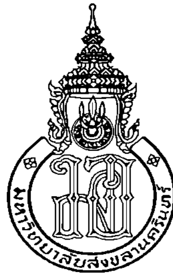

Degradation and Biocompability of Self Fabricated Chitosan Scaffolds in Comparison to Polycarpolactone/Tricalcium Phosphate (PCL/TCP) Scaffolds in Vitro

Chandra Dutt Rai

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Oral and Maxillofacial Surgery

Prince of Songkla University

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Thesis Title	Degradation and Biocompability of Self Fabricated Chitosan Scaffolds in Comparison to Polycarpolactone/Tricalcium Phosphate (PCL/TCP) Scaffolds in Vitro.
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Abstract

Introduction: Chitosan is one of the most extensively studied materials in recent times as an tissue engineering scaffold material. It has a distinctive advantages over the brittle ceramics. It offers a distinct set of advantageous physio-chemical and biological properties that qualify them for a variety of tissue regeneration. The natural polymers like chitosan has property to influence cell morphology,modulation and differentiation.

Objectives: Self fabrication of chitosan scaffolds and determining the degradation rate, biocompability and mineralized nodule formation in comparison to that of the pcl/tcp scaffolds.

Materials and methods : The chitosan scaffolds were fabricated with the freeze dried method with 2% chitosan of 85% of degree of deacetylation. The pcl/tcp scaffolds were procured from Osteopore International; Singapore and the swelling, degradation, cytotoxicity and mineralized nodule formation tests were done comparing the two scaffolds. The sample size for each experiments were three. Swelling studies will be performed by submerging chitosan and PCL/TCP scaffolds in phosphate buffer saline (PBS, pH 7.4) at 37°C for 5 minutes, 10 days and 20 days. The degradation test were done by adding lysozyme on 7th 15th and 30th day. The cytotoxicity test were done with PrestoBlue and measuring the fluorescence photometrically. The mineralized nodule formation were done with Alizarin Red S stain. The color change noted and the absorbance recorded after dissolving the nodules.

Results : The chitosan scaffolds in circular shape measuring 10mm in diameter and 2mm in height were fabricated with the percentage porosity varying from 85% to 94%. Resultant chitosan scaffolds were white and spongy in dry forms. They were smooth and no leaching of particles were found. They could be handled effectively without

causing any deformity and breakage. The swelling ratio and the degradation ratio both were superior for the chitosan scaffolds. Both the scaffolds showed good cell viability and mineralized nodule formation.

Conclusion: The chitosan scaffolds showed comparably good physical characteristics and supported the cell growth and osteogenesis with the osteoprogenetor cells.

Key Words: Scaffolds, chitosan, pcl/tcp, tissue engineering, bone substitute materials, cytoxicity, mineralized nodule.

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Chapter 1

Introduction

Although bone has an inherent property of regeneration; larger bony defects resulting from trauma, pathological and physiological resorption and congenital defects are still the major health problems. The need for bone regeneration in cranial, oral and maxillofacial and orthodontic surgery is still a central clinical issues in regenerative and rehabilitation medicine [1]. The autologous bone graft is still “the gold standard” for bone augmentation and repair technique. It has all the properties of osteogenesis, osteoinduction and osteoconduction; but it also has the drawbacks like, donor site morbidity, surgical complication, cost and the amount of available graft [2-4]. Auto graft is histocompatible and non-immunogenic. The other grafting techniques involve the use of allograft, xenografts and alloplastic materials. The allograft includes demineralized freeze dried bones (DFDB), freeze dried bones (FDB). They carry the risk of immunoreactions and transmission of diseases. They also have less osteoconductive properties because they have no cellular components [5]. The xenografts technique uses demineralized bovine bone (DBB).

The alloplastic bone substitute materials include hydroxyappatite, calcium phosphate, collagens, polymers and bioactive glasses.

Bone Tissue Engineering

Tissue engineering is a multidisciplinary science involving biology, engineering and material sciences. It provides suitable biochemical and physiochemical factors to improve or replace biological function. Langer and Vacanti defined it as, “an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitute that restore, maintain, or improve tissue function or a whole organ”. Tissue engineering is centered on the triads of ECM (scaffolds), cells and biologically active molecules (growth factors).

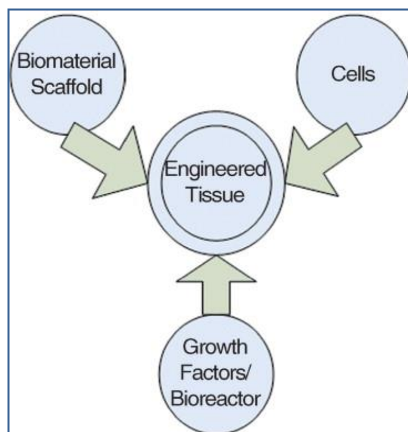

Fig 1. Tissue Engineering Triad

The scientific advancement in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created opportunity to fabricate tissues in the laboratory from ECM(scaffolds), cells and biologically active molecules.

Literature Review

Scaffolds

Scaffolds are artificial structures which acts as extracellular matrix and supports three dimensional tissue formations. Cells can be seeded in the porous structure and it supports the new tissue formation.

Properties of scaffold materials

1. Geometry - Define and maintain the space for tissue regeneration.
2. Mechanical - Maintain temporary load bearing mechanical function.
3. Biologic- Enhance the regenerative capability of chosen biofactor and enhance ingrowths of tissue.

Scaffolds must also possess the characteristic of biocompatibility, biodegrability and controlled degradation rate [6].

Scaffolds Biomaterial "Biomaterials are the materials intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body" (European Society of Biomaterials).

The common biomaterials used as scaffolds are metals, ceramics, synthetic polymers, natural polymers and or the hybrid materials combining two or more biomaterials [7]. Typical metals include stainless steel, cobalt alloys and titanium alloys. Ceramics include hydroxyapatite and calcium phosphate. They have high mechanical stiffness but have low elasticity and hard brittle surface. It has better biocompatibility and stability than polymers and metals. Synthetic polymers include polyesters, PLLA, PGA, PLGA, and PCL. They have reduced bioactivity and their degradation by hydrolysis is not predictable. Natural polymers include collagen, alginate based materials, and chitosan. They are biologically active, have good cell adhesion and growth; but have poor mechanical properties. The hybrid material is a combination of two or more materials; and it enhances and compliments the functionalities. Recently, increasing attention has been paid to bioresorbable composites made of polymers and ceramics for the reconstruction of organs, tissue interfaces and the bones.

PCL/TCP

Recently, increased attention has been paid to the bio- resorbable composites made of polymers and ceramics for reconstruction of multiple tissue organs, tissue interfaces and structural tissues, including bone. The composite scaffolds comprising of PCL/β-TCP provides better mechanical properties and biological responses than when PCL and TCP are used as scaffolds alone [8-9]. Poly- ε- caprolactone (PCL) is semi crystalline aliphatic polyester. It has good biocompatibility and biodegrability; but has poor mechanical strength and the surface chemistry does not promote cell adhesion.

Fig 2. PCL/TCP Scaffolds

Thus, incorporation of calcium phosphate would overcome the shortcoming of hydrophoobicity of pcl and brittleness of ceramic [10]. Compared to pcl scaffolds, composite scaffold containing pcl and tcp, designed for load bearing application, exhibit improved hydrophilicity, compressive modulus and strength. Human alveolar osteoblast shows higher seeding efficiency, better proliferation and earlier expression of bone matrix related protein than cells grown on pcl alone[11]. The pcl/tcp composites show faster degradation rate than pure PCL and strength of 80/20

Pcl/tcp composite is still suitable for human cancellous bone healing support after six weeks degradation; and scaffold fabricated by 3DP porogenic method provide essential capability for bone tissue engineering [12]. This composite proved biocompatible and when loaded with osteoblasts, sustained osteogenic expression [13].However, the brittleness and low biodegrability of this composite restricts it's clinical application.

The pcl/tcp scaffolds have proven records of good clinical applications. The scaffolds which can be fabricated using a rapid prototyping process have been used for bone regeneration. It has a potential to degrade within desired time period of 5-6 months and favorable mechanical properties which can be used for dentoalveolar reconstruction [14]. The hydrophobic surface of the scaffold can be made hydrophilic with improved cell attachment and viability without any loss of mechanical properties with collagen coating [15]. It is an appropriate scaffold for loading MSCs in bone regeneration process [16]. Pcl/tcp scaffold also showed better maintenance of alveolar contour as compared to autogenous particulate bone, though there was minimal bone regeneration within the defect at 6 months in study done in monkeys [17]. In a study done by A. Yeo., et. al. in a minipig model, using pcl/tcp scaffold for guided bone regeneration (GBR), in lateral ridge augmentation, they found good bone formation, though it was inferior to that of the autogenous graft. They concluded that, it has the potential application for lateral ridge augmentation. Similarly, in another study done by Thongchai Nuntanaranont et. al., in rabbits as a sinus lift grafting material, they found that it has a capability for new bone formation. They concluded that pcl/tcp scaffold could be used as an alternate sinus lift grafting material, though the bone formation was less than that of the autogenous bone grafting. In a single case study of a 71 year old female with a failed implants in anterior mandible by Karl-Heinz et. al., in 2008, they

successfully treated the defect with pcl scaffold in six months. It was followed by the successful reinsertion of the new implants.

Chitosan

A number of natural and synthetic polymers have been studied for overcoming the drawbacks of the ceramics. Among them chitosan is the one. It has been studied extensively to be used as bone substitute material ever since it was found to promote growth and deposit mineral rich matrix by osteoblast in culture. It is one of the most promising biomaterials in tissue engineering because it offers a distinct set of advantageous physio-chemical and biological properties that qualify them for a variety of tissue regeneration [18]. Chitosan is a linear polysaccharide obtained from deacetylation of chitin. Chitin is the primary structural polymer of exoskeleton of crustaceans, cuticles of insects and cell walls of fungi [19, 20]. Chitin, poly (β-(1-4)-N-acetyl –D glucosamine) is a natural polysaccharide. If the number of N-acetyl glucosamine is higher than 50%, the polymer is considered as chitin and if the number of N-glucosamine is higher, then it is considered as chitosan. It is a semi crystalline polymer and it's crystallinity depends on degree of deacetylation [21]. Depending on the source and the methods of preparation the molecular weight of chitosan may range from 300-1000kDa., and degree of deacetylation 35% to 95% [51].

The natural polymer like chitosan has property to influence cell morphology, modulation and differentiation [22].

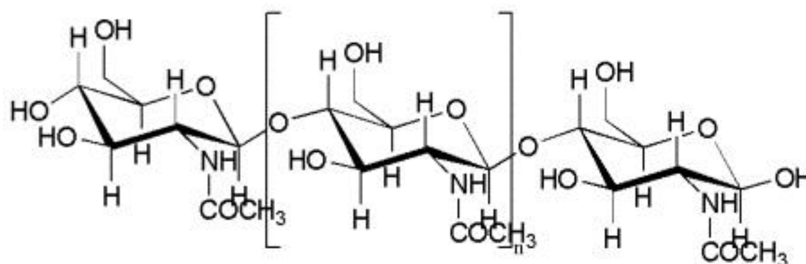

Fig 3. Structure of chitin

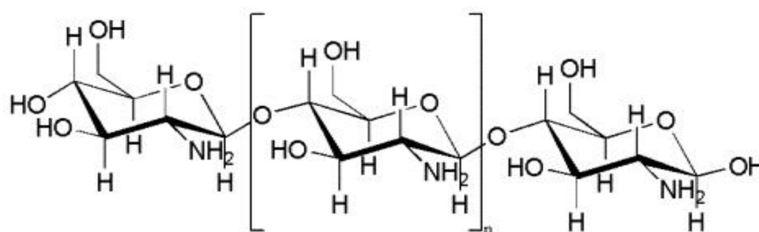

Fig 4. Structure of fully deacetylated chitosan

Chitosan presents good biodegrability, biocompatibility, antibacterial activity and bioadhesive character [23-27]. The antibacterial property of the chitosan is attributed to the cationic amino group in its structure. The cat ion interacts with the anions of the bacterial cell wall and suppresses biosynthesis [52]. Chitosan can be easily modified into various forms like films, fibers, beads, sponges, and more complex shapes for orthopedic treatment [28]. The cationic nature of chitosan is responsible for attracting various negative charged proteoglycans, glycosaminoglycans and other anionic molecules. This property of the chitosan is of interest because the cytokines/growth factors are linked to glycosaminoglycans [20]. Porous materials have a highly significant role in the bone implantation process. Porous chitosan structures can be formed by freezing and lyophilizing chitosan acetic acid solutions in suitable molds [29]. Chitosan have been combined with a variety of materials such as hydroxyapatite, alginate, hyaluronic acid, calcium phosphate, poly (methyl methacrylate), poly-l-lactic acid and growth factors for potential application in orthopedics. They can be fabricated as interconnected-porous structures by using suitable molds [30]. This interconnected porous structure can support numerous cells seeding and thus increasing the cell numbers. Chitosan has been shown to degrade in vivo, which is mainly by enzymatic hydrolysis. The degradability of a scaffold plays a crucial role on the long-term performance of tissue-engineered cell/material construct because it affect many cellular process, including cell growth, tissue regeneration, and host response.

If a scaffold is used for tissue engineering of skeletal system, degradation of the scaffold biomaterial should be relatively slow, as it has to maintain the mechanical strength until tissue regeneration is almost completed. Lysozyme is the primary enzyme responsible for in vivo degradation of chitosan, which appears to target acetylated residues [31]. The final degradation products are biocompatible chitosan oligosaccharides of variable length. The degradation rate is inversely related to the

degree of crystallinity which is controlled mainly by the degree of deacetylation (DD). Highly deacetylated forms (DD>85%) exhibit relatively a low degradation rate and may last several months in vivo, whereas the forms with lower DD degrade more rapidly [32-34]. The degradation rate also inherently affects both the mechanical and solubility properties. Chitosan has been used for bone tissue engineering scaffold extensively and has shown some promising results. But it is still used only in wound dressing and as haemostatic agent in medicine. Chitosan has been used in varieties of applications and has the potentials for several biomedical applications. In the food and beverages, it is being used as dietary fibers, lipid binders, preservatives, thickeners and stabilizers, protective, fungistatic and antibacterial coatings on fruits. It is widely used in cosmetics and toiletries. It has also found uses in the agriculture and water and waste treatment plants. In the biomedical field, the potential use includes surgical sutures, surgical wound dressings, drug delivery system, artificial skin, and gene delivery system.

Rationale of study

The craniofacial defects comprise cranial defects, cleft palate and related defects and mandibular defects. The traditional method of reconstruction of alveolar cleft involves the use of autologous bone grafts which is considered as gold standard.

The autologous bone grafts are derived from iliac crest, fibula, scapula and the ribs.

Allograft though is an ideal graft material; and is considered as the “gold standard” has many disadvantages. The prominent among them being the donor site morbidity, surgical complications, increased length of stay in the hospital with added cost; and the amount of graft material available is limited. Therefore, the search for the alternative with similar properties continues. Pcl/tcp and other ceramics as a graft material have proven clinical efficacy, but it is still expensive and beyond the common people’s affordability. Therefore, search for cheaper alternatives in polymers has gained momentum. Chitosan, being natural polymer which is found abundantly and cheaply has promising aspects as the other alternative. Therefore, self fabrication and comparison of its degradation and biocompatibility with the pcl/tcp scaffolds, which has already established itself in the clinical field, will give us the comparable properties of chitosan scaffolds.

Objectives of the study

1. Self fabrication of the chitosan scaffolds.
2. Comparing degradation and biocompatibility of chitosan scaffolds with pcl/tcp scaffolds.
   a) Swelling test
   b) Degradation study
   c) Cytotoxicity test
   d) Mineralized nodule formation.

Hypothesis

The self fabricated chitosan scaffolds have equally good physical and biological properties in comparison to pcl/tcp Scaffolds.

Chapter 2

Materials and methods

PCL-TCP Scaffold

Standard PCL-TCP (80:20) composite scaffolds with lay down pattern of 0/60/120° and porosity of 70%.were purchased from Osteopore Int. Singapore._They were pretreated with NaOH for 3 hours, followed by rinsing with PBS three times. After that they were sterilized with 70% ethanol for 24 hours and again rinsed with PBS three times. The scaffolds were then dried in humidified atmosphere at 37° C. at 5% CO_2 for 1 hour before the experiment.

Chitosan Powder

Chitosan powder with the degree of deacetylation > 85 and molecular weight ranging from 190-370 kDa was purchased from the local Thai company.
PBS, Chicken white lysozyme and DMEM medium were procured from Sigma Aldrich Chemical Company.

Alizarin Red S Staining kit was procured from Sigma Aldrich and PrestoBlue kits from Thermo Fisher Scientific Company.

Preparation of chitosan scaffolds

In chemical hydrolysis method, four main steps are involved in order to produce chitosan from marine crustacean shell. They are (i) demineralization; (ii) deproteinization; (iii) discoloration and (iv) deacetylation.

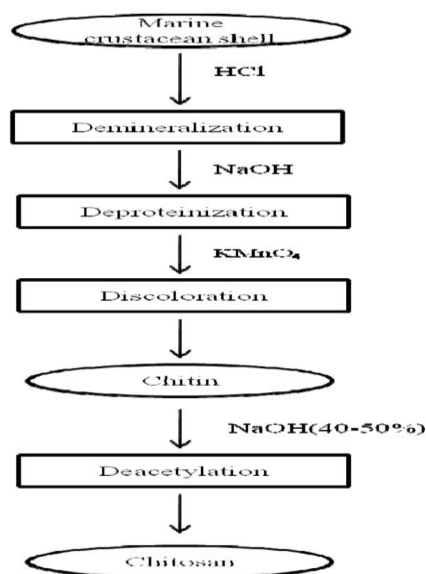

Fig 5. Preparation of chitin and chitosan from marine crustaceans

Food grade 2% chitosan powder with degree of Deacetylation (DD) 85 % were mixed with 0.2 M acetic acid in a concentration of 2% w/v (2 g of powder per 100 ml of acetic acid) and filtrated through 0.45 µm Millipore filter. This solution was then dropped in a glass of 0.05 M NaOH putting on top of a stirrer with the speed of 20,000 rpm to create fibril-like chitosan. Then the product was washed with distilled water to remove excess NaOH and poured in polypropylene cylindrical molds (inner diameter 14 mm, height 7.5 cm.) and centrifuged at the speed of 3,000 rpm for 5 min. The supernatant was rinsed out and the mold was cooled down step by step, starting from 4˚C for 24 hrs. and- 20 ˚C for another 24 hrs. Then they were rehydrated using 96 v/v ethanol for 1 hr, neutralized by 1 M NaOH, and rehydrated again with 70 v/v ethanol for 12 hrs. They were dried in an incubator with the temperature of 25 ˚C for 48 hrs. Each piece of chitosan scaffold was cut at the thickness of 5 mm before submerging in liquid nitrogen for a few seconds to stabilize the pore size. Their surface morphologies were analyzed using an image analysis program from the micro- CT images.

Swelling test

Swelling studies were performed by submerging chitosan and pcl/tcp scaffolds in phosphate buffer saline (PBS, pH 7.4) at 37 °C for 5 minutes, 10 days and 20 days respectively. Three specimens were used at each time point. The scaffolds with the known dry weight were submerged in (PBS, pH 7.4) at 37 °C for the said study period. At

the end of the study period the scaffolds were removed from the medium, rinsed with distilled water and dried with gentle drying with absorbent paper. The swelling were calculated using the following formula.

Sweling ratio = $(W-W_0)/ W_0$

W = wet weight

W_0 = dry weight

Degradation study

The samples were submerged in 2 ml of PBS (pH 7.4) at 37 °C containing lysozyme. The scaffolds with known dry weight were incubated in the lysozyme solution for the period of 7, 15 and 30days days. Three specimens of both the chitosan and pcl/tcp were used at each time period. The lysozyme solution was refreshed at regular intervals. At the end of the study period samples were removed from the medium, rinsed with distilled water and dried to the constant weight. The degradation ratios were examined by weight loss from the formula:

Percentage weight loss = $(W_0 - W_t) / W_0 \times 100$

W_0 denotes the original weight, and W_t is the weight at end of experiment.

Cell viability test

The cell viability assay was carried out with the commercially available Presto Blue Reagent. It is ready to use water soluble preparation. The assay was carried out according to the manufacture's protocol. Each scaffold (n=3) for experimental chitosan and control pcl/tcp scaffolds) were submerged in 1 ml of DMEM media in 24-well-plate for 24 hours. Then the supernatant was extracted and osteoblast-like cell (MG-63) were seeded (12,000 cells/well, 200µg/well) in 96-well plate and incubated for overnight at 37 °C and 5% CO_2. The cell viability tests were done at days 1, 3, 5 and 7.

After the incubation, the cells were stained by adding 20µl of Presto blue reagent to the sample wells. The plates were then incubated for 30 minutes at 37°C and 5% CO_2 in a humidified atmosphere. The cell viability was then determined by measuring the fluorescent signal and absorbance spectroscopy. The absorbance was recorded at 570 nm in universal micro plate reader.

Mineralized nodule formation by Alizarin Red S staining

Alizarin Red S staining was used to measure the mineralized nodule formation. The principle involves the formation of alizarin red s-calcium complex in chelation process when the stain reacts with the cells of osteogenic linage. This can be quantitatively measured by calorimetry, which is the process of calcium deposition by cells of osteogenic linage. The Alizarin Red S stain was obtained from Sigma Aldrich and procedure were carried out according to the manufacturer's standard protocol.

Three numbers of chitosan scaffolds and pcl-tcp scaffolds were used each time. Osteoblast –like cell MG 63 were seeded at the density of 2×10^6 cells/ml/well within the scaffolds in 24-well-plate and 1 ml of DMEM added and cultured at 37°C in an incubator setting in a humidified atmosphere 5% CO_2 until reaching confluence . Then each well was added with 1 ml of DMEM supplemented with 10% FBS, 10mM β glycerophosphate, 100 μg/ml L-ascorbic acid 2-phosphate and 10^{-8} M dexamethasone. Detecting of mineralization during cell differentiation was performed at day 7th, 14th, 21st and 28th.

The medium was aspirated from the well and cells washed with PBS solution and aspirated gently. The cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. The fixative was removed and cells washed 3 times with distilled water. The water was removed completely and stained with 2% alizarin red S solution in water for 20-30 minutes at room temperature. Excess stain was removed by several washes with distilled water. 1 ml of distilled water was added to each well to keep the cells from drying. Then it was observed under microscope. To quantify the stain, the culture was rinsed with ammonium hydroxide and absorbance was measured at 550nm on a multiplate reader.

Data analysis

The data (mean±, standard error obtained) were statistically analyzed by SPSS Version 15.0. Analysis of variance and means were compared with one way ANNOVA. Tukey's HSD test was used for multiple comparisons. The significance was shown by $P<0.05$ at 95% confidence level

Chapter 3

Results

Chitosan Scaffolds

The chitosan scaffolds in circular shape measuring 10mm in diameter and 2mm in height were fabricated in the lab using phase seperation and freeze drying technique. The percentage porosity of the few select scaffolds as calculated from the micro-CT varied from 85% to 94%. The physical and mechanical characterization, pore size determination was not done because these procedures were already done in the same laboratory with the similar procedure earlier [35]. Resultant chitosan scaffolds were white and spongy in dry forms. They were smooth and no leaching of particles was found. They could be handled effectively without causing any deformity and breakage.

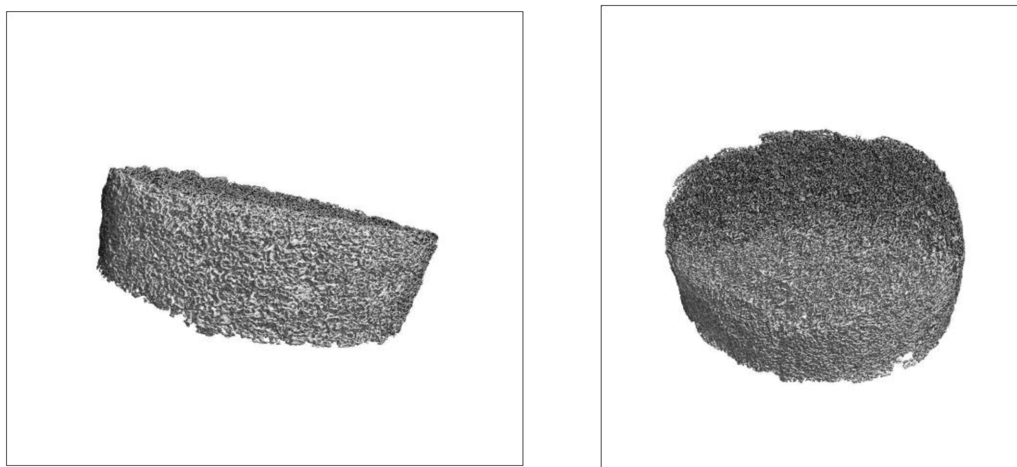

Fig 6. Micro-CT image of chitosan scaffolds

Fig 7. Picture of chitosan scaffolds.

Swelling

The swelling ability of the scaffolds denotes the water absorption capacity of the scaffolds. This also means that the scaffolds can absorb body fluid. This will not only help in maintaining the shape and structure of the scaffolds, it will also help in cell attachment and growth. On the other hand the very high absorption for a long time may cause the scaffolds to expand and deform [36]. The chitosan scaffolds showed a good overall swelling capacity. The swelling ratio at five minutes was recorded at 0.65, 0.96 at 0.96 and it was 1.00 at the twentieth day. The swelling capacities of the pcl/tcp scaffolds were found to be comparatively lesser than that of the chitosan scaffolds. The swelling ratio at five minutes, 10 days and 20 days were 0.11, 0.04 and 0.31 respectively(Table.2). One way ANNOVA and Tukey's HSD test were done to find the correlation among the same group at different test days and also among the two different groups. The level of significance was kept at the p value of ($p>0.05$) at 95% confidence level. No significant difference was observed throughout the period in control group. However, in the tenth day the experimental group showed significant relation in comparison to the control group.

Table 1. Swelling ratio with the mean and SD. (Column with similar superscripts letter are not significant at $p<0.05$ using One way annova and Tukey's HSD test).

Samples	Swelling		Swelling Ratio
	Initial	After	
Experiment: 5min	0.0442 ± 0.0025^{a}	0.0730 ± 0.00340^{ab}	0.65
10 day	0.0471 ± 0.0047^{a}	0.0928 ± 0.00340^{a}	0.96
20 day	0.0418 ± 0.00917^{a}	0.0841 ± 0.02165^{ab}	1.00
Control 5min	0.0406 ± 0.00168^{a}	0.0453 ± 0.00365^{b}	0.11
10 day	0.445 ± 0.00442^{a}	0.0462 ± 0.00297^{b}	0.04
20 day	0.416 ± 0.00299^{a}	0.0545 ± 0.02033^{b}	0.31

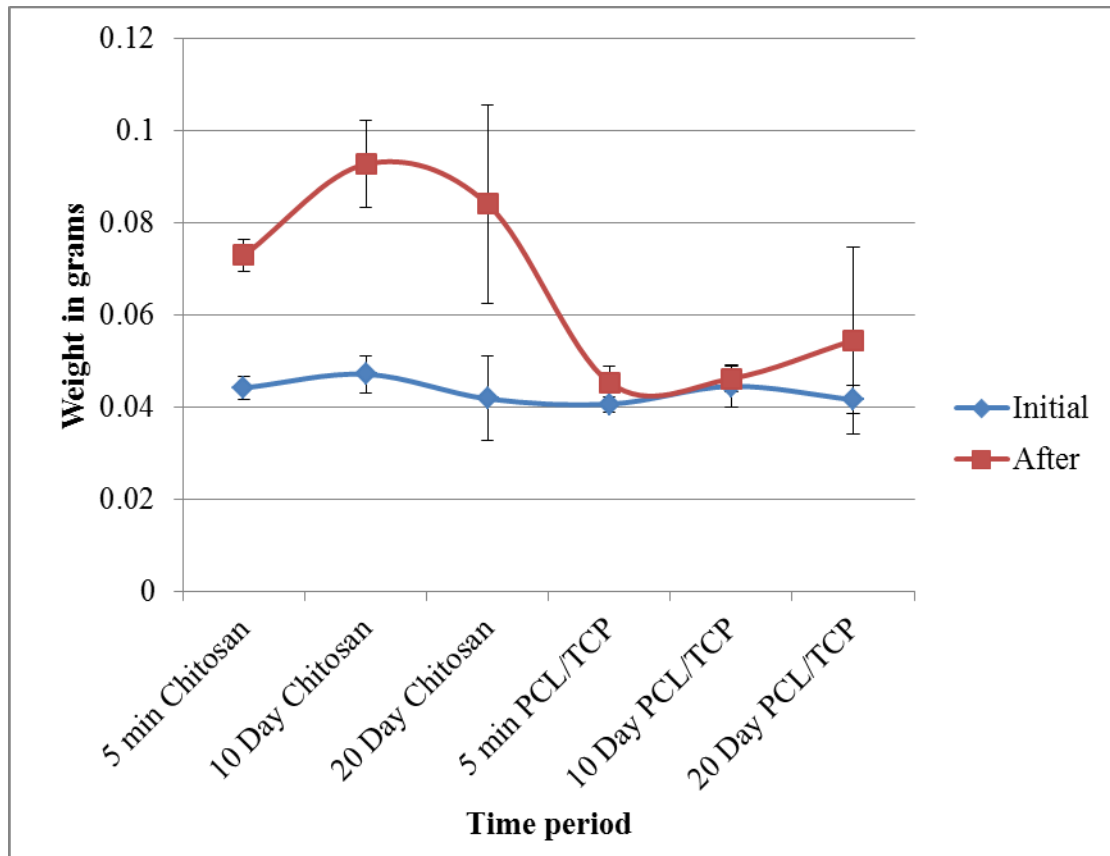

Fig 8. Swelling Ratio of Chitosan Scaffolds Swelling ratios of PCL/TCP Scaffolds.

Degradation

Degradation is one of the prerequisite characteristic a scaffold must have. The ultimate goal of this chitosan scaffold is to undergo a controlled degradation under the influence of body enzyme, viz. lysozyme, so that the cellular growth can gradually replace the scaffold. Therefore, lysozyme was used in this study to investigate the degradation of the scaffolds. Chitosan scaffolds showed insignificant degradation on the first week, but the degradation ratio increased thereafter from second week, which was 13% and on the forth week the degradation percentage reached 28 %. On the other hand, the pcl/tcp scaffolds degradation was insignificant. With no degradation in the first week, it showed only 0.45% degradation at the end of thirty days (Table 2). One way ANNOVA and Tukey's HSD test showed no significance among the control group and the experimental group, except in Day 7 and Day 30 of the experimental group.

Table 2. Degradation percentage with mean and SD. (Column with similar superscripts letter are not significant at $p<0.05$ using One way annova and Tukey's HSD test).

Samples	**Degradation**		**Degradation %**
	Initial	**After**	
Experiment Day 7	0.0447 ± 0.0116^{a}	0.0444 ± 0.0115^{a}	0.67
Day 15	0.0471 ± 0.0037	0.0410 ± 0.0035^{ab}	13.00
Day 30	0.0404 ± 0.0026	0.0288 ± 0.0032^{b}	28.00
Control Day 7	0.0437 ± 0.0028	0.0436 ± 0.0024^{ab}	0.00
Day 15	0.0414 ± 0.0029	0.0409 ± 0.0031^{ab}	0.10
Day 30	0.0415 ± 0.0036	0.041 ± 0.0035^{ab}	0.45

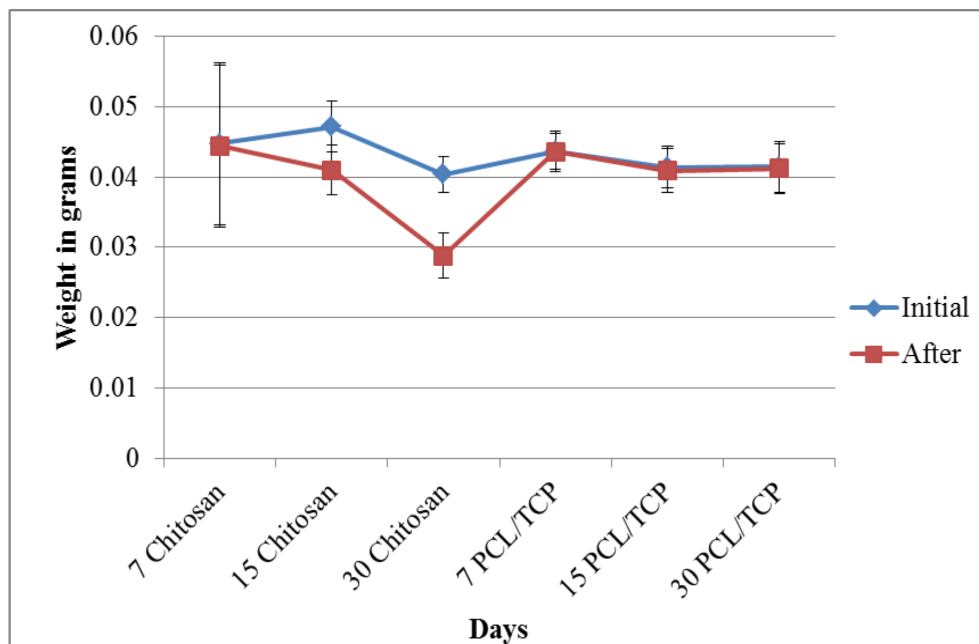

Fig 9. Degradation percentages of chitosan and pcl/tcp scaffolds

Cell Viability Test

Presto Blue assay was used to study the viability of the cells. The change in color from blue to fluorescent red was observed in all the samples. The fluorescence was measured spectrometrically at the wavelength of 570nm. The relative fluorescence unit in day 1 was higher in the chitosan group, though it was higher for pcl/tcp group in the day 3 and day 5. The relative fluorescence unit for chitosan increased till day 3 and then decreased thereafter. The pcl/tcp group showed the highest relative fluorescence unit on day 5 and decrease on day 7. But the control group showed the gradual increase in relative fluorescence unit. In the control group the result between day 1 and day 7 showed significant differences. In the chitosan group the result of between day 3 and day 7 showed significance and in the pcl/tcp group the result between day 1 and day 2 and result between day 1 and day5 showed significance; also the result between day 5 and day 7 showed significance. (Table 3. and Fig 10).

Table 3. Mean fluorescence unit with SD.(Different superscript represent significance at p<0,05.)

	1 day	3 day	5 day	7 day
Control	4996.85±449.96 [a]	6978.576±496.67 [abcd]	7410.208±161.23 [abcd]	9691.241±448.27[de]
Chitosan	6798.99±1165.31 [abc]	9102.079±238.55 [cde]	5869.565±222.27 [ab]	5018.904±561.01[a]
PCL/TCP	5907.37±1042.14 [ab]	11253.938±1793.71[ef]	12441.714±1854.58 [f]	7813.485±217.09[bcd]

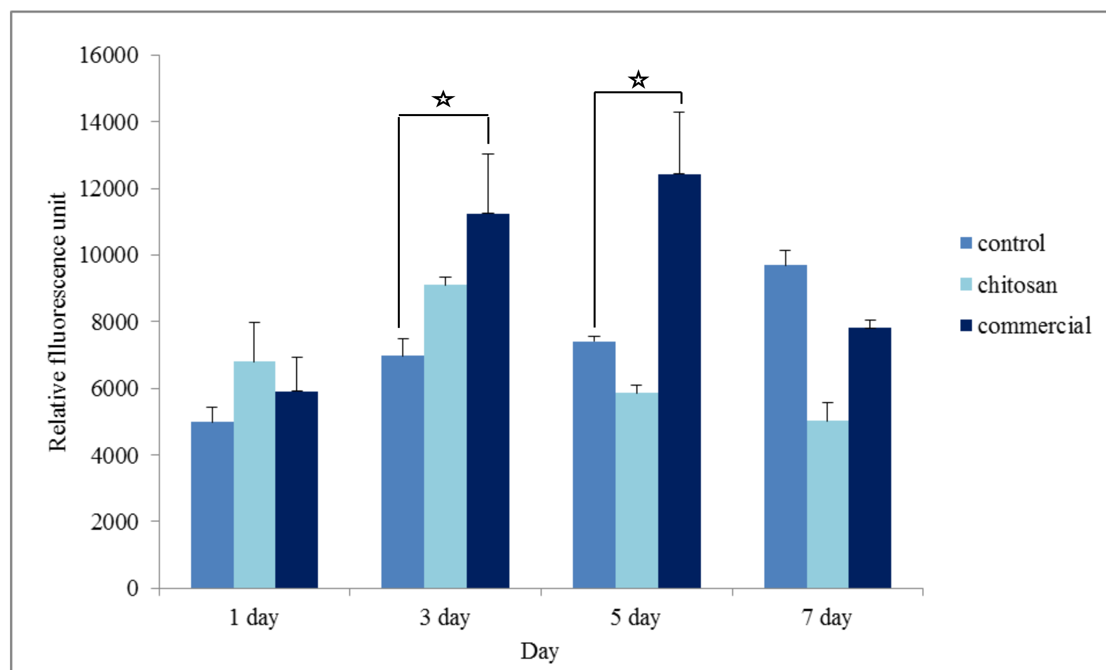

Fig 10. Viability of MG-63 cells, measured by PrestoBlue.

Alizarin Red S Staining

The bright red nodule formation of variable quantity was seen when observed under the microscope in the chitosan scaffolds and the pcl/tcp scaffolds on different test days. No nodule formation on the day 7 and on the control was seen. The nodule formation was seen more in the chitosan scaffolds and it increased with the increase in the time period. The mineralized nodule was dissolved in diethyl sulfoxide and the then the absorbance recorded in a universal multiplate reader in 550 nm. Photo absorbance were found to be insignificant in both the chitosan and pcl/tcp scaffolds in

day 7 and day 14, but on day 21 and day 28 chitosan showed significant photo absorbance. The one way ANNOVA showed significant relationship between day 7 and day 21 and also between day 14 and day 21. Similarly day 28 also showed the same relationship with day 7 and day 14. Pcl/tcp scaffolds showed significant relationship in between day 21 and day 7, and also day 21 and day14. The result of day 21 also showed significance with day 28. (Table 4 and Fig 11)

Table 4. Mean absorbance with SD. (Different superscript represent significance at p<(0,05).

	7d	14d	21d	28d
Control	$100±0.00^{ab}$	$100±0.00^{ab}$	$100±0.00^{ab}$	$100±0.00^{ab}$
Chitosan	$96.376±10.94^{a}$	$187.681±70.25^{ab}$	$589.130±43.42^{d}$	$669.565±5.75^{d}$
Commercial	$113.768±6.64^{ab}$	$187.681±3.32^{ab}$	$352.173±107.45^{c}$	$220.289±46.84^{b}$

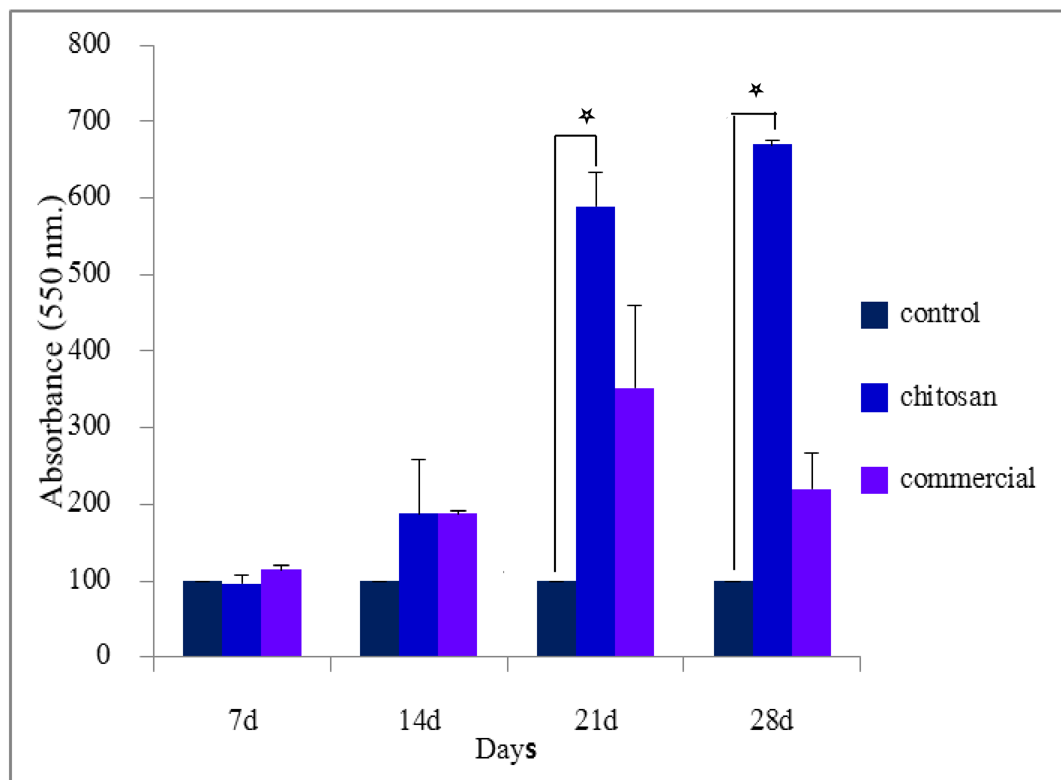

Fig 11. Mineralized Nodule formation with Alizarin Red S Stain.

Chapter 4

Discussion

Chitosan Scaffold

One of the advantages the chitosan has over other scaffold materials is the ease in fabricating into different forms and shapes. It can be shaped into various forms, such as microspheres, sponges, paste, fibers, membranes and porous scaffolds. There are different techniques in fabrication of chitosan scaffolds. The most common techniques are, 1) solvent casting and particulate leaching, 2) phase separation and freeze drying and 3) solution spinning.

For this study the phase separation and freeze drying method was used. In this process the chitosan solution is frozen causing lyophilization, whereby the chitosan acetate phase separates from the ice crystal phase. The sublimation of ice crystals produces porous structure [20, 45]. The limitation with this procedure is that the pore size is not very large and to overcome this solvent exchange/phase separation has been proposed [46, 47]. The choice of solvent is also limited in this process [47].Though the physical and the mechanical characteristic of the resultant scaffold does not vary much this technique was used here for the ease of fabrication and the easy availability of the required equipments.

The porosity of the scaffold fabricated with this technique ranged between 70 to 90 % in different studies. The porosity of the scaffold fabricated for this study was found to be 85 to 95%. The higher porosity with interconnected pores helps in better cell attachment and migration [20] and also fastens the degradation and increases the water uptake capacity [36]. The pore size of the chitosan scaffold used in this study varied from 150µm to 250µm. In other studies with the similar technique varied from 50µm to 200µm [37] and from 60µm to 90µm [38]. The scaffolds should have well interconnected pores to facilitate the cell growth and migration throughout the scaffold surface. This will also facilitate the nutrients transfer and neovascularization [48]. The minimum pore size is considered to be approximately 100-150µm [49]. The larger pore size readily vascularize thus promoting osteogenesis. The smaller pore size

creates hypoxic condition and tends to lead to osteochondrogenesis before the osteogenic process [48]. The high porosity and the bigger pore size facilitates the better osteogenesis but has the compromising effect on the mechanical property[50].The pore size increases as the concentration of the chitosan increases [37] and also the freezing temperature define the size of the pore. The scaffolds fabricated at the higher temperature have larger size than the scaffold fabricated at lower freezing point [39]. The chitosan scaffold has been found to be mechanically weak and unstable, and unable to maintain predefined form as a result of swelling [53]. Therefore, its application in load bearing is limited. The chitosan fiber reinforcement approach was applied to enhance the mechanical properties by Alabama. MZ. et. al. and they found that fiber reinforced chitosan scaffold showed improved tensile and compressive strength [54]. Therefore the mechanical properties of the chitosan scaffolds can be improved by decreasing the pore size and the percentage of porosity. They can be controlled by the molecular weight and the degree of deacetylation of chitosan materials. The pore size and percentage porosity can also be controlled by the freezing temperature in the process. The chitosan fiber reinforcement is the other method of improving the mechanical properties. The combination with other bioceramics and with the polymers is other methods to enhance the mechanical properties of scaffold at the same time retaining the advantages of chitosan.

Swelling

The swelling ability of the scaffolds denotes the water absorption capacity of the scaffolds. This also means that the scaffolds can absorb body fluid. This will not only help in maintaining the shape and structure of the scaffolds, it will also help in cell attachment and growth. On the other hand the very high absorption for a long time may cause the scaffolds to expand and deform.[36]. The larger pore size would take up more water, but the decrease in overall porosity would take up more water because of the increase in overall increase in surface area of the scaffold [36]. In this study the swelling ratio of chitosan was higher than that of the pcl/tcp. It could be attributed to the design of the pcl/tcp scaffold which a mesh work designs.

Degradation

The scaffold should have the property of gradual and controlled degradation, so that it should be able to take the space to be filled by the intended cells gradually. It will also prevent the other unwanted cells to migrate and proliferate in the area. The degradation allows the cells to occupy the area occupied by the scaffold previously gradually. In this study, the degradation of chitosan was found to be slow and gradual and progressing with the time, reaching 28% at 30 days. Some studies reported that the porosity can be controlled by manipulating molecular weight and the degree of deacetylation [43,44]. The scaffolds displaying higher porosity showed higher degradation rate [44]. The pcl/tcp scaffolds in this study showed almost no degradation. This could be because it takes longer for it to start the degradation process. Normally, pcl/tcp takes 9-10 months to start degradation and 12 months to completely degrade [40].

Cell viability

Cell viability was done with the Presto Blue assay. Presto Blue is a resazurin based reagent and is used to assess the cell viability and cytotoxicity. In viable cells the resazurin is reduced to resorufin, causing reagent to change from non-fluorescent to strong fluorescent form. It is non-toxic to the cells, implying that it does not interfere with the normal growth and metabolism of the cells [41]. Presto Blue is highly sensitive and can detect the cells below 100cells per well. The incubation period is also very less requiring only ten minutes. Therefore, it is considered as the fastest live assay. In this study, both the chitosan scaffold and the pcl/tcp scaffolds showed good cell viability, although pcl/tcp showed little more of the viable cells. This implies that both of the scaffolds support the growth and differentiation of the bone forming cell.

Mineralized Nodule formation

Alizarin red S forms the mineralized nodule with the osteogenic cells in a process of chelation with the calcium ions forming alizarin red s- calcium complex. The nodule formation initiates as early as 7 days with human messenchymal cells and increase over to 10 days and decreasing thereafter till the end of lag phase of cell cycle [42]. In this study the nodule formation were not detected in 1 week, but were detected in the subsequent weeks with increase intensities.

Chapter 5

Conclusion

The self – fabricated chitosan scaffold showed comparable physical and biological characteristic with that of the pcl/tcp scaffold. The MG-63 cells were found to be viable and proliferating, which implies that the scaffold is non - cytotoxic. Chitosan scaffold supported the cell to get into osteogenic potential shown by the ability to form the mineralized nodules. Therefore, chitosan scaffold can be equally good as the pcl-tcp scaffold if further improvement in the mechanical properties can be achieved.

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APPENDIX

1. **Biological Materials**
    - MG63 cell line ATTC no. CRL-1427, USA
2. **Equipments and Instruments**
    - Autoclave Hiclave, Model HB-50, Hirayama Mfg. Company, Japan
    - Automatic pipetter, Portable pipette-Aid Model XP, Drummond Scientific Company, USA.
    - Biohazard Laminar Airflow Class II, Astec Micro flow Model ABS1200TCN, Bioquell Lab, UK
    - Biotrak, II microplate reader, Amersham Biosciences, Biochrom Ltd. UK
    - CO_2 Incubator, Series II water Jacketed CO_2 Incubator Model 3111, Thermo Forma, USA
    - Desk Top Centrifuge, Model 5417C, Eppendrof, Germany
    - Hot Air Oven, Model UM500, Memert, USA
    - Microscope, Inverted Nikon TS 100E, Japan
    - Pipetter, Pipetman Gilson, France
    - Refrigerated Centrifuge, Kendro Lab. Products, Germany.
    - Water Bath, Memmert, Germany
3. **Disposable Materials**
    - Well- plates, Nunc, Denmark
    - Centrifuge tubes, Neptune, Switzerland
    - Cryotube, Nunc, Denmark
    - Tissue culture flask, TPP, Switzerland
    - Micropipette tips, Treff AG, Switzerland
4. **Chemical and Reagents**
    - Absolute ethanol, Merck, Thailand
    - Alizarin Red S, Sigma, USA
    - Beta Glycerophosphate, Sigma, USA
    - Dexamethasone, Sigma, USA
    - Dulbecco's Modified Eagle Medium, Gibco, USA
    - Fetal Bovine Serum, Biochrom AG, Germany
    - Lysozyme, Sigma, USA
    - L- Ascorbic acid 2-phosphate, Sigma, USA

- Penicillin G/ Streptomycin/Kanamycin, H&H, Thailand
- Presto Blue Assay, Thermo Fisher, USA
- Phosphate Buffered Saline, Sigma, USA

Vitae

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Higher Graduate Diploma in Clinical Science (Oral and Maxillofacial Surgery)	Prince of Songkla University	2013

Scholarship Awards During Enrolment

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Dental Surgeon, Ministry of Health, Royal Government of Bhutan