THE ANALYSIS OF BOVINE SERUM ALBUMIN BINDING AFFINITY FOR XENOGRAFT COMPARED TO A SYNTHETIC PARTICULATE BONE GRAFT MATERIAL.

A Thesis
Submitted to
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ABSTRACT

WORKING HYPOTHESIS:

Binding of albumin to various bone graft materials is correlated with the surface porosity of these materials, and therefore the binding of albumin to xenograft is stronger than its binding to synthetic bone graft.

NULL HYPOTHESIS: There is no significant difference in the binding strength of albumin to xenograft than its binding to synthetic bone graft materials.

Objectives: The increased availability of commercially produced bone graft materials versus autogenous bone makes these materials a desirable product in guided bone and tissue regeneration procedures. The use of commercially produced bone graft materials also provides the opportunity of the addition of certain biologic materials in order to enhance the healing response and to overcome the predominantly inactive nature of most graft materials on the market. The development of an adequate carrier of biologic agents is a crucial step in the creation of a bioactive graft material. This study uses an easily manipulated model protein to study specific characteristics of protein binding and release on two different bone graft substrates commonly used as calcified scaffolds in guided bone and tissue regeneration. This experiment was completed as a first phase in the establishment of a protocol for the future investigation of other relevant proteins that may be important in bone and tissue regeneration.

Methods: Bovine serum albumin (BSA) dissolved in physiologic buffered saline solution was poured over 100 mg of either xenograft or synthetic particulate grafting

material, and incubated for 24 hrs. at 4°C. The quantity of BSA protein adsorption to the grafting material surface was determined by removing all liquid from the wells after the 24 hr. incubation period, followed by quantification of protein concentrations using the bicinchoninic acid (BCA) protein assay reagent kit. In order to analyze the kinetics of protein release, 1 ml of phosphate-buffered saline (PBS) wash was added to all wells, stirred and removed from each well. This was followed by the addition of 1 ml PBS to all wells and removal of 1 ml of liquid at intervals of 1, 3, and 7 days. Protein concentrations were quantified using the BCA protein assay, and the results were analyzed using a two-way ANOVA.

Scanning electron microscopy was performed on samples of xenograft and synthetic graft particles prior to BSA exposure, as well as at days 1 and 7 following the initial 24 hr. incubation and the subsequent PBS wash. Energy-dispersive X-ray spectroscopy was also used to analyze the elemental components of the xenograft and synthetic graft material after BSA treatment.

Results: Scanning electron microscopy revealed a more porous surface texture and collagenous appearance of the xenograft graft material, versus the synthetic graft. The energy-dispersive X-ray spectroscopy showed a noteworthy difference between the elemental composition of the xenograft and synthetic graft material. A lower concentration of protein was shown in solution after the initial 24 hr. incubation period in the xenograft samples possibly indicating that more protein was bound to the xenograft particles than the synthetic bone. The remaining solution from the xenograft samples throughout the kinetics of release analysis showed more albumin protein released over time as compared to the synthetic graft samples.

Conclusions: This study revealed that xenograft material showed a more porous surface structure and greater binding affinity for bovine serum albumin as compared to the synthetic material. The protocol described in this study is a useful model system for future studies to investigate other proteins involved in wound healing, bone remodeling, and angiogenesis. Protein binding and kinetics of release should be explored on alternative mineralized scaffolds or carrier systems in order to determine an adequate delivery mechanism that allows for sustained release during the optimum time frame for modulation of the healing process. Future experiments should focus on identification of an ideal transport medium for bioactive agents that will direct cells into the osteogenic process to restore new bone and periodontal supporting tissues. The engineering of a material that has the quality of extended release of proteins necessary for the healing cascade has the potential to unlock the key to periodontal regeneration.

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CHAPTER 1

INTRODUCTION

Over the last decade, the use of bioactive agents has become more commonplace in regenerative procedures for intra-osseous bony defects or reconstruction of the alveolar ridge in preparation for dental implants. The volume of dental implants being placed has also dramatically increased in recent years, driving a trend towards the use of commercially available bone graft and synthetic materials to alleviate some of the limitations associated with procurement of autogenous tissue. Autogenous bone grafts require a secondary surgical site, leading to increased patient discomfort as well as limits in the quantity of bone available for harvest. Autogenous bone, however, is the gold standard of treatment for regenerative procedures due to its inherent osteoinductive potential (Misch 2010). Native bone delivers osteogenic vital cells, growth factors and bone morphogenic proteins that allow for quicker healing and incorporation of the graft.

Commercially available bone graft materials are often utilized in regenerative procedures rather than autogenous bone to reduce morbidity and for greater abundance of graft material. Some of the limitations of these graft materials include increased healing time to produce vital bone and a decreased volume of bone growth potential due to the inactive nature of the graft. This type of bone graft acts as a space maintaining, scaffold devoid of growth factors and other osteoinductive components. Previous studies have shown that demineralized freeze-dried bone allograft may have a small occurrence of osteoinductive potential, but these results vary greatly (Schwartz et al 1996). The supposed osteoinductive potential of allogeneic tissues is dependent on the age and health status of

the tissue donor, and cannot be accurately predicted or traced once the donor tissue is processed (Schwartz et al 1998).

Other commonly used particulate graft materials available are xenograft and fully synthetic alloplast. These materials are used in order to provide a longer lasting space maintenance and slower resorbing scaffold as compared to allogeneic products. The xenografts most widely used are of bovine or equine origin. Commercially available bovine xenografts are mineralized freeze-dried bone that has been processed and treated to remove all proteins, lipids and is finalized with a sterilization method. These are used as a calcified matrix for replacement apposition. Fully synthetic alloplast materials are frequently comprised of hydroxyl-apatite with combinations of calcium sulfate or tricalcium phosphate. The hydroxylapatite acts as a long lasting scaffold while the tricalcium phosphate is more rapidly resorbed, allowing for replacement resorption and incorporation of vital bone. Depending on the particle size of the graft material, the resorption and replacement of these scaffolds can take anywhere from six to twelve months. Fully synthetic alloplasts that incorporate more hydroxylapatite have been seen to last longer than xenograft particulates. The porosity and microstructure of xenograft and synthetic alloplast is of interest in this study. One of the primary aims of this investigation is to determine if surface topography of a graft material contributes to more favorable protein binding and biomodification.

A major drawback to the use of commercially available graft materials is their lack of osteoinductive potential. This is where the use of bioactive agents comes into play. If particulate graft materials can be improved to increase biocompatibility and osteoinductive potential, it is possible to decrease the limitations that deem them inferior

to autogenous grafts. The ideal bioactive graft material would be able to deliver biomodiofication agents locally to the graft site to alter the periodontal wound healing response and induce the cascade of bone regeneration. Allograft, xenograft, and alloplastic materials with limited inherent bioactive potential can then be transformed into bioactive regenerative materials.

One obstacle to the transformation of currently used particulate grafts into bioactive regenerative materials is the method of delivery. The development of an adequate carrier of these bioactive agents is a crucial step in the manufacture of a bioactive graft material. The carrier must allow the bioactive agents to be distributed locally at the precise time in the healing process. In order to alter the wound healing and remodeling process, these bioactive agents must be available to initiate the cascade of events that will result in potentiation of bone growth and periodontal regeneration. With the correct bio-modifier, combined with an adequate carrier mechanism, these bioengineered materials could open up a new standard of possibilities in true periodontal regeneration.

The two bioactive proteins selected for study are albumin and amelogenin. Serum albumin is a multi-functional protein found in blood plasma and serum, and is critical to cell health. Serum albumin is used in cell culture media as a supplement to increase growth, productivity, and overall health of cells in culture. Serum albumin is able to deliver nutrients to cells as well as bind toxins, hormones, and growth peptides in order maintain cell stability. Albumin has been widely used in medicine for wound healing applications in burn victims and cases of hemorrhage, malnourishment, and liver and kidney failure (Peters 1995). In vitro studies have shown albumin-coated

mineralized allograft to increase the proliferation of stem cells on the allograft surface without altering the physical characteristics of the bone scaffold (Weszl et al 2011). Albumin coating did not form a monolayer, but infiltrated into the pores of the graft particles. In vivo rat femur studies have shown that serum albumin coating of mineralized bone chips can enhance the remodeling and efficacy by uniting a segmental defect where uncoated allograft did not (Skaliczki G et al 2013). The characteristics of albumin make it a stable protein that can be readily used as a "model" protein. This protein can be manipulated to study specific characteristics in initial studies that may establish a protocol for future investigation of other proteins that contribute to the osteoinductive cascade.

Enamel matrix derivative, trademarked as Emdogain® (Straumann, Andover, MA) is a biologic factor widely used to enhance the potential for regeneration of the periodontal apparatus including cementum, alveolar bone, and periodontal ligament in periodontal defects. It has become increasingly common practice for Emdogain to been used as an adjunct to a variety of bone grafting materials to enhance the effects of standard bone grafting techniques. Emdogain however, was developed with the intention to be used alone in a bony defect without the combination of a barrier membrane or particulate graft material (Struamann, Andover, MA). This addition of Emdogain to grafting procedures nevertheless, in the current literature, has not shown an astounding positive result or enhancement on periodontal regeneration as compared to standard grafting procedures without its use. The predominant enamel matrix protein in Emdogain is amelogenin, which makes up 90% of the composition. The other 10% of the product's composition is proprietary. Emdogain has been shown to elicit TGF-B and BMP-like

activity leading to induction of the SMAD transcription factors. SMADs contribute to the modulation of tissue formation through cell growth, differentiation and development (Wyganowska-Świątkowska M et al 2015). It has been show through in vitro studies, that the current carrier molecule for Emdogain, polyglycolic alginate gel, is made for use without graft particulate and may not be the most adequate delivery method when combined with graft particulate. Previous studies show that when dissolving EMD into a liquid form, the amelogenin proteins were able to adsorb more closely and deeper into surface porosities of particulate bone grafts. When compared to the current gel application, the amelogenins were contained in a thick layer that did not allow the proteins to penetrate or closely adapt into surface porosities (Miron et al 2015).

In this study bovine serum albumin will be used in order to study its binding affinity and release kinetics to two bone grafting materials. The bone graft materials to be examined will be Struamann products boneceramic and xenograft. It is the aim of this study to determine if there is a difference in albumin binding affinity to xenograft compared to a synthetic bone graft material.

CHAPTER 2

MATERIALS AND METHODS

COATING OF GRAFT MATERIALS WITH 1% BSA SOLUTION

Particulate bone graft materials used in this experiment include XenoGraft bovine bone with a granule size of 200-1000 µm (Struamann, Andover, MA) and BoneCeramic Synthetic biphasic calcium phosphate (HA/TCP 60:40) with a granule size of 500-1000 µm (Struamann, Andover, MA). A solution of 1% Bovine serum albumin in phosphate buffered saline solution was prepared. 1ml of prepared 1% BSA solution was poured over 100mg of either xenograft or synthetic particulate grafting material using a twenty four -well plate and all samples were incubated for 24 hours at 4°C. The experiment was replicated using 1ml of sterile water and 1ml of phosphate buffered saline for controls. All experiments were performed individually and all samples were quantified in triplicate.

PROTEIN QUANTIFICATION

To determine the quantity of BSA protein adsorption to the grafting material surface, all liquid was removed from the wells after a 24hr incubation period and the samples were quantified for protein concentration using a BCA protein assay reagent kit.

In order to analyze the kinetics of protein release, 1ml of PBS wash was added to all wells, stirred and removed from each well. This was followed by the addition of 1ml PBS to all wells. Samples were then placed back in 4 °C storage and 1ml PBS was removed at intervals of 1, 3, and 7 days post wash step after the initial 24hr incubation

period. The 1ml samples removed were then quantified using the BCA protein assay regent kit for days 1, 3, and 7 days. BCA results were analyzed using two-way ANOVA.

SCANNING ELECTRIC MICROSCOPY

Scanning electron microscopy was performed on samples of xenograft and synthetic graft particles prior to BSA exposure as well as at the interval of one and seven days post initial 24hr incubation followed by PBS wash. Particulate graft materials were dried and analyzed using environmental scanning electron microscopy for surface texture and porosity variances between materials as well as to determine if surface protein adsorption could be seen. Energy-dispersive X-ray spectroscopy was also used to analyze the elemental components of the xenograft and synthetic graft material.

CHAPTER 3

RESULTS

Scanning electron microscopy images, as seen below, show a vastly different surface archetype and particle morphology between xenograft and synthetic particulate graft materials. The xenograft material is described by the manufacturer as having a rough surface with an open porous structure, which did appear on SEM with a more porous surface texture and collagenous appearance. There was no visible difference on SEM between BSA coated versus non-coated particulate graft in either xenograft or synthetic graft samples (figures 1-3). The energy-dispersive X-ray spectroscopy showed a noteworthy difference between the elemental composition of the xenograft and synthetic graft material, which is listed in below in figures 4-6 and table 1.

The quantity of BSA protein in solution after the 24hr incubation period quantified for protein concentration using the BCA protein assay reagent kit showed a lower concentration of protein in solution in the xenograft samples. The kinetics of release of BSA showed a statistically significant difference in the amount of protein released over time between the xenograft and the synthetic graft materials. There was a lower concentration of protein shown in solution for the synthetic alloplast material than the xenograft after the initial 24hr incubation period and 24hrs after PBS wash with a continuous increase in released protein in solution over time across both graft materials (figures 7-8).

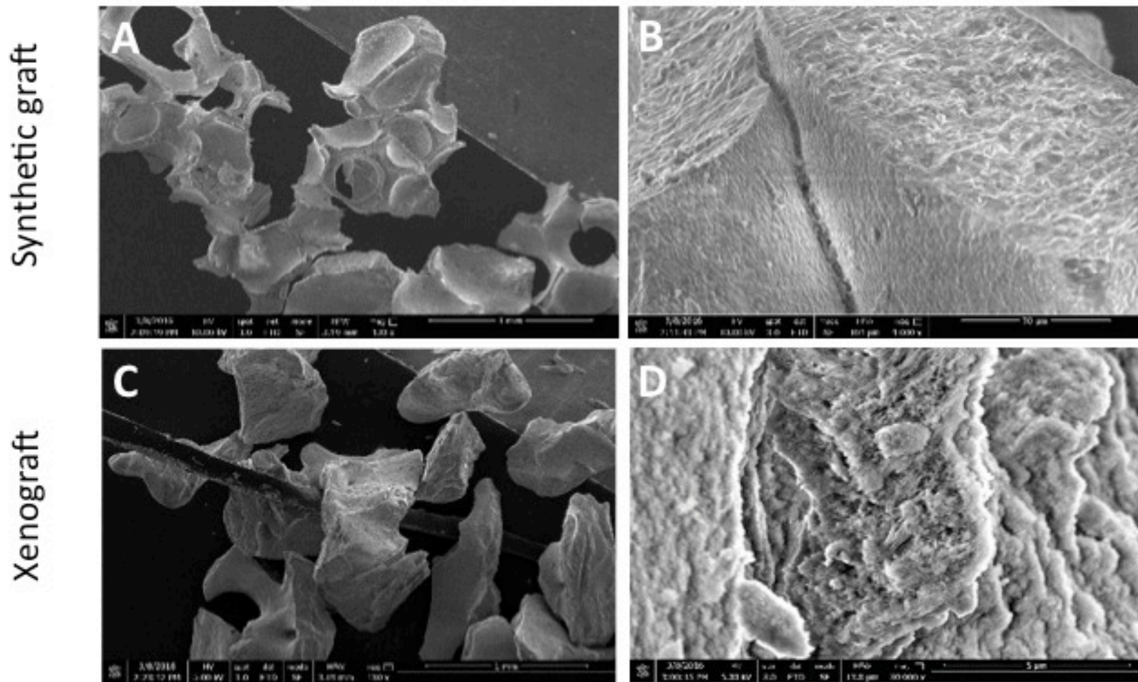

Figure 1. Scanning electron microscopy prior to treatment of graft materials with bovine serum albumin solution.

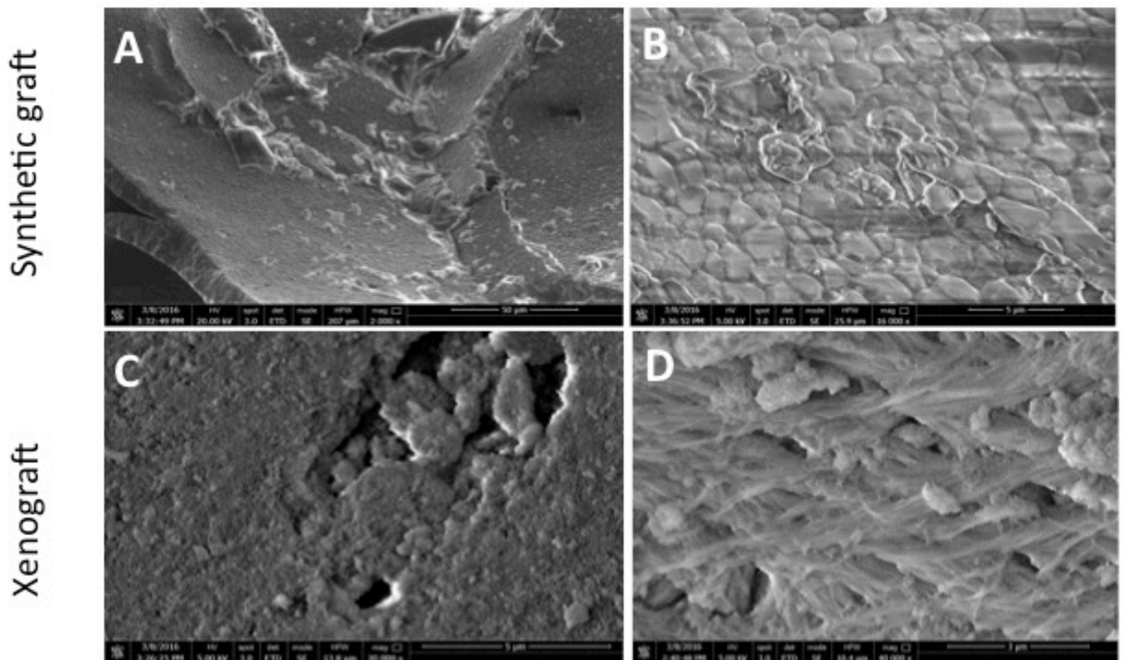

Figure 2. Scanning electron microscopy of graft material treated with the initial 1ml of prepared 1% bovine serum albumin solution incubated at 4°C for 24hr followed

by solution removal, 1ml PBS wash, and addition of 1ml PBS with solution removed after one day.

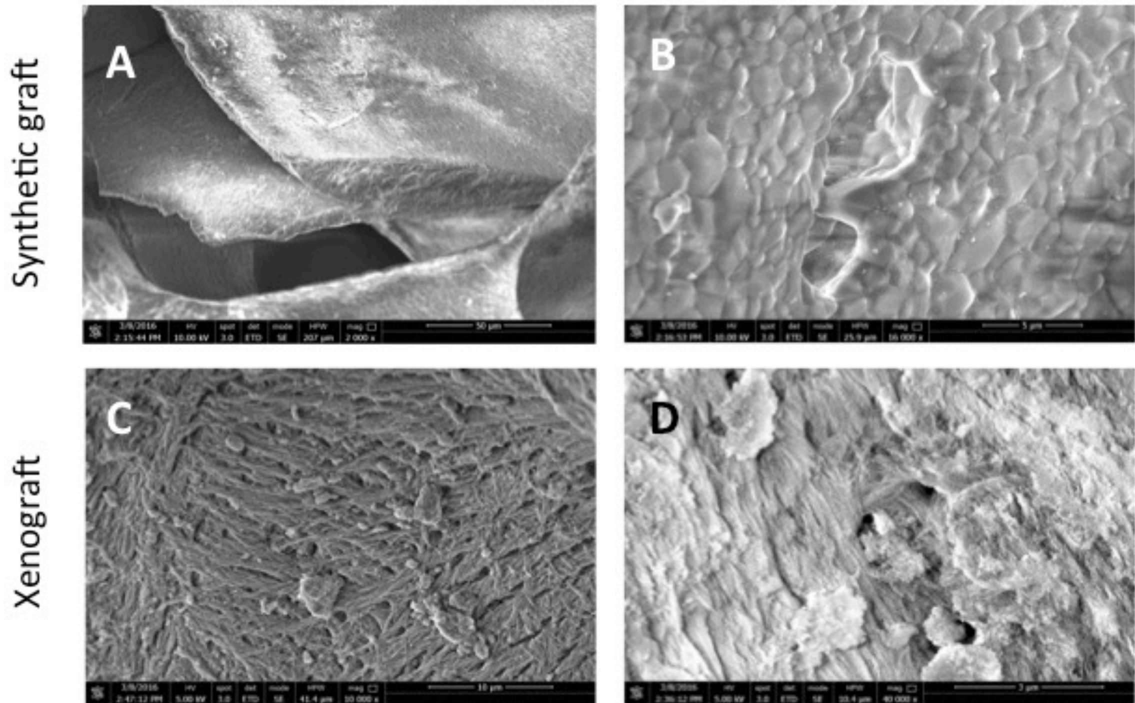

Figure 3. Scanning electron microscopy of graft material treated with the initial 1ml of prepared 1% bovine serum albumin solution incubated at 4 °C for 24hr followed by solution removal, 1ml PBS wash, and addition of 1ml PBS with solution removed after seven days.

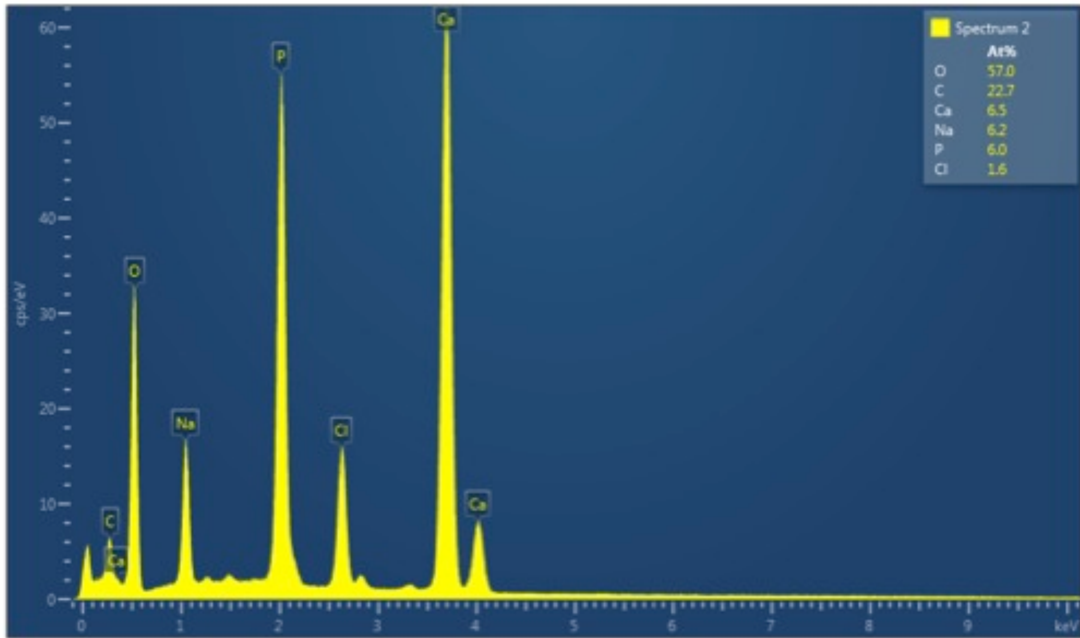

Figure 4. Energy-dispersive X-ray spectroscopy analysis of the elemental components of the xenograft bone graft material.

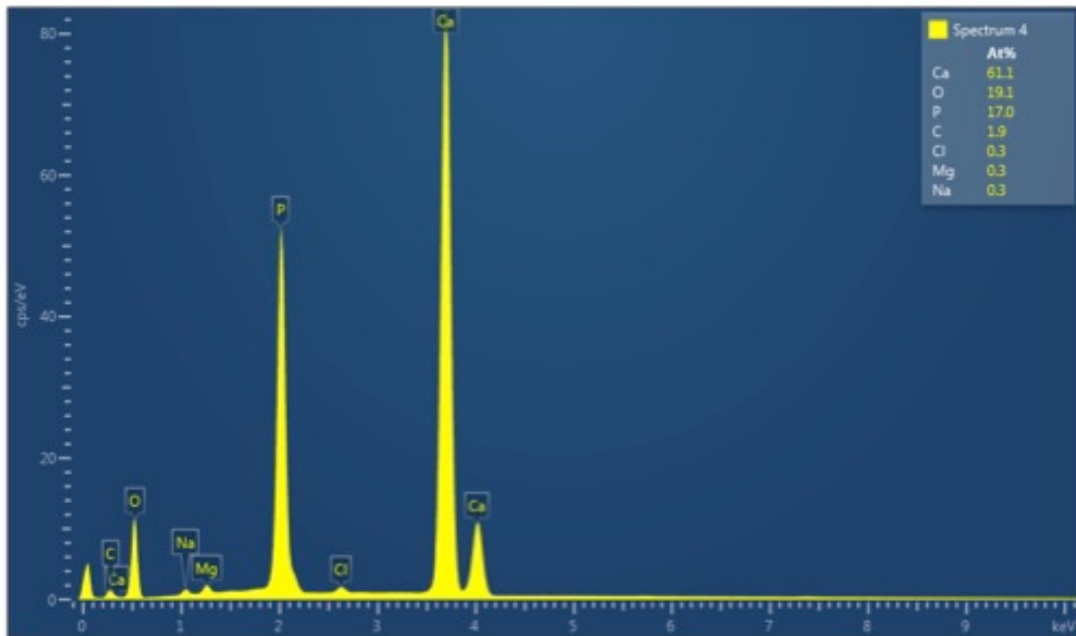

Figure 5. Energy-dispersive X-ray spectroscopy analysis of the elemental components of the Synthetic bone graft material.

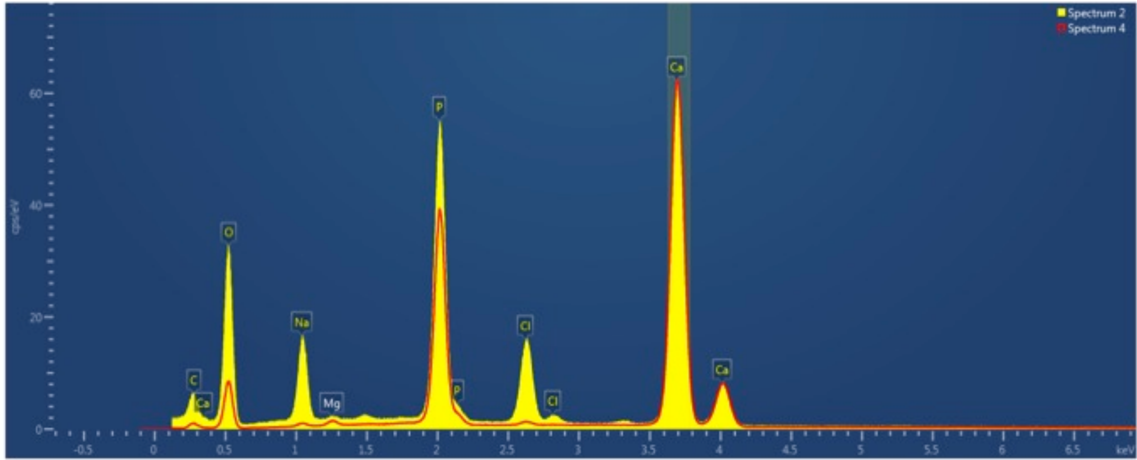

Figure 6. Energy-dispersive X-ray spectroscopy analysis of the elemental components of the xenograft and synthetic bone graft material compared.

Synthetic alloplast (Spectrum 4)			Xenograft (Spectrum 2)		
Elements	Atomic	%	Elements	Atomic	%
Ca		61.06%	O		56.97%
O		19.11	C		22.66
P		17.04	Ca		6.54
C		1.87	Na		6.22
Cl		0.34	P		6.04
Mg		0.33	Cl		1.56
Na		0.26	Total 100.00%		
Total 100.00%					

Table 1. Energy-dispersive X-ray spectroscopy elemental analysis break down.

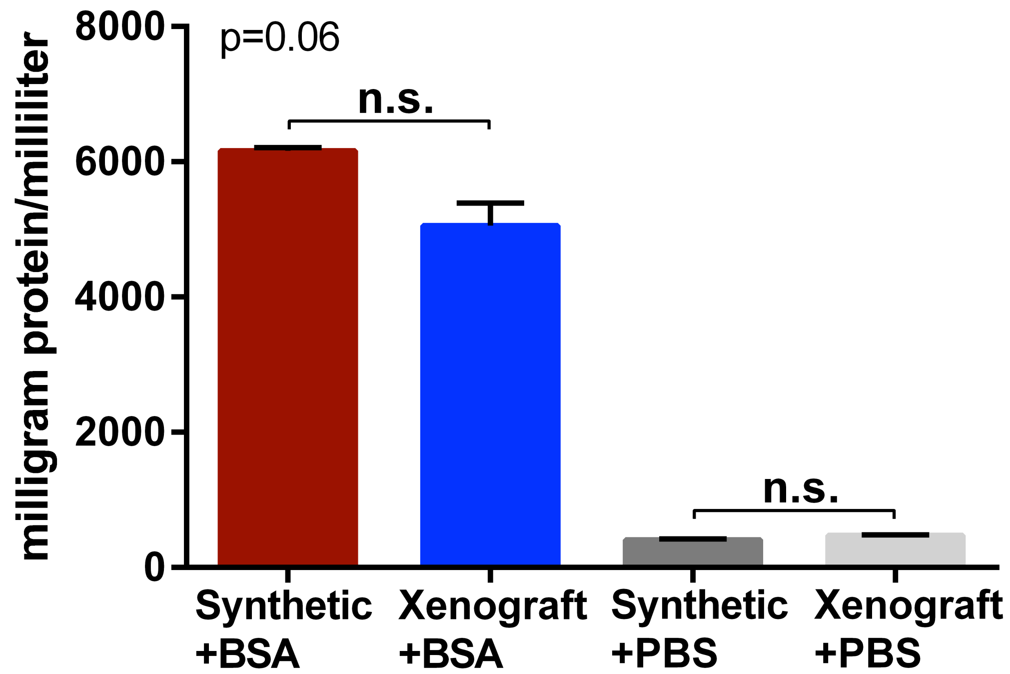

Figure 7. The quantity of BSA protein in solution after the 24hr incubation period quantified for protein concentration using the BCA protein assay reagent kit. Less protein was shown in solution after the 24hr incubation period in the xenograft samples.

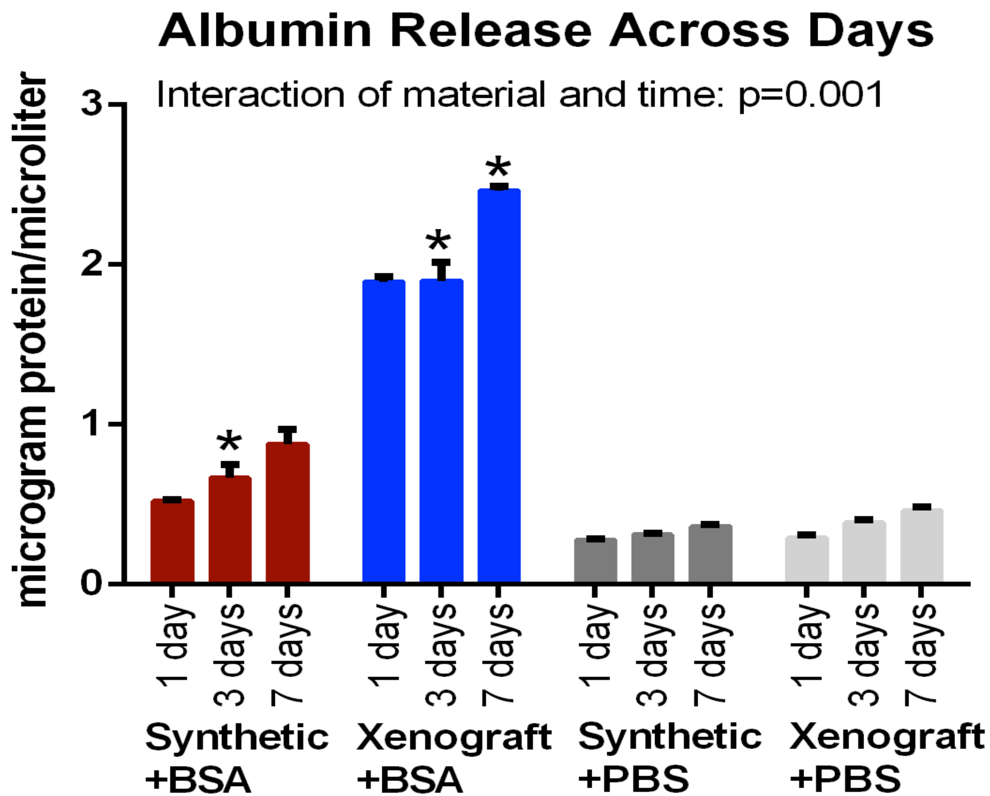

Figure 8. Two-Way ANOVA of the quantity of BSA protein in solution after the 24hr incubation period, PBS wash, and addition of 1ml PBS at intervals of 1,3,7 days. There is a statistically significant difference in the amount of protein released over time between the xenograft and the synthetic graft material. There is a continuous increase in released protein in solution over time across both graft materials.

Raw data Wavelength:595.0												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.148	0.149	0.174	0.245	0.313	0.421	0.534	0.64	0.825	0.948	0.271	0.281
B	0.155	0.146	0.165	0.229	0.291	0.393	0.505	0.615	0.768	0.9	0.243	0.246
C	0.25	0.236	0.243	0.259	0.277	0.366	0.211	0.455	0.363	2.515	2.496	2.454
D	0.142	0.151	0.146	0.152	0.159	0.167	0.173	0.184	0.184	0.191	0.21	0.196
E	0.147	0.147	0.158	0.167	0.177	0.186	0.337	0.217	0.217	0.225	0.272	0.238
F	0.794	0.766	0.808	0.745	0.884	0.744	1.006	0.83	1.026	2.313	1.902	1.931
G	0.138	0.158	0.159	0.18	0.181	0.206	0.204	0.235	0.218	0.226	0.223	0.232
H	0.148	0.152	0.153	0.157	0.166	0.178	0.187	0.196	0.2	0.199	0.204	0.203
A	Blank	Std I	Std H	Std G	Std F	Std E	Std D	Std C	Std B	Std A	EMPTY	EMPTY
B	Blank	Std I	Std H	Std G	Std F	Std E	Std D	Std C	Std B	Std A	EMPTY	EMPTY
C	Syn BSA 1	Syn BSA 1	Syn BSA 1	Syn BSA 3	Syn BSA 3	Syn BSA 3	Syn H2O 7	Syn BSA 7	Syn BSA 7	Syn BSA	Syn BSA	Syn BSA
D	Syn PBS 1	Syn PBS 1	Syn PBS 1	Syn PBS 3	Syn PBS 3	Syn PBS 3	Syn PBS 7	Syn PBS 7	Syn PBS 7	Syn PBS	Syn PBS	Syn PBS
E	Syn H2O 1	Syn H2O 1	Syn H2O 1	Syn H2O 3	Syn H2O 3	Syn H2O 3	Syn BSA 7	Syn H2O 7	Syn H2O 7	Syn H2O	Syn H2O	Syn H2O
F	XBSA 1	X BSA 1	X BSA 1	X BSA 3	X BSA 3	X BSA 3	X BSA 7	X BSA 7	X BSA 7	X BSA	X BSA	X BSA
G	XPBS 1	XPBS 1	XPBS 1	X PBS 3	X PBS 3	X PBS 3	X PBS 7	X PBS 7	X PBS 7	X PBS	X PBS	X PBS
H	XH2O1	XH2O 1	XH2O 1	XH2O 3	XH2O 3	XH2O 3	XH2O 7	XH2O 7	XH2O 7	X H2O	X H2O	X H2O

Table 2. Raw data of the quantity of BSA protein in solution from the BCA protein assay after the 24hr incubation period, PBS wash, and addition of 1ml PBS at intervals of 1,3,7 days.

CHAPTER 4

DISCUSSION

The quantity of BSA protein in solution after the 24hr incubation period quantified for protein concentration using the BCA protein assay reagent kit showed a lower concentration of protein in solution after the 24hr incubation period in the xenograft samples. This was however nearing statistical significance at a P value of 0.06. The need for an antibody linked immune assay was not necessary due to the controls of PBS and sterile water showing no protein contamination or release from bone graft materials, so it is known that all protein concentration is due to 1% BSA solution. This finding proves that the bovine xenograft used in this experiment is free of protein contaminants and does not release protein in solution as stated by the manufacturer to be deproteinized and delipidized as well as terminally sterilized via gamma irradiation. The fact that the xenograft samples showed a lower protein concentration in solution removed in comparison to the synthetic alloplast proves our working hypothesis that albumin binding is dependent on surface porosity of particulate bone graft materials, and therefore its binding to xenograft is stronger than its binding to synthetic bone graft. This is further demonstrated through the scanning electron micrograph images of the surfaces. The surface texture of the xenograft material is much more porous and irregular as compared to the synthetic alloplast, which has a smother outer surface without many crevices. Scanning electron microscopy was not successful in the visualization of albumin protein binding. Further examination using SEM or TEM with gold-labeled albumin would more clearly depict the protein binding interactions on each graft material surface.

The Energy-dispersive X-ray spectroscopy analysis of the elemental components of the synthetic bone graft material revealed a difference in composition of each graft material. The composition of the fully synthetic alloplast graft material is indicated by the manufacturer to be composed of 60% hydroxyapatite, 40% ß-tricalcium phosphate and showed mostly calcium in the spectrum (spectrum 4). The hydroxyapatite component is used to prevent excessive resorption and preserve volume and space maintenance where as the ß-tricalcium component has the potential to resorb much quicker and be replaced by vital natural bone (Straumann, Andover, MA). The xenograft (spectrum 2), however, is primarily composed of oxygen and carbon with a large amount of sodium and chlorine. The incidence of sodium and chlorine are likely due to the physiologic buffered saline solution that precipitated during sample drying that showed a greater affinity for precipitation on the xenograft material as compared to the synthetic.

The kinetics of release of albumin showed a statistically significant difference in the amount of protein released over time between the xenograft and the synthetic graft materials. There was a lower concentration of protein shown in solution for the synthetic alloplastic material than the xenograft after the initial 24hr incubation period and 24hrs after PBS wash but a continuous increase in released protein in solution over time across both graft materials. This result is tending toward the lower available protein bound on the synthetic material so lower levels of albumin were released over subsequent days, with the xenograft having initially bound more albumin protein there is a higher amount in solution released at each interval. The kinetics of release curve for the synthetic material showed a linear pattern while the xenograft indicates an algorithmic curve, which indicates more than one type of binding interaction of album to xenograft material.

This Finding is consistent with the hypothesis that albumin binding is dependent on surface porosity of particulate graft material, but the strength of binding was not determined in this experiment. More sensitive testing measures should be done in future experiments also bringing in other proteins of wound healing to determine their binding potential or possible synergy and significance in the future development of biologically active coated grafting materials. The binding strength and release kinetics will determine if the combination of protein and carrier are optimal for delivery at suitable points in the healing cascade to allow for a modification of response.

CHAPTER 5

CONCLUSIONS

A lower concentration of protein was shown in solution after the 24hr incubation period in the xenograft samples leading us to believe more protein was bound to the xenograft particles. The remaining solution from the xenograft samples throughout the kinetics of release analysis showed more albumin protein released over time due to the increased amount of protein available due to higher binding affinity as compared to the synthetic alloplastic samples.

This study however will act as a model system for future studies to analyze other proteins involved in the wound healing, bone remodeling, and angiogenesis process with analysis of their kinetics of release on alternate scaffold or carriers materials. This will allow for the determination of an adequate delivery mechanism that ideally permits a sustained release over and adequate time frame in order to modulate different phases of the healing process. The ultimate goal will produce a biologically active graft material that causes transformation of the wound healing into a tissue remodeling and growth phase to restore new bone and periodontal supporting tissues.

Future experiments will aim focus at bioactive transport mediums to modulate the healing response that synergistically release adequate concentrations of active proteins over an extended period of time. More sensitive testing measures should be taken when testing future proteins using transmission electron microscopy to analyze how closely bound or adhered proteins are and compare alternate transport mediums followed up with

ELISA for the kinetics of release profile while testing over a longer period of time to determine a longer protein delivery mechanism.

CHAPTER 6

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