FACULTY OF ENGINEERING

DEPARTMENT OF BIOMEDICAL ENGINEERING

GRADUATION PROJECT II

BME402

BIOCERAMIC SCAFFOLDS FOR BONE REGENERATION

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

TE: Tissue Engineering

3-D: Three Dimensional

BMP's: Bone Morphogenic Proteins

DBM: Demineralized Bone Matrix

HAp: Hydroxyapatite

nHA: nanoHydroxyApatite

B-TCP: Tri-Calcium Phosphate

MSC's: Mesenchymal Stem Cells

BM-MSC's: Bone Marrow Mesenchymal Stem Cells

BMAC: Bone Marrow Aspiration

Na_2CO_3 : Sodium Carbonate

SEP : Soluble Eggshell Protein

MW: Molecular Weight

$FeCl_3$: Iron (III) chloride

Nm: nanometers

NaOH: Sodium Hydroxide

PBS: Phosphate-buffered saline

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KEYWORDS

SCAFFOLD*:* A 3-D structure composed of polymer fibers

BIOCERAMIC*:* Bioceramics and bioglasses are ceramic materials that are biocompatible which are inert in the body and used for implants.

SILK FIBROIN*:* Fibroin is an insoluble protein present in silk created by spiders, the larvae of Bombyx mori. Silk in its raw state consists of two main proteins, sericin and fibroin, with a glue-like layer of sericin coating two singular filaments of fibroin called brins.

TISSUE ENGINEERING*:* Tissue Engineering is the study of the growth of new connective tissues, or organs, from cells and a collagenous scaffold to produce a fully functional organ for implantation back into the donor host.

BIOCOMPATIBILITY*:* Ability to be in contact with a living system without producing an adverse effect.

BIODEGRADATION: Is the chemical dissolution of materials by bacteria, fungi, or other biological means. Although often conflated, biodegradable is distinct in meaning from compostable.

BOMBYX MORI: The silkworm is the larva or caterpillar of the domesticated silkmoth. It is an economically important insect, being a primary producer of silk.

CHITOSAN: Is a linear polysaccharide. Chitosan's properties allow it to rapidly clot blood.

SERICIN: Sericin is a protein created by Bombyx mori (silkworms) in the production of silk.

DEGUMMING: Degumming is the first stage in the refining process it is used to separate the gums, phospholipids, proteins

HYDROXYAPATITE*:* Is a naturally occurring mineral form of calcium apatite

CYTOCOMPATIBILITY: Ability for a cell to be compatible

BIOMIMICING: To imitate in same form and appearance of a models, systems, and elements of nature for the purpose of solving complex human problems

REGENERATION*:* Regeneration is the process of renewal, restoration, and growth that makes genomes, cells, organisms, and ecosystems resilient to natural fluctuations or events that cause disturbance or damage.

OSTEOCONDUCTIVE SCAFFOLD*:* Physical, three-dimensional scaffold or matrix to facilitate bone repair.

OSTEOPROGENITOR CELLS*:* Osteoprogenitor cells are types of cells that work in the growth or repair of bones. These cells originate from stem cells and are created by progenitor cells.

OSTEOGENESIS*:* The formation of bone.

OSSIFACTION*:* **Ossification** (or osteogenesis) in bone remodeling is the process of laying down new bone material by cells called osteoblasts.

PROLIFERATION*:* The growth or production of cells by multiplication of parts.

ABSORBANCE: The ability of a layer of a substance to absorb radiation

UV-VISIBLE SPECTROPHOTOMETER: Is an easy and accurate instrument ideal for measuring the absorbance spectra of various chemical and biochemical compounds

ABSTRACT

Bone regeneration as a well-organized physiological process of bone formation can be seen during normal fracture healing, and it involves continuous re-modelling. However, in some complex clinical conditions bone regeneration is required in large quantity, such as for skeletal reconstruction of large bone defects created by trauma, infection, tumour resection and skeletal abnormalities, or cases in which the regenerative process is compromised, including avascular necrosis, atrophic non-unions and osteoporosis. Osteo-progenitor cells and distraction osteogenesis. Improved 'local' strategies in terms of tissue engineering and gene therapy, or even 'systemic' enhancement of bone repair, are under intense investigation, in an effort to overcome the limitations of the current methods, to produce bone-graft substitutes with biomechanical properties that are as identical to normal bone as possible, to accelerate the overall regeneration process, or even to address systemic conditions, such as skeletal disorders and osteoporosis. Regeneration of tissues using cells, scaffolds and appropriate growth factors is a key approach to treatments of tissue or organ failure. Silk protein fibroin can effectively be used as a scaffolding material in these treatments. In tissue engineering applications or even in 3D cell cultures, the biological cross talk between cells and the scaffold is controlled by the material properties and scaffold characteristics. In order to induce cell adhesion, proliferation, and activation, materials used for the fabrication of scaffolds must possess requirements such as intrinsic biocompatibility and proper chemistry to induce molecular bio-recognition from cells.

Materials, scaffold mechanical properties and degradation kinetics should be adapted to the specific tissue engineering application to guarantee the required mechanical functions and to accomplish the rate of the new-tissue formation. For scaffolds, pore distribution, exposed surface area, and porosity play a major role, whose amount and distribution influence the penetration and the rate of penetration of cells within the scaffold volume, the architecture of the produced extracellular matrix, and for tissue engineering applications, the final effectiveness of the regenerative process. Depending on the fabrication process, scaffolds with different architecture can be obtained, with random or tailored pore distribution. In the recent years, rapid prototyping computer-controlled techniques have been applied to the fabrication of scaffolds with ordered geometry. This project reviews the principal polymeric materials that are used for the fabrication of scaffolds and its processes, with examples of properties and selected applications. (Dimitriou *et.,al* 2011)

1. INTRODUCTION

Every day thousands of surgical procedures are performed to replace or repair tissue that has been damaged through disease or trauma. The developing field of tissue engineering (TE) aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue. This project describes the functional requirements, and types of materials used in developing state of the art of scaffolds for tissue engineering applications.

Disease, injury and trauma can lead to damage and degeneration of tissues in the human body, which necessitates treatments to facilitate their repair, replacement or regeneration. Treatment typically focuses on transplanting tissue from one site to another in the same patient (an autograft) or from one individual to another (a transplant or allograft). While these treatments have been revolutionary and lifesaving, major problems exist with both techniques. Harvesting autografts is expensive, painful, constrained by anatomical limitations and associated with donor-site morbidity due to infection and hematoma. Similarly, allografts and transplants also have serious constraints due to problems with accessing enough tissue for all of the patients who require them and the fact that there are risks of rejection by the patient's immune system and the possibility of introducing infection or disease from the donor to the patient. Alternatively, the field of tissue engineering (a phrase that is interchangeably used with regenerative medicine) aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function.

The field relies extensively on the use of porous 3D scaffolds to provide the appropriate environment for the regeneration of tissues and organs. These scaffolds essentially act as a template for tissue formation and are typically seeded with cells and occasionally growth factors, or subjected to biophysical stimuli in the form of a bioreactor; a device or system which applies different types of mechanical or chemical stimuli to cells. These cell-seeded scaffolds are either cultured *in vitro* to synthesize tissues which can then be implanted into an injured site, or are implanted directly into the injured site, using the body's own systems, where regeneration of tissues

or organs is induced *in vivo*. This combination of cells, signals and scaffold is often referred to as a tissue engineering triad.

The limited supply of donors and increasing morbidity have put new demands on (TE) tissue engineering as a treatment of organ failure. The TE approach involves regenerating tissue within suitable scaffold with the goal of implanting the constructed tissue at the target site.

The regeneration of functional tissue requires a suitable microenvironment that closely mimics the host site for desired cellular responses. Such an environment is typically provided by 3-D tissue engineering scaffold that acts as an architectural template. Apart from biocompatibility, which is the essential prerequisite for any biomaterial, matching the degradation time with that of the tissue regeneration is also a critical requirement for a cell scaffolding material. Such a match can maintain the mechanical properties and structural integrity of the engineered tissue in all stages of its regeneration process. In addition, degraded products of the biomaterial should be safely metabolized and cleared from the host body. Materials like polymers, metals and ceramics are widely used as cell scaffolds for tissue engineering. Both synthetic and natural polymers have been trialed, though each has its own limitations. While the former allows easy processing and modifications, the later offers better cyto- and bio-compatibility. There is no universal biomaterial that meets the scaffolding requirements for all the tissues. Different issue constructs require biomaterials with specific physical, mechanical and degradation properties. Hence there is on-going search for universal biomaterial for regeneration therapy. (Kundu *et al.*, 2013)

1.1 SOURCES OF SILK FIBROIN

Silk proteins are present in glands of silk producing arthropods (such as silkworms, spiders, scorpions, mites and bees) and spun into fibers during their metamorphosis. Silkworm's silk is an established fiber extensively used in the textile industry. On the other hand, the cannibalistic nature of spiders restricts the commercial production of spider silk. Additionally, the yield of fiber from a single silk cocoon is 600–1500 m, compared to only ~137 m from the ampullate gland of a spider and ~12 m from the spider web. Spider silks are also heterogeneous in nature. Therefore, silk based biomaterials are commonly prepared from silkworm silk. Of note is the silk produced by Bombyx mori, a member of the Bombycidae family. B. mori silk is also known as mulberry silk. Another silk producing family is Saturniidae and the silk is known as non-mulberry silk. Silk has several major advantages over other protein based biomaterials, which are derived from tissues of allogeneic or xenogeneic origins. As such, the risk of infection is high for those materials. Processing of such materials is also expensive due to the stringent protein isolation and purification protocols. In contrast, silk is an established textile fiber and nearly 1000 metric tons of silk are produced and processed annually. Silk fiber purification is routinely carried out using a simple alkali or enzyme based degumming procedure, which yields the starting material for sericin free silk based biomaterials. It is also economically advantageous to use silk for biomedical applications, because of available large scale processing infrastructure of traditional silk textile industries.

Figure 1. Cocoons From Bombyx Mori

1.2 SOURCES OF CHITOSAN

Chitin or poly (β-(1→4)-N-acetyl-D-glucosamine) is a natural polysaccharide of major importance, first identified in 1884. This biopolymer is synthesized by enormous number of living organisms and it belongs to the most abundant natural polymers, after cellulose. In the native state, chitin occurs as ordered crystalline micro-fibrils which form structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. So far, the main commercial sources of chitin are crab and shrimp shells.

1.3 PROPERTIES OF SILK FIBROIN

1.3.1 MECHANICAL PROPERTIES

Mechanical properties Silk offers an attractive balance of modulus, breaking strength, and elongation, which contributes to its good toughness and ductility. Silk fibers are tougher than Kevlar, which is used as a bench mark in high performance fiber technology. The strength-to-density ratio of silk is up to ten times higher than that of steel. Spider silk fibers in particular have high extensibility and exhibit marked strain hardening behavior. Strain hardening refers to increase in stress as a fiber is extended in the plastic region beyond the yield point. Such behavior is particularly important for energy absorbing materials. Stress–strain curves of the wild silkworm silk fibers exhibit shape and strain hardening similar to that of spider or dragline silks. Considering the good strength and toughness of silk fibers, it is no surprise that silk has been exploited to develop scaffolds for load bearing tissue engineering. However, in current design of silk based biomaterials, the wide choice of mechanical properties available from using different silk types is not fully explored. A biomaterial implant fails either due to poor mechanical properties needed for the application, or higher than optimum mechanical properties due to inappropriate stress concentration at the implant tissue interface. Therefore, variations in mechanical properties of different type of silk provide good choice to match material properties. In general, the wide choice of extensibility and elasticity of silks, good strength and strain hardening from different varieties of silk provide outstanding advantage to developing a range of silk based biomaterials.

1.3.2 BIOLOGICAL PROPERTIES

1.3.2.1 BIOCOMPATIBILITY

The long history of success of silk sutures has made silk a known biocompatible material. But, like any other non-autologous biomaterials causing foreign body response, some adverse immunological events associated with silk proteins cannot be ruled out, particularly due to its non-mammalian origin. Some incidents of delayed hypersensitivity of silk sutures in rare cases are suggested to be due to the presence of silk gum-like protein sericin. Further studies employing isolated silk sericin and sericin based biomaterials have provided no clear evidence to suggest sericin as the source of adverse effects. Detailed investigations are needed to specifically identify the source of any cyto-toxic non fibroin elements in silk and develop appropriate diagnostic method.

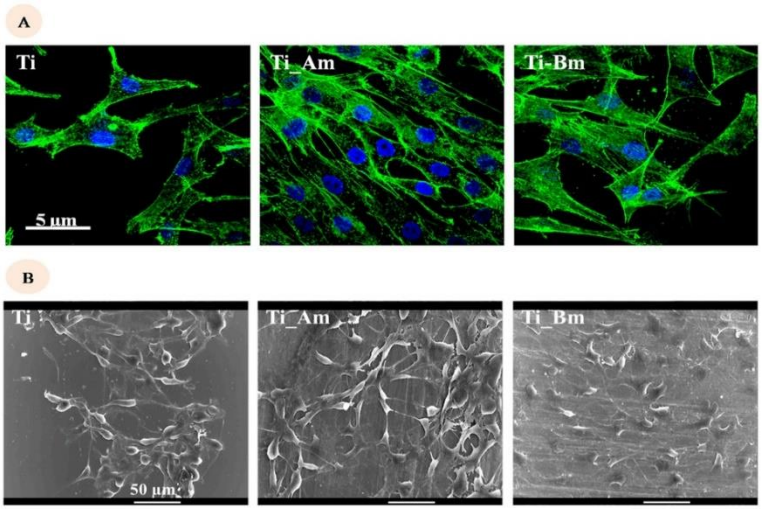


Figure 2. Electron Microscopy of scaffolds

1.3.2.2 BIODEGRADIBILITY

The silk biodegradation is studied based on mass loss, change in morphology and analysis of degraded products in-vitro. Similarly, degradation is tested in animal models by testing mechanical properties of silk after implantation for certain time and studying structural integrity by histological examinations, fluorescent staining and various biochemical assays. As implanted construct, regenerated silk fibroin biomaterial degrades much faster than fibers. The degradation rate is dependent upon the secondary structure of silk resulting from preparation of regenerated silk materials. The term biodegradability is often used to discuss the disintegration of silk materials.

According to the definition of Vert et al., biodegradability is the degradability of an implantable polymer by biological elements giving fragments, which can move away from the site through fluid transfer but not necessarily from the body. On the other hand, bio-sorption is total elimination of the initial foreign material either through filtration or metabolization of the degraded bio-products. (Kundu *et.,al* 2012).

2. MATERIALS AND METHOD

2.1 SYNTHESIS OF PURE SILK FIBROIN

In other to make bone scaffolds from silk based materials, there are general procedures to follow, below are a list of step by step procedures to processing bone scaffolds:

2.1.1 Cleaning and cutting – The cocoons gotten from the Bombyx-mori (silkworm) have to be cut (for measurement purposes and as to allow the degumming process to be faster) into smaller bits and cleaned to remove any unwanted dirt and impurities.

Figure 3. Cleaned & Cut Cocoons

2.1.2 Degumming -- Degumming is the process of removing the sericin (a glue protein and a sticky substance) produced by the silkworm that holds the strands of silk together. This is done by adding 3g of cocoons (chopped and cleaned) and 400ml of Na_2CO_3 into a conical flask which are then electro-spurned with a magnetic stirrer for Three (3) hours in three rounds, the electro-spurned fibers are washed with deionized water after each round in other to completely remove sericin.

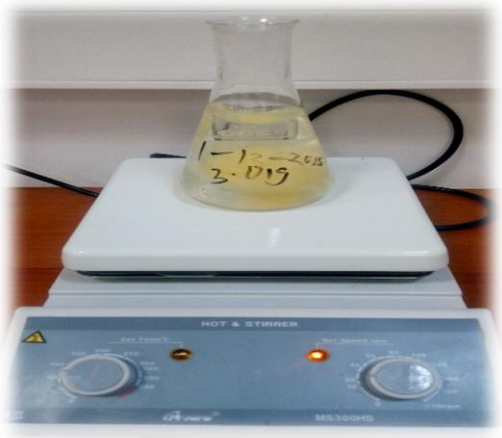


Figure 4. Degumming Process (Electrospinning)

Preparation of 0.1mol sodium carbonate (Na_2CO_3)

5.3g Na_2CO_3 + 500ml deionized water

2.1.3 Drying and picking – After the process of degumming, we washed the degummed fibers and dried for two days. The dried fibers were picked in other to obtain strands of fibers that are not stocked together.

Figure 5. Picked Fibers

2.1.4 Dissolution – We prepared an electrolyte solution and then by electrospinning we dissolved the picked fibers in the electrolyte solution. To produce various other material formats, degummed silk fiber is dissolved to obtain silk solution. In some cases, where it is difficult to dissolve fibers, fibroin can be extracted directly from glands of silkworms using an appropriate

buffer solution. Different solid forms are then prepared from silk solution by liquid solid phase transfer. Both native fibers and materials regenerated from silk solution are used for tissue engineering.

Figure 6. Dissolution

Preparation of electrolyte solution

29.15ml Ethanol

27.75ml Calcium Chloride ($CaCl_2$)

36ml deionized water

2.1.5 Dialysis – We added the solution into a semi-permeable membrane and we put it into a deionized water in a beaker. Placed it on a stirrer for nine hours in three rounds (no heat applied) with a change of water after each round.

The silk fibroin is water soluble in its α-helical and random coil forms. Solubility can be maintained over days and even weeks depending on the storage temperature, pH and concentration of silk solution. Hence, silk based systems can be prepared using water based solution under mild manufacturing conditions such as room temperature, neutral pH and without application of high shear force. Such conditions are favorably exploited for loading sensitive drugs into silk implants. Mild processing conditions are also helpful for photonic or electronic devices or biosensors, which may be incorporated within a silk based system or coated with silk for improved bio-integration

in-vivo. Conformational transition of α-helix and random coil to highly stable β-sheets is required in silk products to provide good resistance to dissolution, thermal and enzymatic degradation. This can be achieved through water vapor annealing, mechanical stretching and ultrasonic treatments, hence avoiding the use of harmful chemicals. These processing advantages and good structural stability make silk a promising polymeric system for bio-related applications.

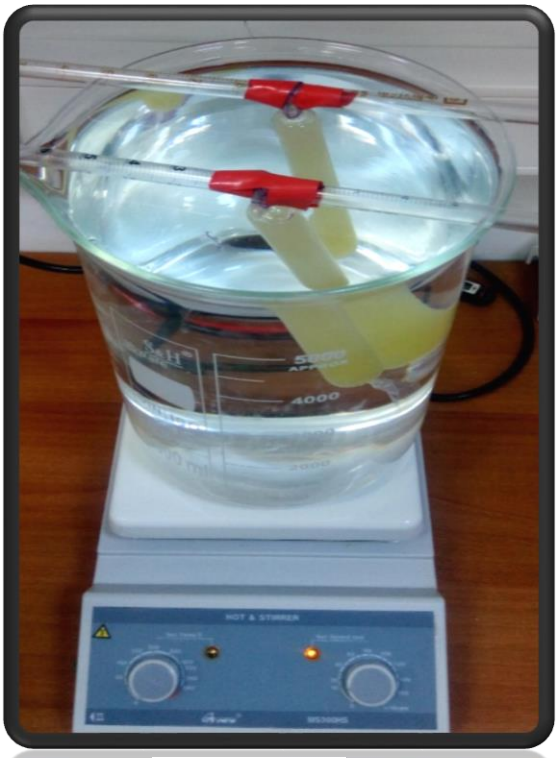


Figure 7. Dialysis

2.2 EGGSHELL

Hydroxyapatite is a calcium phosphate ceramics material that is found to be very crucial as a biomaterial because of its osteophilic nature and its incorporation into bone tissues. The use of calcium phosphate salts to successfully replace and augment bone tissue is known for many years since it was discovered that calcium phosphate plays a major role in the inorganic phase of hard tissues, bones, and teeth which necessitates medical researchers to consider this salt as a way of improving the healing process. Natural bone is made up from inorganic/organic composite mainly nanostructure hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and collagen fibers. Hydroxyapatite (HAp) forms the major inorganic portion of bone and teeth tissue and it is a key requirement for developing bone grafts. Hydroxyapatite has a number of applications in tissue engineering and it is both bioactive and biocompatible with similar chemical composition and biological affinity to the mineral part of bone. Bone mineral is a salt where the ratio of calcium to phosphate (C/P) is 1.5 to 1.7 and the hydroxyapatite C/P ratio is generally assumed to be 1.67 which is within the range. HAp has shown excellent biocompatibility with mostly soft tissues in skin, muscle, and teeth gums. This compatibility with biological tissues has made HAp regarded as superior to other artificial materials. This has qualified HAp to be an ideal implant or an implant component material in orthopaedic and dental surgery.

Figure 8. Raw Egg Shell

Figure 9. Egg Shell Powder

2.3 BIOCERAMICS

In our project, we modified a porous silk fibroin (SF) scaffold with soluble eggshell membrane protein (SEP) with the aim of improving the cell affinity properties of the scaffold for tissue regeneration. This was achieved by combining particles of hydroxyapatite and the solution of dissolved silk fibroin after which the mixture was subdued under 60 degrees Celsius heat in other for it to evaporate.

10ml silk fibroin solution + 0.5g egg shell powder + 60℃ heat

The existence of SEP on the scaffold surface and the structural and thermal stability can be confirmed by energy-dispersive X-ray spectroscopy, X-ray diffraction, Fourier transform infrared spectroscopy, differential scanning calorimetry, and thermogravimetric analysis. Using cell culture study, it should indicate a significant improvement in the cell adhesion and proliferation of mesenchymal stem cells (MSCs) on the SF scaffold modified with SEP. The cytocompatibility of the SEP-conjugated SF scaffold can be confirmed by a 3-(4,5-dimethyltriazol-2-y1)-2,5-diphenyl tetrazolium assay. Thus, this project demonstrates that the biomimic properties of the scaffold could be enhanced by surface modification with SEP. (Sah *et al.*, 2015).

Figure 10. Bioceramic (Eggshell+Silk Fibroin)

2.4 CHITOSAN AND EGG SHELL POWDER

We describe a comparative assessment of the structure–property–process relationship of three-dimensional chitosan–nanohydroxyapatite (nHA) and pure chitosan scaffolds in conjunction with their respective biological response with the aim of advancing our insight into aspects that concern bone tissue engineering. High- and medium-molecular-weight (MW) chitosan scaffolds were fabricated by evaporation. The nanocomposites were characterized by a highly porous structure and the pore size is expected to be in a similar range for the scaffolds with different content of nHA. A combination of X-ray diffraction, Fourier transform infrared spectroscopy and electron microscopy should indicate that nHA particles were uniformly dispersed in chitosan matrix and there was a chemical interaction between chitosan and nHA. Using the swelling test, the water uptake ability of composites decreased with an increase in the amount of nHA, while the water retention ability was similar to pure chitosan scaffold. The observations related to well-developed structure morphology, physicochemical properties and superior cyto-compatibility suggest that chitosan–nHA porous scaffolds are potential candidate materials for bone regeneration although it is necessary to further enhance the mechanical properties of the nanocomposite. (Han *et al*., 2009)

Eggs of Gallus gallus were collected. Egg shells were isolated and cleaned with deionized water and then dried. Raw egg shell powder was mixed with 2ml chitosan solution. The chitosan solution was obtained by measuring 2g of chitosan into 25ml 0.1mol ascetic acid and then dissolved with a 60℃ heat applied.

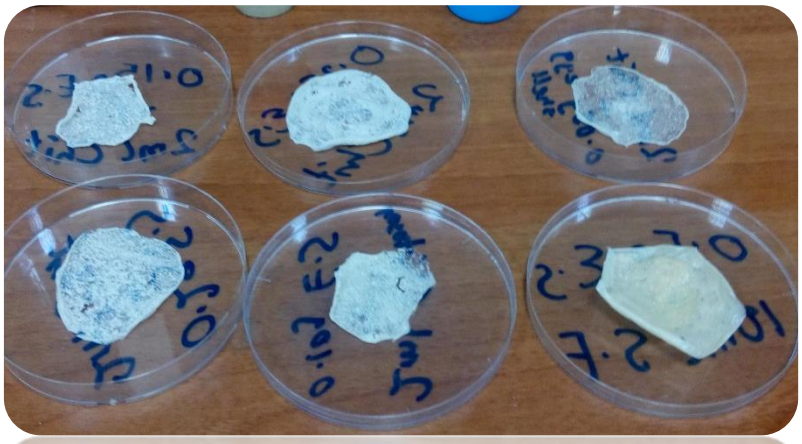


Figure 11. Different Ratios of Eggshell+2ml Chitosan

3. RESULTS

3.1 Swelling Test

This was done to determine the swelling capacity of the scaffolds by using different sample solutions. From this we observed that the water ability of composites decreased with an increase in the amount of nHA (eggshell). Below are the table of results of different samples,

Table 1: RESULTS FOR THE SWELLING TEST OF 0.5g EGG SHELL + 10ml SILK FIBROIN

W_0=DRY WEIGHT =0.03g, T_S=START TIME

Figure 12. Swelling Test Samples

Ethanol Solution (70%)					0.1M Phosphate Buffer Solution			
	Time	T_s	W			Time	T_s	W
T_1	5mins	11:00	0.04		T_1	5mins	11:01	0.04
T_2	10mins	11:06	0.06		T_2	10mins	11:07	0.05
T_3	15mins	11:21	0.07		T_3	15mins	11:22	0.06
T_4	30mins	11:50	0.08		T_4	30mins	11:51	0.07
T_5	45mins	12:35	0.06		T_5	45mins	12:36	0.06

T_6	75mins	13:50	0.05		T_6	75mins	13:51	0.05
T_7	105mins	15:35	0.05		T_7	105mins	15:36	0.05

0.1M HydroChloric Acid					Deionized Water			
	Time	T_s	W			Time	T_s	W
T_1	5mins	11:02	0.04		T_1	5mins	11:03	0.04
T_2	10mins	11:08	0.06		T_2	10mins	11:10	0.05
T_3	15mins	11:23	0.07		T_3	15mins	11:25	0.06
T_4	30mins	11:52	0.08		T_4	30mins	11:54	0.07
T_5	45mins	12:37	0.06		T_5	45mins	12:39	0.06
T_6	75mins	13:52	0.05		T_6	75mins	13:54	0.05
T_7	105mins	15:37	0.05		T_7	105mins	15:39	0.05

0.1M NaOH			
	Time	T_s	W
T_1	5mins	11:04	0.05
T_2	10mins	11:014	0.06
T_3	15mins	11:29	0.07
T_4	30mins	11:59	0.07
T_5	45mins	12:40	0.07
T_6	75mins	13:56	0.06
T_7	105mins	15:42	0.06

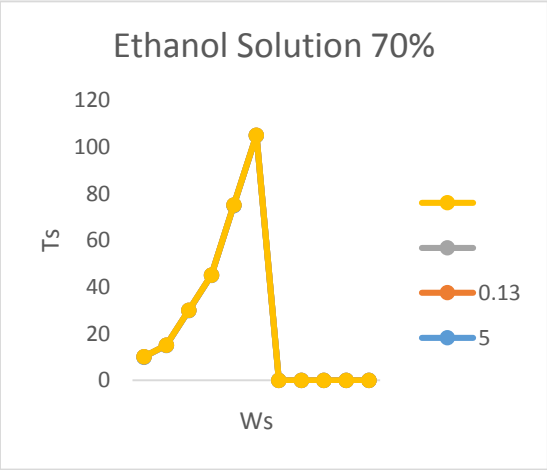
Swell ratio = $Ws-W_o/W_o*100$

TABLE 2: RESULTS FOR THE SWELLING TESTS OF 0.05g EGGSHELL + 2ml CHITOSAN

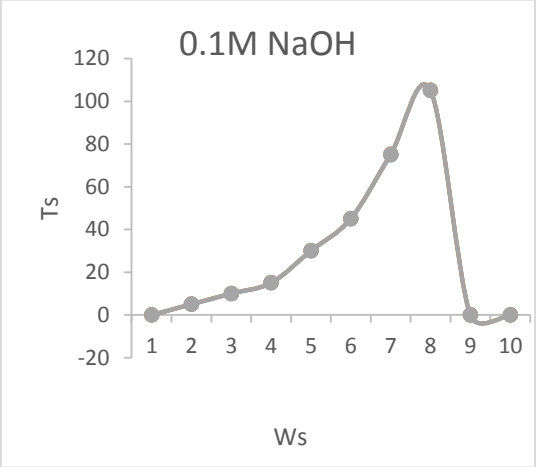
W_0=DRY WEIGHT=0.03g, T_S= START TIME

Ethanol Solution (70%)					0.1M Phosphate Buffer Solution			
	Time	T_s	W			Time	T_s	W
T_1	5mins	10:00	0.06		T_1	5mins	10:01	0.09
T_2	10mins	10:06	0.05		T_2	10mins	10:07	0.10
T_3	15mins	10:21	0.05		T_3	15mins	10:22	0.09
T_4	30mins	10:50	0.05		T_4	30mins	10:51	0.08
T_5	45mins	11:35	0.04		T_5	45mins	11:36	0.07
T_6	75mins	12:50	0.04		T_6	75mins	12:51	0.06
T_7	105mins	14:35	0.04		T_7	105mins	14:36	0.05
Deionized Water					0.1M NaOH			
	Time	T_s	W			Time	T_s	W
T_1	5mins	10:02	0.13		T_1	5mins	10:03	0.05
T_2	10mins	10:08	0.14		T_2	10mins	10:10	0.06
T_3	15mins	10:23	0.13		T_3	15mins	10:24	0.06
T_4	30mins	10:52	0.14		T_4	30mins	10:53	0.07
T_5	45mins	11:37	0.16		T_5	45mins	11:38	0.07
T_6	75mins	12:52	0.17		T_6	75mins	12:53	0.06
T_7	105mins	14:37	0.18		T_7	105mins	14:38	0.06

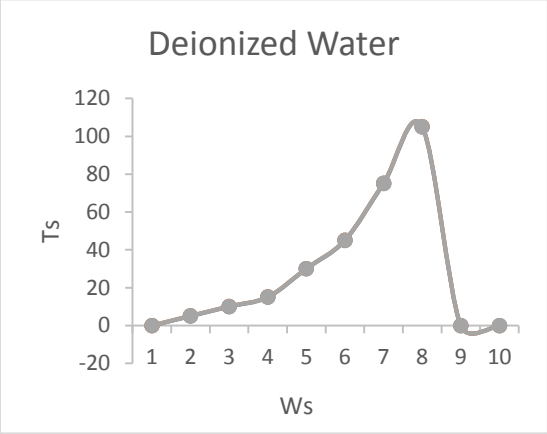
Graphical representations



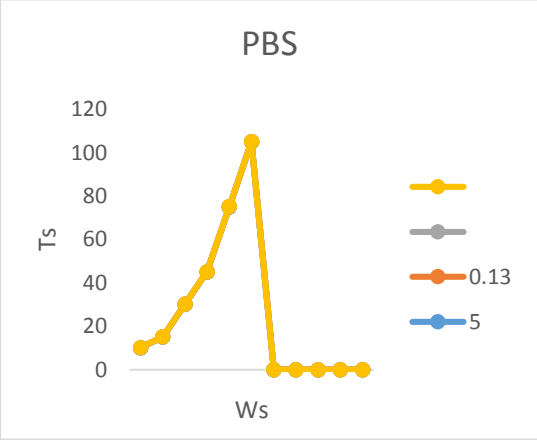
a) Figure 13. Ethanol Solution



b) Figure 14. 0.1M NaOH



c) Figure 15.Deionized Water



d) Figure 16. PBS (Phosphate-buffered saline)

3.2 Absorption Test

Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as the beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

A spectrophotometer is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. Depending on the range of wavelength of light source, it can be classified into:

UV-visible spectrophotometer: which uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
A UV-Visible Spectrophotometer is used to measure the absorbance of each of the samples. The UV-VIS Spectrophotometer is an easy and accurate instrument ideal for measuring the absorbance spectra of various chemical and biochemical compounds. It has a USB interface allowing it to plug directly into a computer for data collection. Several apparatus are used for the setup; A pair of glass cuvettes, solvent bottle, beakers, and the sample solution.

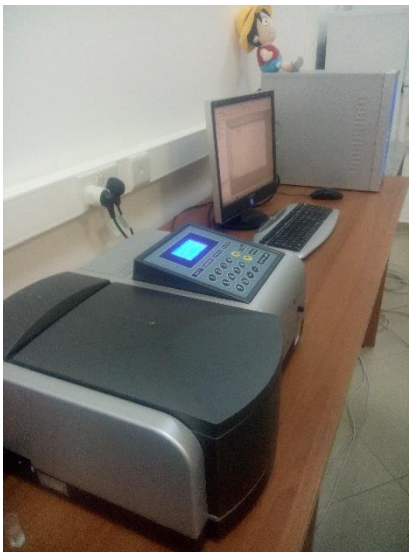


Figure 17. UV-Visible Spectrophotometer

Prepare an Iron (III) chloride solution

- 200ml distilled water
- Weigh the sample 1.622g $FeCl_3$ of 0.05M and add into the distilled water
- Put a magnetic bar and place on a magnetic stirrer for some time at 30rpm with no heat (zero ˚c), until it becomes homogenous. (This process could take up to an hour)

Calculations for the $FeCl_3$

200ml =0.2l

Molar mass of $FeCl_3$ = 162.35747 g/mol

0.05M x 0.2l

n=0.01mol

Molecular weight (mw) of $FeCl_3$ =162.2g

n=m/mw

0.01mol*162.2g/mol

Mass=1.622g $FeCl_3$

Figure 18. Preparation process for $FeCl_3$

Procedures

- Using the UV spectroscopy to measure the absorbance of the solution ($FeCl_3$ solution + 0.024g silk fibroin film).
- Firstly use the cuvettes with a little amount of water to test.
- Press AutoZero and then OK on the desktop which is connected to the UV-Visible Spectrophotometer.
- Measure the absorbance of the solution by using a pipet to draw the solution then empty into a cuvette and place inside the UV spectrophotometer. GOTO Wavelength on Desktop, enter a wavelength of 356nm which is the best for this particular solution.
- After a few seconds the absorbance will be displayed on the screen as 3.128
- *How is the absorbance measured?* Holding the cuvette by the two opaque sides, insert the cuvette into the UV spectrophotometer. The absorbance is measured as light passes through the transparent sides of the cuvette.
- Weighing a little portion of silk fibroin film I got 0.024g which I used for the experiment.
- 10ml solution + 0.024g silk fibroin film at 11:00am leaving it for 10minutes.
- 1ml of (solution + silk fibroin film) place inside the UV-Visible Spectrophotometer and measure absorbance which was 0.201
- Leave solution for another 15minutes and take 1ml of the solution and place in UV-Visible Spectrophotometer and measure the absorbance which was 0.183
- Leave solution for 30minutes and take 1ml of solution and place in the UV-Visible Spectrophotometer and measure the absorbance which was 0.182
- Leave solution for 1 hour and take 1ml of solution and place in the UV-Visible Spectrophotometer and measure absorbance which was 0.203
- Leave for 1hour: 30minutes and take 1ml of solution and place in the UV-Visible Spectrophotometer and measure absorbance which was 0.213
- Leave for 2 hours and take 1ml of solution and place in the UV-Visible Spectrophotometer and measure absorbance which was 0.261

Table 3: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.024g SILK FIBROIN FILM

Start time 11:00am

Time (minutes)	**Absorbance**
10	**0.201**
15	**0.183**
30	**0.182**
60	**0.203**
90	**0.213**
120	**0.261**

SECOND ABSORPTION TEST FOR EGGSHELL+CHITOSAN

5 Different ratios of Eggshell +2ml Chitosan were all tested taking a little portion of each.

0.15g eggshell+2ml Chitosan

0.309g eggshell+2ml Chitosan

0.2g eggshell+2ml Chitosan

0.25g eggshell+2ml Chitosan

0.05g eggshell+2ml Chitosan

I. $FeCl_3$ solution+ Chitosan Film

Chitosan Film composes of 2ml chitosan solution + 0.15g eggshell + 10 drops glycerin

0.0184g chitosan film

First measure the absorbance of $FeCl_3$ solution which was 0.226

10ml $FeCl_3$ solution + 0.0184g chitosan film at 11:32am leaving it for 10minutes.

Take 1ml of ($FeCl_3$ solution + 0.0184g chitosan film) place inside the UV-Visible Spectrophotometer and measure absorbance 0.334

Leave solution for another 15minutes and take another (1ml $FeCl_3$ solution + 0.0184g chitosan film) solution sample and place in UV-Visible Spectrophotometer and measure the absorbance 0.371

Do same for all other $time_{(s)}$ below;

Table 4: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.0184g CHITOSAN FILM

Start time 11:32am. Date 29-04-2016

Time (minutes)	**Absorbance**
10	**0.334**
15	**0.371**
30	**0.367**
60	**0.378**
90	**0.384**
120	**0.402**

II. From 0.309g Egg Shell +2ml chitosan**;**
0.0245g chitosan film was taken and used below

Measure absorbance of $FeCl_3$ and it was 0.671

10ml $FeCl_3$ solution+ 0.0245g chitosan film; for all the times below take 1ml and measure its absorbance.

Table 5: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.0245g CHITOSAN FILM

Start time 10:33am Date 03-05-2016

Time (minutes)	**Absorbance**
10	**0.127**
15	**0.130**
30	**0.157**
60	**0.163**
90	**0.295**
120	**0.332**

III. (0.2g Egg Shell +2ml chitosan); **0.0240g chitosan film** was used

Measure absorbance of $FeCl_3$ and it was 0.098

10ml $FeCl_3$ solution+ 0.0240g chitosan film; for all the times below take 1ml and measure its absorbance.

Table 6: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.0240g CHITOSAN FILM

Start time 10:45am Date 18-05-2016

Time (minutes)	**Absorbance**
10	**0.190**
15	**0.120**
30	**0.122**
60	**0.203**
90	**0.221**
120	**0.272**

IV. (0.25g Egg Shell +2ml chitosan); **0.0195g chitosan film** was used

Measure absorbance of $FeCl_3$ and it was 0.181

10ml $FeCl_3$ solution+ 0.0195g chitosan; for all the times below take 1ml and measure its absorbance.

Table 7: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.0195g CHITOSAN FILM

Start time 11:02am Date 18-05-2016

Time (minutes)	**Absorbance**
10	**0.184**
15	**0.125**
30	**0.190**
60	**0.261**
90	**0.297**
120	**0.360**

V. (0.05g Egg Shell +2ml chitosan); **0.0212g** was used

Measure absorbance of $FeCl_3$ and it was 0.126

10ml $FeCl_3$ solution+ 0.0212g chitosan; for all the times below take 1ml and measure its absorbance.

Table 8: RESULTS FOR THE ABSORPTION TESTS OF $FECL_3$ SOLUTION + 0.0212g CHITOSAN FILM

Start time 9:36am Date 23-05-2016

Time (minutes)	**Absorbance**
10	**0.106**
15	**0.124**
30	**0.120**
60	**0.126**
90	**0.128**
120	**0.124**

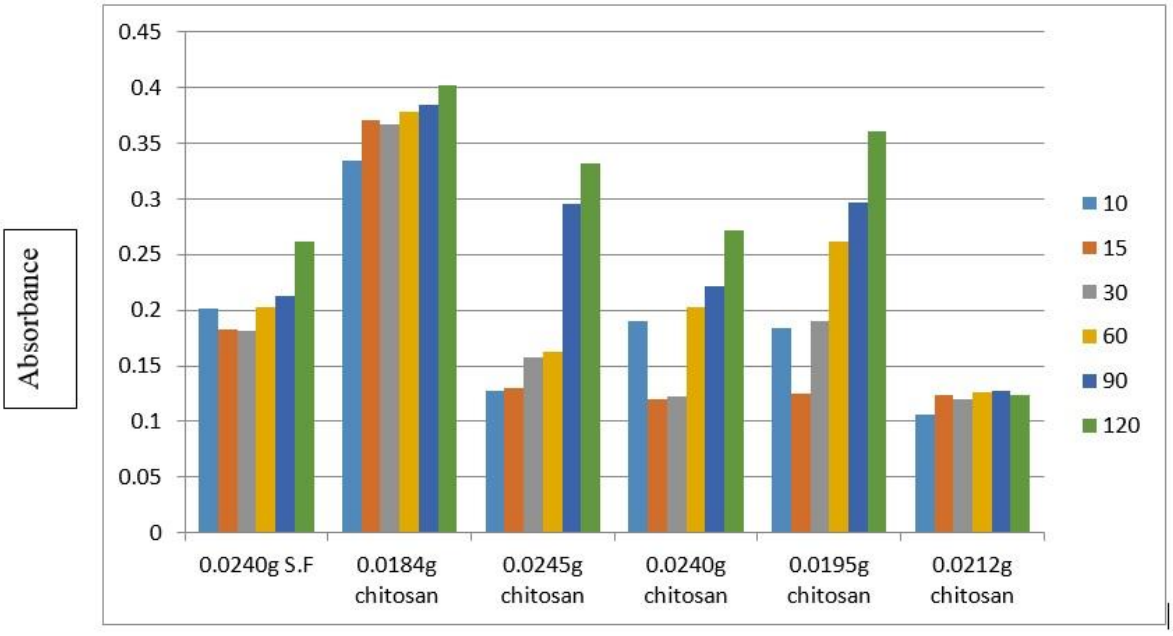


Figure 19: A Graph of Absorbance against Time showing the above tables

3.3 Discussion

- With electrospinning and evaporation, we got a three dimensional bio ceramic scaffolds. HA nanoparticles were dispersed on it, this indicates a polymer matrix showing that the structure has high porosity and large specific surface, providing good conditions for cell attachment, profileration and spreading.

- With the swelling test, we determined the swelling capacity of the scaffolds by using different sample solutions. From this we observed that the water ability of composites decreased with an increase in the amount of nHA (eggshell). We also observed a change in weight which indicates active degradation.

- $FeCl_3$ was added to the various films to check the absorbance by the use of a UV-Visible spectrophotometer. $FeCl_3$ is a very compatible reagent with bioceramic and the result gotten from the test shows that with an increase in Time, the Absorbance capacity of the films increases. Also because of $FeCl_3$ rich crystal chemistry and its ability to absorb large molecules in their micropores they were prepared and used for this test.

- Chitosan dissolves in acidic mediums like $FeCl_3$ but because of the presence of eggshell which has a rigid property it did not dissolve completely.

- 0.0184g of chitosan from the graph above shows a very good linear increase in absorption over time.

4. Future prospect and Conclusion

Tissue regeneration for therapeutics is very critical and target specific. To complement the functionality of living systems, the constructed tissue must successfully interact with the body's immune system. Silk based designs allow easy control on matrix morphology, degradation rate and conformal adhesion to underlying tissues with low immune-toxicity and good biocompatibility (Rangam et al., 2010)

Recent advancements in understanding silk structure and processing open up new opportunities in the use of various forms of silk in tissue regeneration. Silk systems will be particularly useful for applications where slow biodegradation and good mechanical properties are critically required, such as for bone, ligament and muco-skeletal tissues. Successful applications of silk based materials in tissue engineering depends on further understanding of the long term biocompatibility, biodegradability and its degraded products, along with the ability to tune silk morphologies for tissue specific requirements. Hybrid materials, incorporating 100% silk in different matrix morphologies show promising results in this regard. (Shi *et al.*, 2010)

Silks are fibrous proteins, which are spun by a variety of species including silkworms and spiders. Silks have common structural components and have a hierarchical structure. Silkworm silk must be degummed for biomedical applications in order to remove the immunogenic sericin coating. It may subsequently be processed into a variety of forms, often via the formation of a fibroin solution, including films, fibers and sponges, and used in combination with other materials such as gelatin and hydroxyapatite.

Silkworm silks have been reported to support attachment and proliferation of a variety of cell types. Silks have subsequently been investigated for use in tissue engineering. This project provides a general overview of silk biomaterials, discussing their processing, biocompatibility and degradation behavior and paying particular attention to their applications in tissue engineering.

The excellent cell adhesion property of the processed silk fibroin gave us an ability to produce a water resorb able bio-ceramic scaffold which could be used for bone regeneration. This regenerative property was contributed and enhanced by eggshell powder combined with it which

contains hydroxyapatite whose chemical components are much more similar to those of a natural bone.

From this, it was understood and observed that silk fibers are excellent biomaterials used for regeneration, culturing and or general replacement of defected cells and tissues because of their ability to mimic the natural biological system of animals and humans with less biocompatibility issues hence proving to be very vital in the future for bioengineering and tissue engineering as a whole.

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