Research Article

Ovalbumin-Based Porous Scaffolds for Bone Tissue Regeneration

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Cell differentiation on glutaraldehyde cross-linked ovalbumin scaffolds was the main focus of this research. Salt leaching and freeze drying were used to create a three-dimensional porous structure. Average pore size was $147.84 \pm 40.36\,\mu$m and $111.79 \pm 30.71\,\mu$m for surface and cross sectional area, respectively. Wet compressive strength and elastic modulus were 6.8 ± 3.6 kPa. Average glass transition temperature was $320.1 \pm 1.4°$C. Scaffolds were sterilized with ethylene oxide prior to seeding MC3T3-E1 cells. Cells were stained with DAPI and Texas red to determine morphology and proliferation. Average cell numbers increased between 4-hour- and 96-hour-cultured scaffolds. Alkaline phosphatase and osteocalcin levels were measured at 3, 7, 14, and 21 days. Differentiation studies showed an increase in osteocalcin at 21 days and alkaline phosphatase levels at 14 days, both indicating differentiation occurred. This work demonstrated the use of ovalbumin scaffolds for a bone tissue engineering application.

1. Introduction

Autogenous bone is the most preferred bone grafting material. However, limitations and complications from using autografts include a limited quantity and chronic donor site pain [1, 2]. This has led to the need for an ideal bone graft substitute. An ideal substitute must have enhanced capabilities to reduce or eliminate the need for an autograft altogether [3] and are necessary to provide support, fill voids, and enhance biologic repair of defects.

The need for tissue-engineered constructs is increasing and advances in the field have led to the use of scaffolds, cells, and factors to regenerate organs and tissues [3]. The integration of the biological, physical, and engineering sciences will create the new constructs that regenerate and restore the functional state of damaged tissues [4]. Using tissue engineered constructs such as biobased scaffolds as bone graft substitutes has emerged as an approach to regenerate bone.

Tissue-engineered constructs, specifically biopolymers, can promote successful bone healing when originating from natural proteins found in the body. Ovalbumin (OA) is being used in this study because it is a biopolymer found in chicken egg whites, has a molecular mass of 45 kDa, and is comprised of 386 amino acids with 10% of the amino acid sequence conserved when compared to human serum albumin. OA is comprised mainly of α-helix and β-sheet, but when introduced to an alkaline environment (pH $>$ 7), it transforms to a predominantly β-sheet structure [5]. It can be used to create biocompatible scaffolds that aid in osteoblast adhesion and mineralization into 3D structures [6]. Ovalbumin is more readily available and cheaper ($40/kg, Sigma Aldrich) than using synthetic or other natural biopolymers [7].

Ovalbumin contains nineteen lysines per OA molecule which are necessary for chemical crosslinking with a common agent, glutaraldehyde (GA) [8]. Glutaraldehyde crosslinking is governed by reactions with ε-amino groups of lysines (Figure 1). The GA crosslinking process has been shown to alter cellular response due to its cytotoxicity [9] and may alter osteoblastic responses through modification of the scaffolds [10]. However, it has been previously reported that using GA as a crosslinker for other biopolymer scaffolds such as collagen, alginate, and keratin has not affected
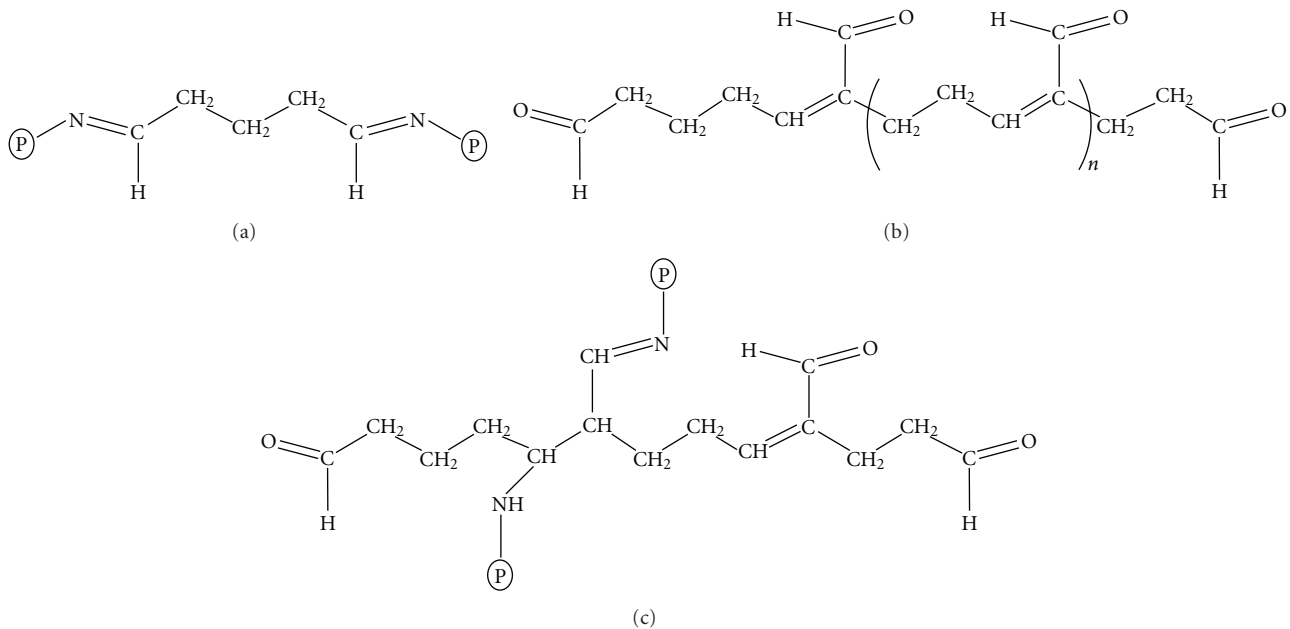
(a)

(b)

(c)

FIGURE 1: (a) Schiff base formation obtained by crosslinking of lysine residues from two protein (P) molecules by monomeric glutaraldehyde. (b) The structure of polymeric glutaraldehyde. (c) Suggested end product obtained from reaction between polymeric glutaraldehyde and lysine residues from the cross-linked proteins16 .

biocompatibility [11–13]. Therefore, small concentrations of GA will be used to prevent cytotoxicity, and OA scaffolds may also be created using this method.

Both a material and biological perspectives are needed to fully understand interactions in the body to make biomaterial scaffolds successful. From a material perspective, the extent of how surface properties and material characteristics affect cell behavior must be determined. It is also important to know the parameters that govern positive cell response to a biomaterial and what *in vitro* models reproduce these parameters in order to conduct successful research [14]. Using these results as a guideline, a biobased scaffold for osteoblast adherence and proliferation can be envisioned. biobased polymers need to be researched for further tissue engineering advances, specifically their use in bone regeneration [15]. This study aims to create a biobased OA scaffold and investigate the effects of adhesion, growth, and differentiation of preosteoblast cells on these structures.

2. Materials and Methods

2.1. OA Solution. To create an OA solution, 5.0 g OA from chicken egg white, grade II (Sigma Aldrich) and 0.01 g dithiothreitol (Sigma Aldrich) minimum 99% titration were dissolved in 30 mL borate buffer (pH 9.5) and 50 mL deionized (DI) water. The solution was stirred overnight at room temperature and dialyzed using snake skin dialysis tubing in water at room temperature for three days. The water was changed twice a day for the duration of the dialysis. The dialyzed solution was then stored in the refrigerator until use.

2.2. Scaffold Fabrication. Scaffolds were fabricated using sodium chloride salt porogen, OA solution, and GA (Sigma-Aldrich, Grade I, 25% in H_2O) crosslinker. One gram sieved salt with particle sizes 90–150 μm was measured into wells of a 12-well plate. Two and a half milliliters dialyzed OA solution was pipetted over the salt of each well plate. Ten percent GA to OA solution by volume was pipetted into the wells to introduce crosslinking. Each well was stirred to ensure equal distribution and placed on a shaker overnight to allow for crosslinking.

Once cross-linked, the scaffolds were removed from the well plates and placed in 150 mL of 100 mM glycine (Sigma Aldrich) solution at 35°C to terminate GA crosslinking. After one hour the scaffolds were taken out of the glycine solution and placed in a beaker of DI water to commence the salt leaching process. The beakers were placed on a shaker and the scaffolds remained in DI water for three days and water changed twice a day during the process.

After the salt leaching process the scaffolds were placed in the −80°C freezer overnight. The next day they were placed in a lypholizer at −80°C and 10 torr overnight to remove excess water. After 24 hours the samples were removed and placed in a desiccator until use.

2.3. Percent Crosslinking. A trinitrobenzenesulfunic acid (TNBSA) assay as described by Hermanson [16] was performed to measure the number of ε-amino groups in the scaffolds to determine percent crosslinking. Scaffolds with 10% GA to OA solution by volume crosslinking as well as OA powder (as previously discussed) as a control were used in the study. Briefly, 11 mg dry scaffold was placed in a 50 mL screw cap tube with 1 mL of 4% $NaHCO_3$ and

1 mL 0.5% TNBSA. The vial was placed in a water bath with a stirring bar at 320 rpm at 40°C for four hours. Three milliliters of 6 M HCl was added to the vial to hydrolyze the reaction and then the vial was placed in the autoclave for one hour at 120°C and 15–17 psi to hydrolyze and dissolve the protein. After autoclaving, the solution was diluted with 20 mL DI water and read on a UV-vis spectrophotometer at 350 nm.

2.4. Scaffold Morphology. The surface and cross-sectional area scaffold morphologies were viewed using the FESEM (Leo/Zeiss 1550, Munich, Germany). Samples were sprayed with 15 μm thick conductive Gold-Palladium coating under vacuum in an argon atmosphere. Samples were observed in the FESEM under vacuum at 5 kV.

Average pore size was determined by viewing FESEM images in a Q analysis program and measuring the diameter of 25 different pores. Pores were identified as areas of void space.

2.5. Mechanical and Thermal Testing. Scaffolds were mechanically tested by compression using an Instron 5869 with a 100 kN load cell. Compression tests were carried out on samples with approximately twenty millimeter diameters and approximately seven millimeter heights at a crosshead speed of 2 mm/minute. Scaffolds were submerged in phosphate buffered saline (PBS) solution for two days, completely wetting the samples to allow for wet compression testing.

Differential scanning calorimetry (DSC) thermal measurements were performed on wet scaffolds and a noncross-linked OA film as the control with a Netzsch 889 analyzer from 30°C to 350°C at a heating rate of 10°C/minute under a helium atmosphere. The temperature was decreased to room temperature at a rate of 10°C/minute. Thermal analysis was carried out using the Proteus Thermal Analysis software.

2.6. In Vitro Evaluation

2.6.1. Proliferation Studies. All scaffolds were sterilized with ethylene oxide prior to cell culture. Scaffolds were placed in 48-well plates containing MC3T3-E1 preosteoblasts suspended in 0.5 mL medium (10,000 cells/scaffold) for 4 hours and 96 hours. MC3T3-E1 cells were cultured in expansion medium containing α-minimum essential medium (MEM) plus 10% fetal bovine serum (FBS) and 1% antibiotic/antiomycotic.

Cell staining [17] consisted of fixing the cells for 5 minutes using 0.5% by volume Triton X-100, 4% by volume formaldehyde and 1 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$ in phosphate buffered saline at pH 7.4. The scaffolds were then rinsed and postfixed for 20 minutes in the same fixative as before without Triton X-100. Cells were stained in the dark for 1 hour with 2 μM Sulforhodamine 101 Phalloidin (Texas Red) and 6 μM 4′,6-diamidino-2-phenylindole (DAPI) in PBS containing 1 mM Ca^{2+} and 2 mM Mg^{2+} After 5 minutes of air drying, the scaffolds were mounted on glass slides with Vectashield containing DAPI. Cell number was determined using confocal microscopy.

2.6.2. Differentiation Studies. To induce osteoblastic differentiation, cells were seeded and cultured for 3, 7, 14, and 21 days on scaffolds. Three scaffolds and cross-linked films were tested per time period with an empty well as a control. MC3T3-E1 preosteoblast cells were cultured on ethylene oxide sterilized 5.5 mm diameter scaffolds as well as cross-linked films. A 48-well plate with 0.5 mL expansion medium (per well) supplemented with ascorbic acid (50 mg/mL) and β-glycerol phosphate (10 mmol/L) to enhance osteoblast differentiation was used. The medium was changed every 3 days during the differentiation studies. To determine if differentiation occurred, scaffolds were kept for ALP level analysis using a reagent assay.

OCN levels were measured for each time interval using an enzyme-linked immunosorbant assay (ELISA) kit (Biotechnologies, Inc.) according to the manufacturer's directions

2.7. Statistics. All experiments were performed with three scaffolds and cross-linked films unless otherwise specified. The results were reported as mean ± standard deviation. Statistical significance was determined by single factor analysis of variance (ANOVA) with $P < .05$

3. Results

3.1. Percent Crosslinking. Using the TNBSA assay, percent crosslinking averages for the scaffolds were determined. Moles of lysine present were calculated using average absorbencies for the scaffolds. The percent crosslinking was calculated by using the average moles of lysine at 350 nm for the OA powder control and 10% GA to OA solution by volume scaffolds. It was determined that the scaffolds had a percentage crosslinking of 35 ± 9%.

3.2. Scaffold Morphology. SEM analysis of the scaffolds allowed for morphology and size of pores to be evaluated. A porous structure was viewed for both surface and cross-sectional area (CSA) of the scaffolds and average pore size was determined. Average pore size for the surface was 147.84 ± 40.36 μm and 111.79 ± 30.71 μm for the CSA. No significant difference was found between the surface and CSA. Figure 2 shows a representative image for pore size and morphology of the scaffolds.

3.3. Mechanical and Thermal Tests. Scaffolds were tested wet to determine the effect on ultimate compressive strength. The average ultimate stress and elastic moduli for the scaffolds were 6.8 ± 3.6 kPa. Wet scaffolds exhibited elastic behavior.

Using the Proteus Thermal Analysis software, the glass transition temperature (T_g) of the scaffolds and OA powder control were found. The control had a T_g of 240 ± 35°C and the scaffold, 320.1 ± 1.4°C.

3.4. Cell Studies

3.4.1. Proliferation Studies. Cells were stained with DAPI and Texas Red to look at cell morphology on the scaffolds. Nuclei were stained blue due to the DAPI binding to the DNA while
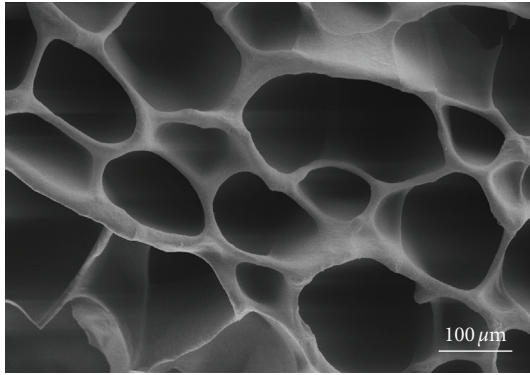
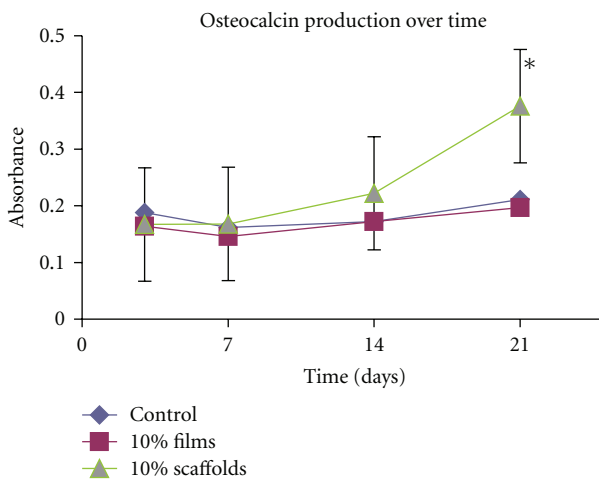FIGURE 2: FESEM scaffold image illustrating scaffold morphology and pore size. Image is taken at 200x.

FIGURE 3: Scaffolds at 21 days showed a significant increase in OCN levels when compared to the control and 10% cross-linked film.
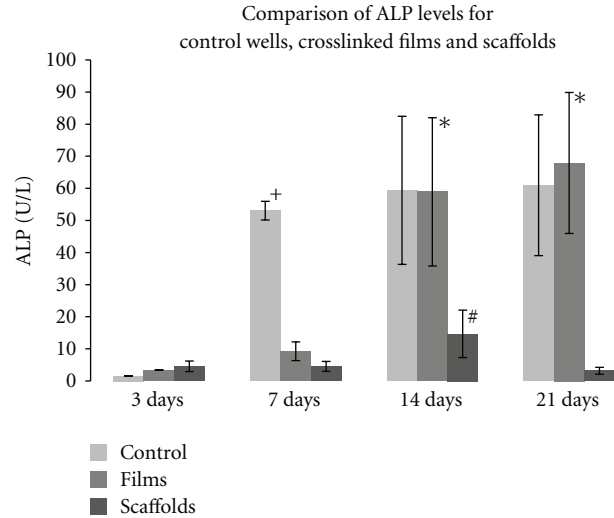

FIGURE 4: A significant increase in ALP in the control well was seen at 7, 14, and 21 days when compared to the control well at 3 days. ALP levels for cross-linked films at 14 and 21 days showed a significant increase compared to cross-linked films at 3 and 7 days. Scaffolds showed a significant increase at 14 days compared to scaffolds at 3 and 7 days with a downregulation at 21 days.

Texas Red binds to the F-actin of cells staining it red. Because of the scaffolds autofluoresce, it was impossible to see the stained cell body to determine morphology along the scaffold pores. However, cell numbers for both the 4-hour and 96-hour time intervals could be determined by counting the stained nuclei. At four hours the average number of cells on the scaffolds was 60.8 ± 18.9 cells per image and at 96 hours the average number of cells was 153 ± 4.8 cells per image, a twofold increase. Cell numbers between time intervals were significant.

3.4.2. Differentiation Studies. Differential studies compared scaffold OCN levels at 3-, 7-, 14-, and 21-day time intervals to standard solutions. OA powder as a control (as previously mentioned) and 10% by volume GA to OA solution cross-linked films were also compared to a standard curve found from the average standard absorbances calculated. Absorbance for all samples was determined and compared. Scaffolds at 21 days showed a significant increase in OCN levels when compared to the control and cross-linked film (Figure 3).

A significant ALP increase was seen in the control well at 7, 14, and 21 days when compared to the control well at 3 days. ALP levels for cross-linked films at 14 and 21 days showed a significant increase compared to cross-linked films at the 3- and 7-day time intervals. Scaffolds showed a significant increase at 14 days when compared to scaffolds at 3 and 7 days and a downregulation of ALP production was seen at 21 days (Figure 4).

4. Discussion

Although GA is a common crosslinking agent, the mechanism and chemistry involved in the crosslinking reaction is not yet fully understood [18]. It has been shown that varying GA concentration has an effect on crosslinking [19]. At low concentrations of GA, it is more probable for GA to crosslink with lysines in OA molecules because the amount of lysines is equal to or greater than the amount GA molecules present. At higher concentrations of GA, it is more probable for GA to react with itself because the amount of GA molecules is larger than the amount of lysines present to crosslink. Therefore, there is a limit to how much GA is able to crosslink with lysine molecules. This explains why only 35% crosslinking was observed in the scaffolds and confirms that an excess of GA was used.

The average pore size of the scaffolds in this study corresponds with those reported in the literature [20–22] that have been shown as optimal pore size for cell adhesion, proliferation, and differentiation [23]. The wet compressive mechanical properties of the scaffolds fell below the actual compressive strength of bone. Compact bone is known to have a compressive strength of 150–250 MPa due to variability in bone density [24, 25]. Although the ideal

mechanical strength of biomaterial scaffolds has not yet been determined, previously researched scaffold compressive strengths have fallen within a 2–45 MPa range [26–28]. The compressive modulus for bone has been measured to be 5–20 GPa while biomaterial scaffolds vary from 60 MPa–15 GPa [27]. Although OA scaffolds are lower in compressive strength and modulus than other biobased scaffolds as well as natural compact bone, it is not fully understood to what extent scaffolds must mimic natural bone mechanical properties. They have, however, demonstrated to be a promising substrate for cell growth and bone regeneration as shown by the cellular studies and sponge-like characteristics of the scaffolds.

Only glass transition temperatures were observed in the control and scaffold; therefore, it can be concluded that the scaffolds were amorphous. The glass transition temperature of the control was determined to be 240°C; so an increase in T_g to 320°C for the scaffolds confirms crosslinking occurred.

Using confocal images, cell numbers for the scaffolds at 4 hours and 96 hours were determined and a significant increase between the 4-hour- and 96-hour-cultured scaffolds was seen. Because of this significant increase, it was determined that the preosteoblast cells responded positively to the scaffold and surrounding environment and reached a proliferative state. Alkaline phosphatase studies showed a significant increase in cross-linked films at 14 and 21 days in comparison to cross-linked films at 3 and 7 days. For the scaffolds, a significant increase in ALP was seen at 14 days; however a decrease in ALP production was seen at 21 days. Osteocalcin differentiation studies showed an increase in OCN levels in scaffolds cultured for 3, 7, and 14 days when compared to the control well and cross-linked films; however no significant difference was found. There was a significant increase in OCN levels in the scaffolds cultured for 21 days when compared to the cross-linked film and control well at the same time interval. It has been shown that at two weeks *in vitro*, ALP is increased and then downregulated when mineralization begins. This corresponds with the scaffold OCN data showing a significant increase of OCN at 21 days indicating the beginning of mineralization. The significant increase in OCN levels at 21 days and the downregulation of ALP after the two-week time period indicate that differentiation occurred.

5. Conclusion

After scaffold fabrication, morphology and pore size were determined and all scaffolds had a CSA and surface porous structure comparative to porous scaffolds used in previously reported literature. Glutaraldahyde was used as the crosslinker to create the 3D porous structure and material characterization was conducted on the scaffolds. A TNBSA assay was conducted to determine percent crosslinking of the scaffolds, and it was determined that GA crosslinking reaction did occur. However, 100% crosslinking was not reached due to GA intermolecularly crosslinking with itself, not allowing for chemical interaction with all lysines present

in OA molecules. Also, an average glass transition temperature, compressive strength, and compressive modulus were found for the scaffolds. Although the average compressive strength and modulus is lower than bone, the needed compressive strength for scaffolds is not known and therefore the scaffolds cannot be ruled out as a possible substrate for cell growth and differentiation. In fact it was shown through cell studies that despite low compressive strength and modulus, MC3T3-E1 preosteoblast cells did increase in number and therefore a proliferative state of the cells was reached. Differentiation studies showed a significant increase in OCN levels at 21 days for scaffolds. A significant increase in ALP at 14 days was seen for the scaffolds as well as a decrease at 21 days corresponding with the increase in OCN at 21 days. These results signify the beginning of mineralization and support the idea that differentiation did occur. Overall OA scaffolds have shown to be a promising 3D material construct to induce the proliferation and differentiation of MC3T3-E1 preosteoblast cells for bone tissue regeneration.

Acknowledgments

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