and urethra, and liver as well as composite systems like a human phalanx and joint on the basis of scaffold biomaterials from polymers, ceramic, metal, composites and

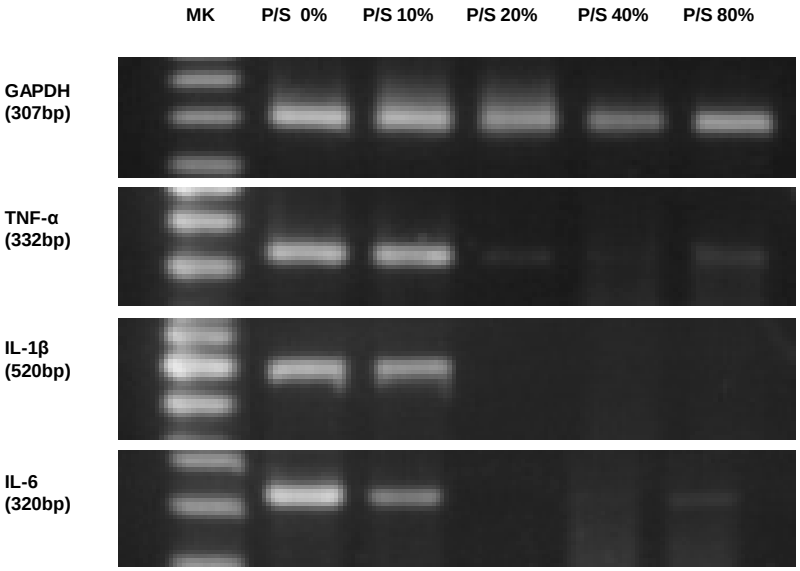


**Fig.16** *Representative* gene expression band as IL-6, IL-1□ , TNF-□ and GAPDH. These bands were normalized andshowed in Figure 17.

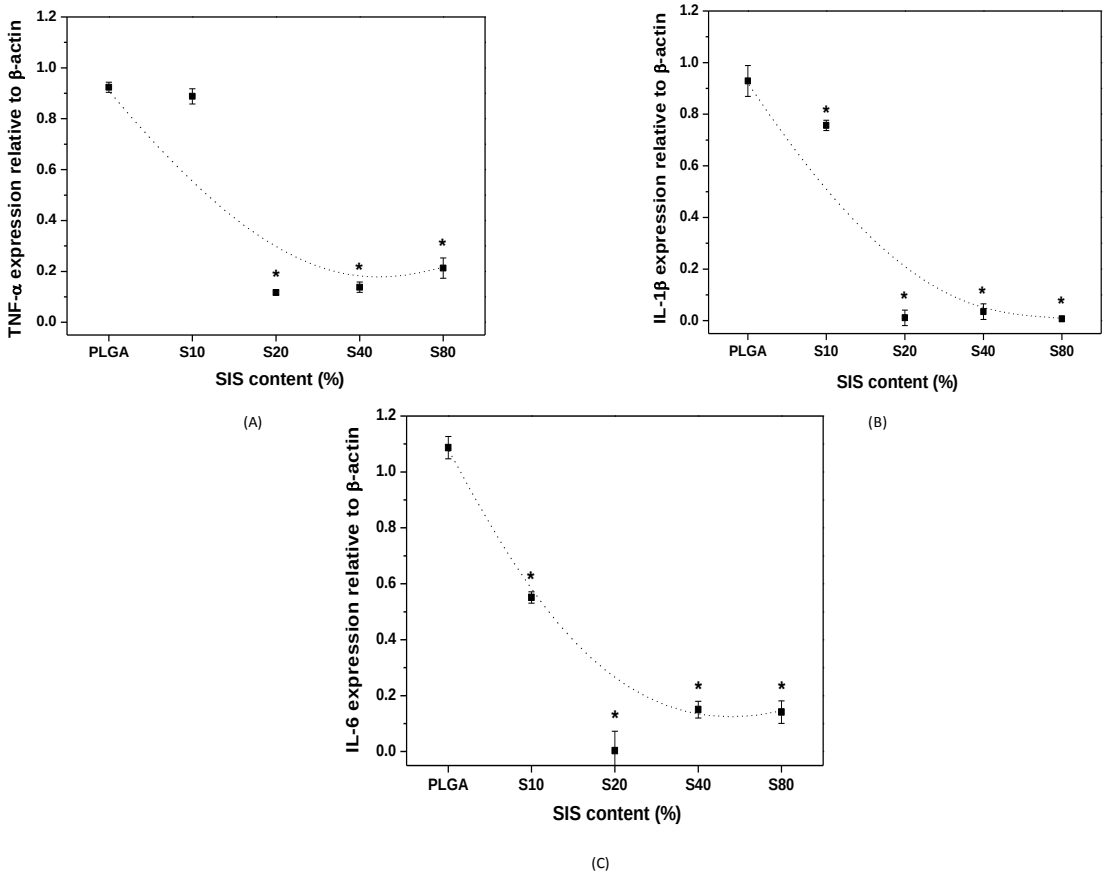


**Fig. 17** The expression of TNF-α IL-1β and IL-6 mRNA in in vivo fell continuously with an increase in content of SIS in PLGA. (A) The significant decrease of a TNF-α expression in each scaffold as SIS contents increases from 0 to 80%. (B) IL-1β mRNA expression decreased markedly on 40% and 80% SIS/PLGA film. (C) IL-6 mRNA expression decreased markedly on 40% and 80% SIS/PLGA film.*Corresponds to P<0.05 in comparison with PLGA film.

its hybrids. The prerequisite physicochemical properties of scaffolds are (i) to support and deliver for cells, (ii) to induce, differentiate and conduit tissue growth, (iii) to target cell-adhesion substrate, (iv) to stimulate cellular response, (v) wound healing barrier, (vi) biocompatible and biodegradable, (vii) relatively easy processability and malleability into desired shapes, (viii) highly porous with large surface/volume, (ix) mechanical strength and dimensional stability, (x) sterilizability and (xi) do not induce inflammatory reaction and fibrotic capsule .[3,4]

From this point of view, the design and control over precise biochemical signal is needed by the combination of scaffold matrix and bioactive molecules including genes, peptide molecules and cytokines. Moreover, the combination of the cells and redesigned bioactive scaffolds has attempted to expand to a tissue level of hierarchy. In order to achieve this goal, the novel hybrid scaffold biomaterials, the novel scaffolds fabrication methods and the novel characterization methods must be developed.

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