DEVELOPMENT OF CHITOSAN/ALGINATE/SILVER NANOPARTICLES HYDROGEL SCAFFOLD FOR SOFT TISSUE ENGINEERING APPLICATIONS

ROSLINDA HANI BINTI RAMLI

UNIVERSITI TUN HUSSEIN ONN MALAYSIA

DEVELOPMENT OF CHITOSAN/ALGINATE/SILVER NANOPARTICLES HYDROGEL SCAFFOLD FOR SOFT TISSUE ENGINEERING APPLICATIONS

ROSLINDA HANI BINTI RAMLI

A thesis submitted in
fulfillment of the requirement for the award of the
Degree of Master of Mechanical Engineering with Honours

Faculty of Mechanical and Manufacturing Engineering
Universiti Tun Hussein Onn Malaysia

AUGUST 2017

This thesis is dedicated to

My father, Ramli B. Mohammed

My mother, Rosiyah Bt. Suratman

Husband, Muhammad Basry Bin Mahsun

Little caliph in my womb that go through this thesis writing and project with me and safely born during my thesis writing period, Muhammad Haqeem Naufal Bin Muhammad Basry

My siblings, Roslina Hanim Ramli, Rafizul Hafiz Ramli and Rafirul Hanif Ramli

My family in-laws

My Project Supervisor and Co-Supervisor, Associate Professor Dr. Anika Zafiah Mohd Rus and Associate Professor Dr. Soon Chin Fhong

And all my colleagues and friends.

LIST OF ASSOCIATED PUBLICATIONS

Conference Papers

1. Synthesis of Chitosan/Alginate/Silver Nanoparticles Hydrogel Scaffold. Ramli, R. H., Soon, C. F., & Rus, A. Z. M. (2016). Green Design and Manufacture Internationaal Conference, Phuket, 1-2 May 2016. In *MATEC Web of Conferences* (Vol. 78, p. 01031). EDP Sciences.
2. Characterisation of silver nanoparticles produced by three different methods based on Borohydride reducing agent. Ramli, R. H., Soon, C. F., & Rus, A. Z. M. (2016). Green Design and Manufacture Internationaal Conference, Phuket, 1-2 May 2016. In *MATEC Web of Conferences* (Vol. 78, p. 01032). EDP Sciences.

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah that I finally able to finish my master's study. First of all, I would like to express my sincere appreciation to my supervisor and co supervisor, Associate Prof. Dr. Anika Zafiah Mohd. Rus and Associate Prof. Dr. Soon Chin Fhong for the support given throughout the duration for this research. Without their continuous support and patience throughout this research and thesis writing, I will not be able to complete it on time.

I also would like to express my appreciation especially to MyBrain15, Universiti Tun Hussein Onn Malaysia and GIPS Vot U337 for funding me financial support during the study. Many thanks to all my colleagues in this university that always help me and provide any support throughout this research, Arina Basyirah, Nurul Hamizah, Wong and Leong. I would also like to thank to the technicians involved in this research, Madam Faezahana, Miss Isrihetty and Mr. Fazlan.

In addition, I would like to thank to Microelectronics and Nanotechnology-Shamsudin Research Centre (MiNT-SRC) and Faculty of Mechanical Engineering (FKMP) of UTHM for providing the facilities for me to conduct this research. Appreciation also goes to everyone involved directly or indirectly towards the compilation of this thesis. Last but not least, author would specially thank to beloved parents, husband, families and friends. Without their moral support, author will not have the courage to continue the study and completed the thesis.

ABSTRACT

A biodegradable scaffold in tissue engineering serves as a temporary skeleton to accommodate and stimulate new tissue growth. Alginate (Alg) and chitosan (Chi) are both popular materials applied as biomaterials or bioimplants. However, Alg derived from brown algae is highly compliant and easily decomposed in fluid, whilst Chi derived from shrimp shells has weak strength. In rectify these problems, the development of Chi and Alg based biodegradable scaffolds incorporated with silver nanoparticles (AgNPs) with enhanced mechanical properties and biosafe function is proposed. Different ratios of chitosan/alginate (Chi/Alg) were prepared and the effect of different ratio (1:1, 1:2 and 2:1) to the mechanical, biological properties with and without AgNPs and keratinocyte cell growth were investigated. The preliminary result of FTIR, UV-Vis, XRD, FESEM and EDS proved the production of silver nanoparticles. Meanwhile, FTIR, swelling/degradation, DMA, TGA, DSC, FESEM and MTT assay was conducted to study the properties of Chi/Alg based scaffold. FTIR analysis shows the crosslinking of Chi/Alg based scaffold. Swelling/degradation and DMA shows Chi/Alg and chitosan/alginate/silver nanoparticles (Chi/Alg/AgNPs) has adequate swelling and compressive modulus that exceed the epidermis' Young modulus, thus able to provide mechanical support upon application. Meanwhile, the thermal analysis revealed that the onset decomposition temperature of scaffold were at around 70 ºC which is due to the loss of water present in the scaffold thus thermally safe for soft tissue application. Based on FESEM result, there are different in surface structure of Chi/Alg based scaffold. Finally, with the incorporation of 0.3 % PVP synthesised AgNPs in Chi/Alg based scaffold, cells are able to live up to 14 days. As a result, Chi incorporation in the Alg and AgNPs improved physical, mechanical properties of hydrogel itself and provide biosafe environment during the study.

ABSTRAK

Di dalam kejuruteraan tisu, perancah atau acuan biodegradasi berfungsi sebagai struktur sementara untuk menampung dan merangsang pertumbuhan tisu baru. Antara bahan yang sering digunakan sebagai bio-bahan serta bio-implant adalah alginate (Alg) dan chitosan (Chi). Namun begitu, walaupun sifat Alg dan Chi yang kedua-duanya berasal dari bahan organik iaitu alga coklat dan kulit udang, Alg sangat mudah melarut di dalam cecair manakala Chi mempunyai kekuatan yang rendah. Maka, penggabungan Chi dan Alg serta nanopartikel perak (AgNPs) bagi membentuk perancah biodegradasi dilakukan untuk meningkatkan sifat mekanikal serta fungsi keselamatan biologi. Chitosan/alginate (Chi/Alg) dengan berbeza nisbah (1: 1, 1: 2 dan 2: 1) disediakan sama ada dengan penambahan atau tanpa penambahan AgNPs dan kesannya terhadap sifat mekanikal, biologi serta pertumbuhan sel keratinocyte telah disiasat. Keputusan awal FTIR, UV-Vis, XRD, FESEM dan EDS membuktikan penghasilan AgNPs. Manakala ujian FTIR, degradasi, DMA, TGA, DSC, FESEM dan MTT assay telah dijalankan untuk mengkaji sifat-sifat perancah biodegradasi Chi/Alg. Analisis FTIR menunjukkan Chi/Alg telah bergabung. Keputusan kadar pengembangan di dalam cecair dan DMA menunjukkan Chi/Alg dan Chi/Alg/AgNPs mempunyai kadar pengembangan yang seimbang dan modulus mampatan yang melebihi mampatan epidermis kulit dan seterusnya mampu member sokongan mekanikal sewaktu aplikasi penggunaan. Sementara itu, terma analisis mendedahkan bahawa suhu permulaan penguraian perancah berada di sekitar 70 ° C yang disebabkan oleh kehilangan air di dalam perancah, maka ia selamat digunakan. Berdasarkan keputusan FESEM, permukaan Chi/Alg juga berbeza. Tambahan lagi, penambahan 0.3 % PVP AgNPs dalam perancah Chi/Alg tidak memberikan kesan kepada sel kerana sel mampu untuk hidup sekurang-kurangya selama 14 hari. Kesimpulannya, gabungan Chi dalam Alg dan AgNPs telah meningkatkan sifat fizikal, sifat mekanik hidrogel sendiri dan menyediakan persekitaran biologi yang selamat semasa kajian.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DMA	-	Dynamic Mechanical Analysis
FTIR	-	Fourier Transform Infrared Spectroscopy
DSC	-	Differential Scanning Calorimetry
TGA	-	Thermogravimetric Analysis
AFM	-	Atomic Force Microscopy
SEM	-	Scanning Electron Microscopy
PEG	-	Polyethylene glycol
DI water	-	Deionized water
PLA	-	Poly(lactic acid)
PGA	-	Poly(glycolic acid)
PLGA	-	Poly(lactic-co-glycolic acid)
AgNPs	-	Silver nanoparticles
Chit	-	Chitosan
Alg	-	Alginate

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Research background

Every year, a large number of individuals are suffering from tissue impairment and organ malfunction due to the accident and illness (Adhikari *et al*., 2016). This impaired tissues and aged cells are restored by the self-healing ability of human body. However, the restore capacity of these mature tissues is frequently inadequate if the injury caused severe damage (Upadhyay, 2015). Hence, synthetic devices, donor organs as well as autologous transplants are utilised to substitute unfixable harmed tissues and organs. However, these procedures failed to substitute the organ failure completely. Thus, the development of alternative strategies was caused by demand for transplantation of organs and tissue exceeding the supply (Rouchi & Mahdazvi-Mazdeh, 2015). Additionally, Ministry of Health (Malaysia) communicated that, up to 31 January 2015, practically 19 353 patients require organ transplantation and the organ transplantation rate in Malaysia is the lowest in the world which is 0.68 donor for every one million people. This issue is caused by the supply which is the donor organ that unable to fulfil the demand which is the increasing number of patients (Kementerian Kesihatan Malaysia, 2017).

At regular intervals based on U.S. Department of Health & Human Services, transplant waiting list is expanding and averagely 21 patients die while waiting for the transplantation (U.S. Department of Health & Human Services, 2016). The deficiency of organs or effective organ replacement has caused thousands die every year while waiting for transplantation. Approaches such as substituting impaired organ through mechanical approach (heart lung bypass machines and dialysis) and artificial devices (joint substitution) are short-term solutions used by the clinicians.

However, these approaches do not allow the patients to completely recover from their injury and may have several limitations to conduct their habitual activity. These methods also may cause the state of being infected and refusal act of the body to the foreign device upon transplantation (Orlando *et al*., 2013). These issues need to be addressed by new development in the area of tissue engineering. Research on designing manufactured biomaterials that can supplant harmed or injured tissue for short-term and long-term substitution was conducted due to the necessity for substituting damaged tissue in human body (Park, 2011).

Biomaterial is any biocompatible material, natural or synthetically, that is used to supplant or part of an organ or its tissue, while in private contact with living tissue (Chen & Thouas, 2014). Biomaterials moreover defined as any substance (other than a prescription) or blend of substances, originated naturally or synthetically, which can be used for any time period, with clinical trials taken into account or as a part of a structure which treats, extends, or replaces any tissue (Censi, 2010). Hydrogel frameworks are one of various kinds of biomaterials that has been utilised broadly as part of tissue building applications (Park, 2011).

Due to its favorable characteristics, hydrogels have been applied widely in biomedical such as tissue engineering as well as drug delivery. Their high water content renders them great with living tissues and proteins and their viscoelasticity minimises damage to the encapsulating tissue. Hydrogels are commonly appealing in field of tissue engineering due to their mechanical properties alike to the natural tissues. The biodegradability properties of hydrogels permit the hydrogels transplantation into human body, without the need of second surgery after the hydrogel degraded in the body because the extracellular matrices by the incorporated cells after a period of time will replace the degraded hydrogel (Zhao *et al*., 2015, Chai *et al*., 2017).

In this project, chitosan/alginate based hydrogel with the inclusion of silver nanoparticles was proposed for application as a bio-scaffold in tissue engineering. Chitosan and alginate are natural occurring polymers and offer several advantages to mimic natural extracellular matrix (ECM) in the body. However, these two materials presented have certain drawbacks when used independently (Lanza, 2011; Kim, 2013; Szymanska, 2015). Silver nanoparticles were reported with antibacterial properties in past research (Thomas, 2015). When chitosan and alginate are mixed and incorporated with silver nanoparticles, it is believed that the produced hydrogel

composites scaffold will improve the mechanical properties, biocompatibility, biodegradability and cell growth during the *in-vitro* study as well provide biosafe environment for the growth of seeded human keratinocyte cell lines.

1.2 Problem statement

Alginate and chitosan has been utilised broadly as hydrogel framework either separately or by blended with different materials, for example, hyaluronidase enzyme core-5-fluorouracil-loaded chitosan/PEG/gelatine polymer nanocomposites (Rajan *et al.*, 2013), Chitosan-nanohydroxyapatite (Roy & Sailaja, 2015), Polycaprolactone-alginate (PCL-alginate) (Kim & Kim, 2015), and hydroxyapatite/chitosan-alginate (Han *et al*., 2010) composite scaffolds for applications in tissue engineering. Chitosan has been broadly utilised as a part of the composites blended with collagen, coral and hydroxyapatite to grow new frameworks for tissue designing applications, however the mix of chitosan/alginate has not been much investigated (Lanza *et al*., 2011). The major disadvantages of using chitosan alone as biopolymer are its weak physical strength (Kim, 2013), high swelling tendency (Agarwal & Murthy, 2015) and the restricted solubility of chitosan (Kim, 2013).

In overcome these problem, some chemical modification of chitosan either on $–NH_2$ groups of glucosamine units or on –OH groups of the polymer, cross-linking and the incorporation with other materials was conducted to improve its properties to mimic the structure of the tissue (Saikia *et al*., 2015; Aryaei, 2014; Jayakumar *et al.*, 2011, Rodríguez-Vázquez *et al*., 2015). However, chitosan was found as an attractive natural biopolymer as it resembles glycosaminoglycan (GAGs) structure which is the main component of extracellular matrix (ECM) and its hydrophilic nature aids in cell adhesion, proliferation as well as differentiation. Besides that, chitosan also has aggregated polymeric chains which are compact thus provide stability to the scaffold in terms of application to be used in tissue engineering (Jayakumar *et al.*, 2011). In contrast, despite of being non-immunogenic, biocompatible and gentle gelling properties of alginate, alginate will be exposed to high degradability when exposed to fluid. The loss of divalent cations from alginate to the surrounding medium is uncontrollable, thus it caused hydrogel with unpredictable degradability ability, limited stability for long term effect as well as limited strength and toughness that

mainly depends on alginate's chemical structure (G content effect on stiffness) (Lee & Mooney, 2012; Sun & Tan, 2013).

In this research study, the development of chitosan/alginate hydrogel biocomposites for soft tissue engineering application mainly to improve the mechanical properties of the produced hydrogel itself will be conducted. This biocomposite with properties that complements each other is expected to improve mechanical and biophysical properties of the hydrogels. Silver nanoparticle is a non-toxic material and highly effective for antimicrobial activities. An addition of silver nanoparticles to the chitosan/alginate composites would create a compatible environment for the growth of seeded human keratinocyte cell lines.

1.3 Hypothesis of the research

The development of chitosan based hydrogel as natural based scaffold shows tremendous growth in the tissue engineering field. The disadvantage of chitosan in tissue engineering scaffolds is its limited solubility (Kim, 2013), stability and weak mechanical properties with range of 2.5 kPa modulus at 0.4 wt % of chitosan hydrogels (Mushi, 2014). Chitosan is an attractive natural biopolymer as it resembles GAGs of ECM that enhance the cell adhesion, proliferation and also differentiation to be used in tissue engineering application. However, chitosan has weak mechanical properties, limited solubility and stability (Kim, 2013). In contrast, even though alginate has simple gelling ability, biocompatible and non-immunogenic properties, alginate's drawback such as unpredictable degradability due to loss divalent cations and mechanical properties that depends on chemical structure of alginate has brought the effort to incorporate alginate with other materials to overcome its drawback (Lee & Mooney, 2012; Sun & Tan, 2013). Thus, by incorporating these two materials, chitosan/alginate hydrogel were believed to enhance the biocompatibility, solubility, and mechanical properties of the biocomposite to be used in soft tissue engineering application. Silver nanoparticles further added to improve the properties of hydrogel while ensuring the hydrogel is biosafe to be applied as implants later.

1.4 Aim of research

The aim of this research is to develop renewable source for damaged tissue with biocompatible, biodegradable and tunable mechanical properties. In this work, hydrogel scaffolds by using natural (chitosan & alginate) polymer derivatives with incorporation of silver nanoparticles were synthesised and characterised for tissue engineering applications.

1.5 Research objective

i. To synthesise silver nanoparticles (AgNPs).
ii. To synthesise chitosan/alginate hydrogel scaffolds with incorporation of silver nanoparticles.
iii. To characterise physical and mechanical properties of chitosan/alginate hydrogel with/without inclusion of silver nanoparticles.
iv. To investigate the effect of an addition of silver nanoparticle in chitosan/ alginate with the seedings of human keratinocyte cell lines (HaCaT).

1.6 Scope of research

This research focused on developing hydrogel scaffold by mixing different biopolymers with different ratio and examining the mechanical and biophysical properties of the chitosan/alginate scaffold with an inclusion of silver nanoparticles. The silver nanoparticles also synthesised based on previous research (Malina *et al*., 2012) and applied in this work. Functional groups of the scaffold were studied using Fourier Transform Infrared Spectroscopy (FTIR). While mechanical properties of materials were measured by Dynamic Mechanical Analyses (DMA). Thermal stability and decomposition of the hydrogel were conducted by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) respectively. Molecular surface structures were investigated by using Field Emission Scanning Electron Microscopy (FESEM). Apart from that, cell viability was studied by using MTT assay analysis. Swelling and degradation of the produced hydrogel were also being investigated.

1.7 Thesis contribution

In this dissertation, biosafe chitosan/alginate composites with the incorporation of silver nanoparticles finally produced. The characterisation of chitosan/alginate and chitosan/alginate/silver nanoparticles by different methods has been investigated and the main contribution of this research is by investigating the effect of addition of silver nanoparticles into chitosan/alginate based biocomposites to the human skin cells seeding as well as the study of its mechanical properties that has not been explored before. This result can be further used for other applications of tissue engineering.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Chapter 2 presented the literature studies on tissue engineering, especially in soft tissue engineering (Section 2.2). In this sub section, the literature studies presented the history of tissue engineering, the approach and the materials that were incorporated in the area. The structure of skin and human keratinocytes were discussed in Section 2.3. Section 2.4 presented the physical and chemical properties of hydrogels and natural extracellular matrix (ECM) while Section 2.5 and 2.6 discussed on the natural biomaterials and synthetic biopolymers respectively. Section 2.7 meanwhile elaborated the biomaterials used in tissue engineering which chitosan (Section 2.7.1) and alginate (Section 2.7.2). Next, silver nanoparticles and its mechanism on antibacterial were discussed on Section 2.8. The mechanical and physical characterisation techniques such as thermal analysis, chemical analysis, mechanical analysis and electron microscopy were discussed in Section 2.9. And finally the summary of literature review is discussed on Section 2.10.

2.2 Tissue engineering

Currently, a large number of individuals are waiting for donor tissues because of mishaps or different illnesses. A variety of surgical techniques combined with the therapeutic medications have been developed to treat these individuals. The treatments incorporated includes the transplantation of patients or donors' organs or tissues such as liver, support devices utilized for instance kidney dialysis as well as the simulated prosthesis implantation for hip joints (Bhat & Kumar, 2012). Even though these treatments were beneficial in saving countless lives as well as improve millions lives, several challenges were associated to these. Several difficulties such as donor site morbidity likely to occur by patient's healthy tissue were transplanted to damaged tissue of the body such as face reconstruction from the rib. Allograft is the transplantation from one individual to the patient's body, while xenograft is the transplantation from animal to the patient's body. However, both is extremely limited by the shortage of donor, transplantation immunological reaction and infectious disease transmission. The long haul implantation of non-degradable engineered materials into the body can prompt irritation around the inserts and possible mechanical disappointment of the materials, and ended up with requiring re-surgery (Varkey *et al*., 2015).

The term tissue engineering was presented in 1987 amid a meeting of the National Science Foundation. It is the utilisation of standards and techniques of life sciences and engineering toward the major comprehension of structure-capacity connections in ordinary and neurotic mammalian tissue, and the advancement of organic substitutes to re-establish, keep up, or enhance tissue capacity (O'brien, 2010). The term of tissue engineering best defined by the principle integration approach of engineering and biology to generate renewable source of transplantable tissues outside the body with the main objective is to gain knowledge to better manage tissue repair within the body (Enderle & Bronzino, 2012).

The latest and developed way to deal with supplant the structure and elements of lost tissues, while going around the intricacies of current treatments, is to re-develop or build these structures utilising blend of materials, bioactive atoms, and cells. This tissue building idea is arranged into three methodologies; conduction, acceptance and cell transplantation (Garg, 2010).

In conduction approaches, biodegradable materials are surgically embedded at the site of the harmed tissues to make a space into which cells in the neighboring tissue can relocate. While the materials will degrade, these cells can then recover the tissue. Several of bioactive molecules were sent to damage tissue site to stimulate target cells and enhance the cells regeneration (induction phase). Cells' long term stimulation in the neighbouring tissue was provided by the molecules that released out of a material carrier in a sustained manner. Finally, in place of bioactive molecules, tissue-specific cells or stem cells may be transplanted to the desired site, using an appropriate vehicle to grow the desired tissue (cell transplantation) (Steinhoff, 2016).

Figure 2.1 depicts the details of tissue engineering approach. Firstly, it involved the transplanted cells that are harvested from either patient or donor tissue biopsy and then the cells was expanded in vitro (artificial environment outside a living organism) in a desired number (El-Sherbiny *et al*., 2013). Then the cells are incorporated into three-dimensional polymeric network scaffold that is difference of origin however mimic the function of natural extracellular matrices as found in the tissues. After that, these polymeric scaffolds was responsible in the delivery of the cells to the desired site in the patient's body and as a result give space to regenerative tissue, and possibly control the structure and capacity of the built tissue (El-Sherbiny *et al*., 2013). The desired tissue or organ structure can be created by means of the expansion and separation of the transplanted cells and collaborating host cells. Acceptance and cell transplantation approaches have been generally used in reproducing different tissues, including the artery, skin, ligament, bone, bladder, liver, tendon, nerve, digestive tract, heart valve and tendon (Dumitriu, 2004).

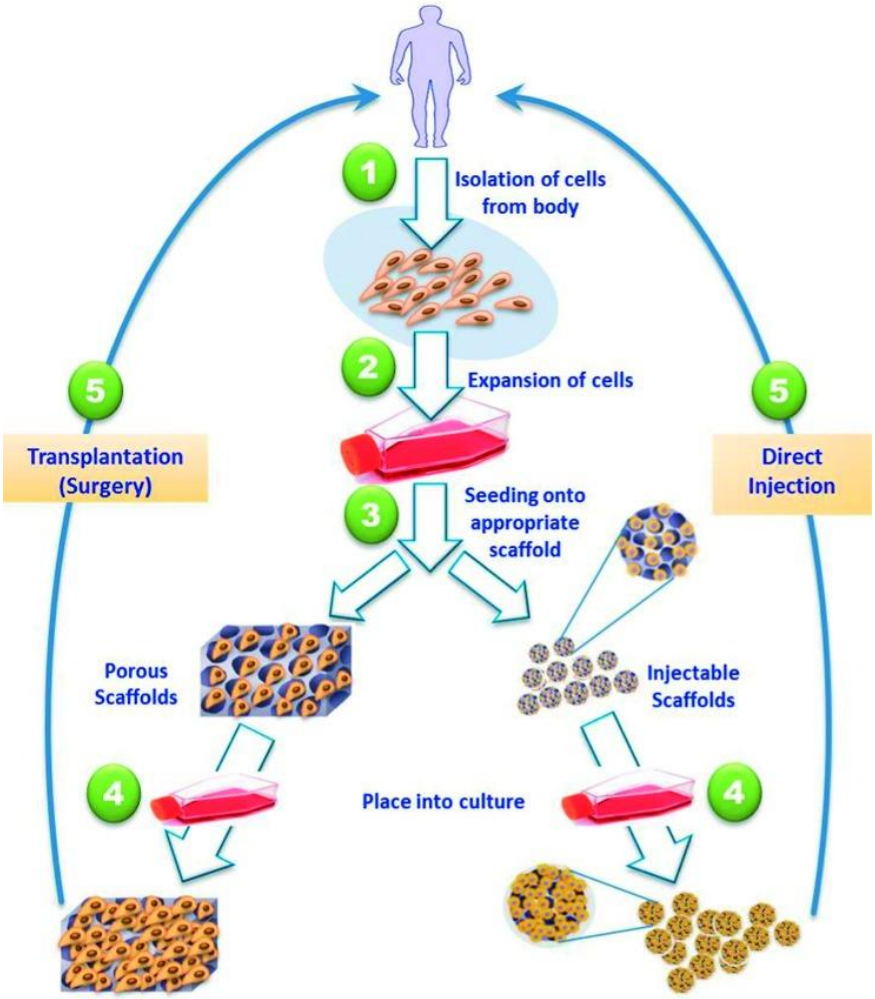

Figure 2.1: A schematic diagram for tissue engineering approaches (El-Sherbiny *et al*., 2013)

Three dimensional polymeric scaffolds are vital in determining the achievement and success in the approach of tissue engineering. The material used as scaffold in the tissue engineering ought to execute in cells and bioactive molecules transportation to the desired site in the patient's body, function in the cell's regulation by being a synthetic extracellular matrix (ECM) and as a template that enhance tissue growth. For this reason, different types of natural and synthetic biodegradable materials has been utilised and developed (Dhandayuthapani *et al.*, 2011). The material selection for the construction of polymeric scaffolds that evoke desired as well as specific biological response includes the profile of degradation, mechanical properties, ease of processing and biochemical response (Rodríguez-Vázquez *et al*., 2015). Nonwoven lattice or fibrils, hydrogels and porous scaffolds were variety of physical structures which are the processed product of materials that

has been utilised in tissue engineering. Prior to the implantation stage, the isolated cells from patient's body are expanded and seeded on the porous scaffold or nonwoven fibrils. While for hydrogel form, polymer solutions often mixed with the cells for gelling purposes then followed with infusion into the body by injectable hydrogel method (El-Sherbiny *et al*., 2013). Polymeric frameworks are not just physical layouts, rather they are intended to effectively control cell growth and advance tissue development by specific cell recognition sites presentation that convey the cells to the desired site in the body (Burdick & Mauck, 2010).

2.3 Skin and human keratinocytes

The cell is the fundamental unit of life. Microorganisms, for example, microscopic organisms, yeast, and amoebae exist as single cells. By complexity, the grown-up human is comprised of around 30 trillion cells (1 trillion = 1012) which are for the most part sorted out into aggregates called tissues (Bolsover *et al.,* 2011). The main individual to watch and record cells was Robert Hooke (1635-1703) who discovered the cells (open spaces) of plant tissues (Bolsover *et al*., 2011).

Skin is the largest organ of the body, with surface area of 1.8 m^2 and making up 16 % of body weight. The main functions of the skins are as environment physical barrier, control the inward and outward passage of water and other substances, and protect the skin from micro-organisms, ultraviolet radiation, toxic substances and mechanical damage. Epidermis, dermis and subcutis are three structural layers that developed the skin structure. Epidermis which is the skin's external layer play an important role as physical and chemical barrier, inward and outward between the body and environment. Skin's structural was supported by the deeper layer that is called the dermis and the important fat embedded in the loose connective tissue layer, which is subcutis or also called as hypodermis (Dai, 2017).

The significant function of skin is also to act as a boundary to the outside environment. Skin shields the body from erosion and mechanical effects while keeping unsafe chemicals, microscopic organisms, infections, and bright (UV) radiation from entering the body. It likewise avoids water misfortune and directs body temperature by blood stream and the vanishing of sweat.

The skin is made out of various layers: the epidermis, and dermis. The epidermis, the

furthest layer of the skin, is between around 0.1 mm and 0.3 mm thick. The stratum corneum (horny cell layer) frames the peripheral layer of the epidermis and extents in thickness from 0.01 mm to 0.03mm. The dermis is between 1.0 mm and 3.0mm thick (Walters & Roberts, 2002).

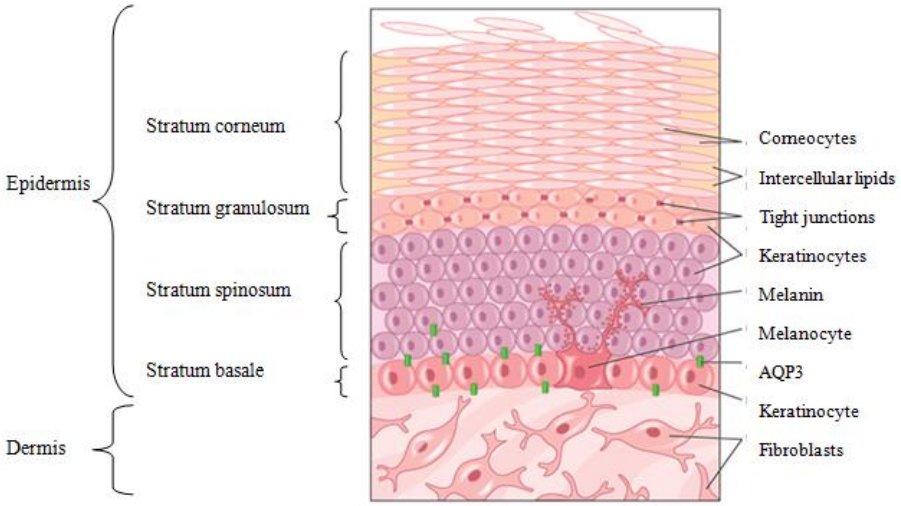

Figure 2.2: Structure of epidermis (Brandl & Beutler, 2013)

As shown in Figure 2.2, the epidermis is divided into four sublayers. From the base (deepest), these sublayers are the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Dai, 2017).

Epidermal cells (keratinocytes) created in the stratum basal and this keratinocytes will move upward to the external surface. During this process, keratinocytes' structures and physiological capacities will be changed. One cycle takes around 28 days. On the other hand, the function of to prevent toxic and harmful substance entrance and to prevent body dehydration are the role play of stratum corneum as skin's barrier function. In the stratum granulosm, there is structure that is called as tight junctions and these structures play similar role as skin's barrier function as in stratum corneum. Meanwhile, intercellular lipids surround the stratum corneum and plays function in maintaining moisture of the skin (Dai, 2017).

Cornified layer surface that was covered by the intercellular lipids also act as protective film. Melanocytes that are located on the epidermis' basal layar produced dark skin pigment called as melanin and diffuse to the skin surface. At the point when skin is presented to UV radiation, melanocytes begin to create melanin to shield the skin cells from UV harm (Dai, 2017). AQP3 is an aquaglyceroporin,

capacity in transporting both water and glycerol at moderate rates (K. Brandl & Beutler, 2013).

Dermis is the second layer of skin, underneath the epidermis. The dermis is chiefly comprised of extracellular grid (ECM), an extracellular piece of human tissue that is to a great extent made out of collagen, hyaluronic acid and elastin. The dermal ECM is delivered for the most part by dermal fibroblasts. The tension, strength and elasticity were provided by tough network of collagen filaments structure. Meanwhile, the hyaluronic acid is effective to maintain the moisture and can hold several times its weight in water (Walters & Roberts, 2002).

2.4 Hydrogel

Hydrogels have been utilised to repair and help recovery of an assortment of tissues, for example, vasculatre, bone and ligament (Zhu, 2010). Prior to its similarities on function and structure to natural extracellular matrices (ECM), hydrogels has become an attractive structure to be utilised in tissue engineering scaffolds (Burdick & Mauck, 2010). Hydrogels is consisted of highly swollen three-dimensional (3D) interconnected systems with dimensions of macroscopic. The presence of crosslinks in the hydrogels made it insoluble hydrophilic building blocks and through its elastic networks, it permits dispersion of nutrients and cellular waste (Burdick & Mauck, 2010; Zhu, 2010).

Hydrogel permits the cells to adhere, proliferate as well as differentiate onto the hydrogels due to its property that able to absorb water up to 10-20 % and thousand times of their dry weight (Yahia *et al*., 2015). The hydrophilic groups presence for instance –OH, –CONH– and –COOH allow its water absorbance property. The properties such as mechanical properties, biocompatibility, surface morphology, and porosity are influenced by hydrogels water content (Singh *et al*., 2011). The vital properties that hydrogels need to fulfil to be utilised as tissue engineering scaffold are the hydrogels need to be biodegradable and biocompatible, have high porosity as well as interconnected pore structure thus permits the diffusion and dispersion of metabolites and nutrients, during implantation, hydrogel also need to have adequate mechanical properties to allow any surgical handling, able providing mechanical support, and able to transmit external forces from the

environment to the tissues effectively over a drawn out timeframe as well as promote targeted cell interactions or capacities by temporal and spatial fashion of biological cues (Burdick & Mauck, 2010; O'brien, 2010). Previous work reported also emphasizes the importance of scaffold properties such as biocompatibility (Thi Hiep *et al*., 2017), porous structure and size (Maji *et al*., 2016; McHugh *et al*., 2013) to be applied in tissue engineering.

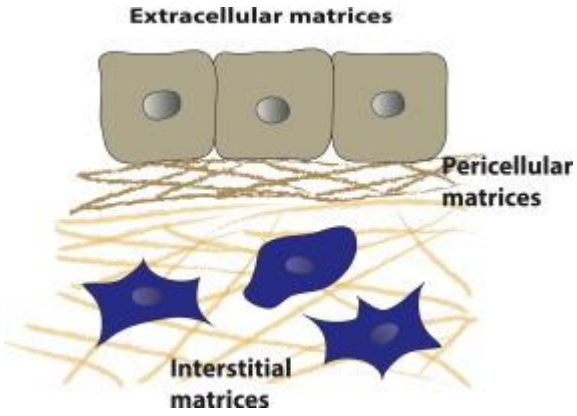

Figure 2.3: The matrices in the extracellular matrices (ECM) (Theocharis *et al*., 2016)

Extracellular matrix (ECM) is a non cellular components, three-dimensional macromolecular network that is composed of glycosaminoglycans (GAGs), collagens, laminins, fibronectin, elastin and other glycoproteins (Theocharis *et al*., 2016). Cells in the matrix mostly produced macromolecules that further formed extracellular matrices (ECM). Matrix arrangement as wells external matrix orientation can be controlled by the cystoskeleton orientation inside the cells (Yue, 2014). Based on the structure and composition as shown in Figure 2.3, ECM is classified into two main type which is called as interstitial and pericellular matrices. Cells are surrounded by the interstitial matrices while cells have an intimate contact with the pericellular matrices. The capability of each components in ECM such as collagens, GAGs, laminin, elastin, proteoglycans (PGs), fibronectin (FN) and matricellular proteins that consist of various interacting sites with different specificities to bind with each other enable the formation of three-dimensional network that associated with resident cells. Besides, the trigger of various biological activities and signals are provided by ECM to the cells is essential behavior for tissue homeostasis and normal organ development (Theocharis *et al*., 2016).

In addition, hydrogels nature can be classified into two different groups which are natural and synthetic hydrogels. Meanwhile, based on mechanism of cross-linking of hydrogel, hydrogels can be classified into two different groups which is physical and chemical hydrogels as shown in Figure 2.4 and Table 2.1. The structure of physical hydrogel involved the entanglement and secondary forces only however for chemical hydrogel, cross-linker hold and tighten the entanglement and secondary forces. Hydrogel's network interacted by the transient junctions such as ionic bond, hydrogen bond and van der waals interaction in physical hydrogel thus it is naturally safe but mechanically weak and these network may undergo changes in the external environment. On the other hand, permanent junction such as covalent bonding or the existence of crosslinker hold the hydrogel's network in chemical hydrogel thus make it unsafe with mechanically strong (Hiemstra, 2007; Schwartz, 2008).

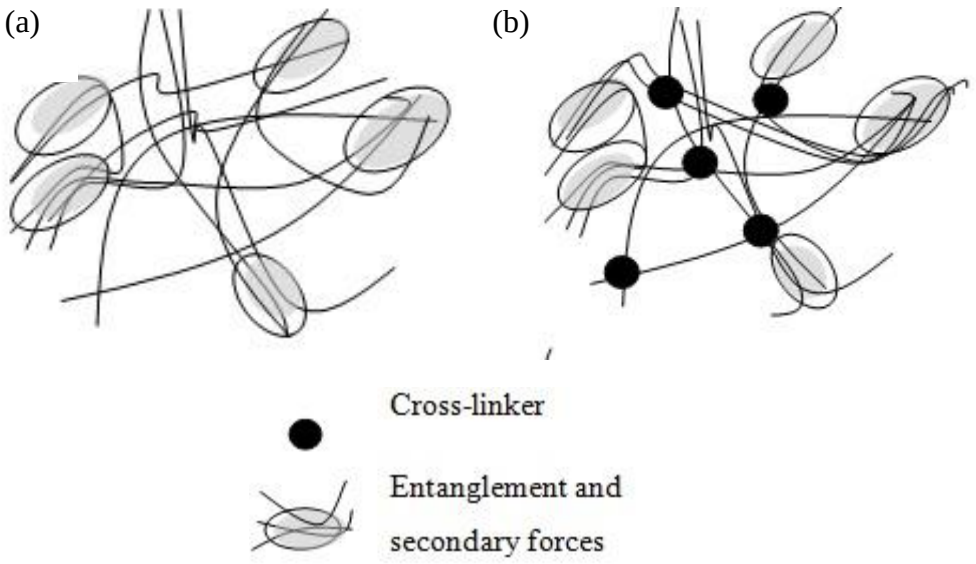

Figure 2.4: The structure of (a) physical and (b) chemical hydrogel

Table 2.1: Classification of hydrogel

Specification	Physical hydrogel	Chemical hydrogel
The interaction of bonding in the hydrogel	• Transient junctions-polymer chain entanglements, ionic bond, hydrogen bond, van der waals interaction, hydrophobic interaction (Hiemstra, 2007; Schwartz, 2008)	• Permanent junction-covalent bonding between hydrophilic macromolecules crosslinking (Hiemstra, 2007; Schwartz, 2008) • Achieve this cross-linking using glutaraldehyde, genipin and ultraviolet irradiation have also been employed (Hiemstra, 2007; Schwartz, 2008)
The safety of hydrogel in tissue engineering applications	• Safe- Proceeds under mild conditions, thus allowing immobilization of proteins (Hiemstra, 2007; Schwartz, 2008)	• Unsafe- safety concern due to involve usage of toxic chemical cross-linker (Hiemstra, 2007; Schwartz, 2008)
The stability of hydrogel	• Mechanically weak, changes in external environment (i.e. pH, temperature, ionic strength) give rise to disruption of hydrogel network (Hiemstra, 2007; Schwartz, 2008)	• Mechanically strong hydrogel network • Ability to swell in water while maintaining that network

Several advantages properties for instance biodegradability, biocompatibility, non-toxic and so on can be offered by natural polymers derived hydrogels. It includes polysaccharides natural polymer such as agarose, alginate, chitosan and proteins such as gelatin and collagen. While chemical polymerisation is utilised to form synthetic polymer based hydrogels and its functionality and degradability is able to be tailored and modified to suit the requirements to be used in the various applications (Hiemstra, 2007; Hu, 2011).

2.5 Natural biopolymers in human physiological system

Non-indispensable prosthetic devices such as wooden legs and metallic dentures can be considered as early endeavours of biomaterials utilisation in reconstructive

medicine (Meyer *et al*., 2009). Due to its various point of interest for instance the biocompatibility, controllable mechanical properties as well as biodegradation and ease of processing enable wide utilisation of biopolymers as most common scaffold material. Biopolymers can be classified as natural or synthetic based on its origin (Cao, 2011).

Natural biomaterials consist of polymers derived from natural sources. Collagen is the most widely recognised characteristic biomaterial. Other types of naturally derived biopolymers are such polysaccharides (alginate, chitosan, chitin, and hyaluronic acid, and so on), fibrin, laminin and fibronectin. Their utilisation is appealing in tissue designing, since they can be redesigned *in vivo*. Moreover, the relative preservation of extracellular lattice proteins in various phylogeny bunches permits embedded characteristic biomaterials to react to and incorporate with cells by means of held bioactive flagging particles (Vishwakarma A. *et al*., 2014).

Polypeptides and polysaccharides are two major classes of natural biopolymers that mostly used as scaffolds (Zhu & Marchant, 2011). The attractive properties of natural biopolymers to be used as scaffolds are its biocompatible, enzymatic biodegradable as well as consisted of bio-functional molecules that aid and promote cells to adhere, proliferate and differentiate (Dhandayuthapani *et al*., 2011). However, natural biopolymers also have its disadvantages. The function of natural biopolymers may be inhibited by its enzymatic degradation properties that depending upon the application. In addition, the degradation rates of natural biopolymers are also uncontrollable as the enzymatic activity between hosts is varied thus caused difficulties for lifespan determination of natural biopolymers in vivo. Moreover, low mechanical strength of natural biopolymers also one of its drawback, but its structural stability can be enhanced by cross-linking (Fisher *et al*., 2007).

The major constituents present in natural tissues are protein or amino acids and it is also has ability of natural degradation. This type of protein-based materials commonly utilised in suturing applications as scaffold material, drug delivery applications and so on. The examples of protein based natural polymers include collagen, gelatin, elastin, silk, albumin, fibrin and keratin while polysaccharide based natural polymers include chitosan and alginate (Kumbar *et al*., 2014).

2.6 Synthetic biopolymers for application in tissue engineering

The attractive properties of synthetic biomaterials have caused the increasing use of synthetic biomaterials in tissue engineering for biomedical applications. Its mechanical as well as chemical properties are more uniform and more predictable and controllable. Besides, synthetic polymer also immunogenicity free and the properties or structure can be designed to fulfill specific purposes and functions. The properties of synthetic polymers can affect the behaviors itself. Properties such as the temperature of melting and glass temperature, molecular weight, crystallinity, side groups and also its behaviors are determined by the selection of different monomers, initiators and reaction conditions, as well as the presence of additives (Kumbar *et al*., 2014).

Polyglycolic acid (PGA), polylactic acid (PLA) and its copolymers such as poly($_{D,L}$-lacgtide-co-glycolide) (PLGA) have the ability to degrade over time, thus it was reported that its widely utilisation as a scaffold for adipose tissue engineering (Gentile et al., 2014). The degradation properties of polyglycolic acid, polylactic acid and its copolymer was caused by changing of monomer's molar mass and molar ratios as well as ester bonds bulk hydrolytic degradation (Gentile *et al*., 2014; Kumbar *et al*., 2014). Besides that, some of synthetic polymers that used as scaffolds in tissue engineering field includes poly(propylene fumarate) (Trachtenberg *et al*., 2017), poly(anhydrides), polyorthoesters, poly(trimethylene carbonate), poly(phosphazenes), polyurethane, polyhydroxyethylmethacrylate and polyurethane amide (Patel *et al*., 2011; Guo & Ma, 2014). US Food and Drug Administration (FDA) has approved these synthetic biopolymers for human clinical use while for many years, it has been utilised for surgical sutures (Zhang, 2016).

Prior to its biodegradability, biocompatibility, physiochemical properties that are well-defined (molecular weight), low possibility to transfer diseases, tailorable mechanical properties and ease of processing, synthetic polymers is an important material to be used in regenerative medicine. The advantages of synthetic polymers are the cheaper price, manufactured in large quantities uniformly, longer time-shelf and also comparable and controllable physiochemical as well as mechanical properties (degradation rate, elastic modulus and tensile strength) as compared to the biological scaffold (Dhandayuthapani *et al*., 2011). The drawbacks related to the synthetic biopolymers are its degradation by-products that may cause undesired body

response, hydrophobic, and to modulate the behavior of cells, synthetic polymers' characteristic which is bio-inert surface, lack of recognizable cell and physiological parameters. Synthetic polymers show limited biological properties as compared to the natural polymers. In addition, the presence of chemical cross-linker or initiator has caused the presence of impurities that may affect the behavior of cells or may cause inflammation in vivo (Sethuraman *et al*., 2016).

2.7 Biopolymers for application in tissue engineering

Biopolymers that are also known as bio macromolecules are produced either by plants, animals or microorganism. Due to its attractive properties, biopolymers have been utilised widely in food and textile industries, biosensors, medical, water treatment, drug delivery and tissue engineering. The requirements of biopolymers to be used in tissue engineering is the ability to allow the regulation of cell proliferation, deliver the signal for differentiation of cells as to substitute the function of natural extracellular matrix (ECM). Besides being a biocompatible, biodegradable, biopolymers also need to mimic the mechanical, biochemical and structural of natural ECM (Thomas *et al*., 2013).

2.7.1. Chitosan

In this research work, natural biopolymers of chitosan and alginate were used as a mixture to improve the properties of produced scaffold itself to be used in tissue engineering. By using an alkaline solution, chitosan is produced through the deacetylation of shellfish-derived chitin (Alasalvar *et al*., 2011). Rouget first discovered on 1859 that chitosan solubility in organic acid when he boiled chitin in potassium hydroxide. Chitosan name was attributed by Hopper Seyler on 1894. Next, the discovery of chitosan structure was resolved on 1950 as shown in Figure 2.5 (Habibi & Lucia, 2012). β(1-4) linked 2-acetamido-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose units composing the chitosan structure. Deacetylation degree of chitosan mainly determines its crystallinity and chitosan is a semi-crystalline polymer (Wong, 2013). The major advantage of chitosan is its structure that is similar to element presence in natural extracellular matrix (ECM) which is

glycosaminoglycans (GAGs). Thus, chitosan will functioned and share similar activity as natural ECM (Tan & Marra, 2010).

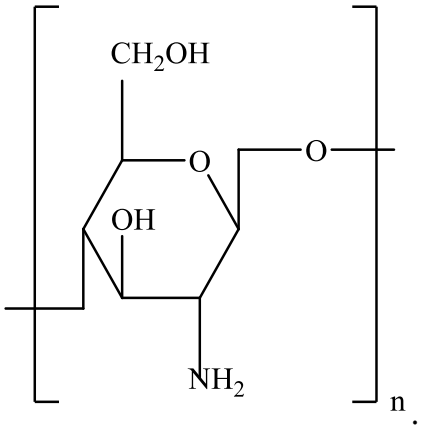

Figure 2.5: Chemical structure of chitosan (Kyzas *et al*., 2013)

Chitosan was found to be soluble in the aquous acids (pH < 6.3) however it is insoluble neither in organic solvents nor water. In solution, chitosan exhibited high charge density that is caused by free amine groups' protonation on the backbone chain. The antibacterial activity and the interactions with negatively charged cells are caused by the high charge density as well as its nature of cationic in chitosan structure. Chitosan seems to be appealing material to be used in tissue engineering due to its biocompatibility and biodegradability (Badawy & Rabea, 2011). Moreover, there is natural enzyme in vivo, which is called lysozyme and chitosan has the ability to degrade by this enzyme. The acetyl residue amount can affect the rate of biodegradation of chitosan (Costa-Pinto *et al*., 2014). Furthermore, variety of mechanical as well as physical properties of chitosan can be tailored by the chemical modification on the active amino groups of chitosan (Badawy & Rabea, 2011). As a result, chitosan is important and attractive material to be utilised as a scaffold in the tissue engineering due to its biodegradability, pH dependent solubility, ease of processing as well as biocompatibility (Ebnesajjad, 2012). However, even though various developed techniques have been conducted, its limited solubility has also caused several challenges and problems to be used in tissue engineering (Burdick & Mauck, 2010).

Based on previous work done (Zhao *et al*., 2011), the technique of solid-liquid phase transition was used to fabricate the chitosan acetate nano-fibers. The

chitosan acetate structure found to be affected by the phase separation temperature. Nano-fibrous structure of chitosan acetate was formed by lower quenching temperature and chitosan concentration found to affect chitosan acetate structure more than acetic acid concentration (Zhao *et al.,* 2011).

The production of artificial skin scaffold was produced by chitosan with a porous sponge structure. The solutions of citric and acetic acid were used to produce the chitosan scaffold. Acetic and citric acid was used to dissolve chitosan powder and it is froze at -27°C for one day and lyophilized for 3 days to obtain microporous films of the scaffolds. The result shows the fibrous network pore and pore surrounded by cell walls was produced by chitosan/citrate scaffold and chitosan/acetate scaffold respectively. For outer skin regeneration, it is found that both were effective. However, chitosan/citrate provides better facilitation in wound healing (Tanigawa *et al*., 2008).

2.7.2 Alginate

On the other hand, alginate which is one important material in this research is capsular polysaccharides in some bacteria soil, also found primarily as marine brown seaweed structural component and it is also naturally derived biopolymers. (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers is main component that composing the alginate structure. The structure of alginate involved the arrangement of M and G units which whether it is repeating (-M-M- or -G-G-) or alternating (-M-G-) blocks. The sources from which alginate is isolated will determines the amount and distribution of each unit. M and G units strongly influence the alginate and alginate-based scaffold properties which is swelling, transmittance and viscoelasticity. Salt formations such as sodium alginate (as shown in Figure 2.6) are due to the capability of carboxylic groups in its structure, in which sodium monovalent ions ionically attached to the carboxylic groups (Cao, 2011).

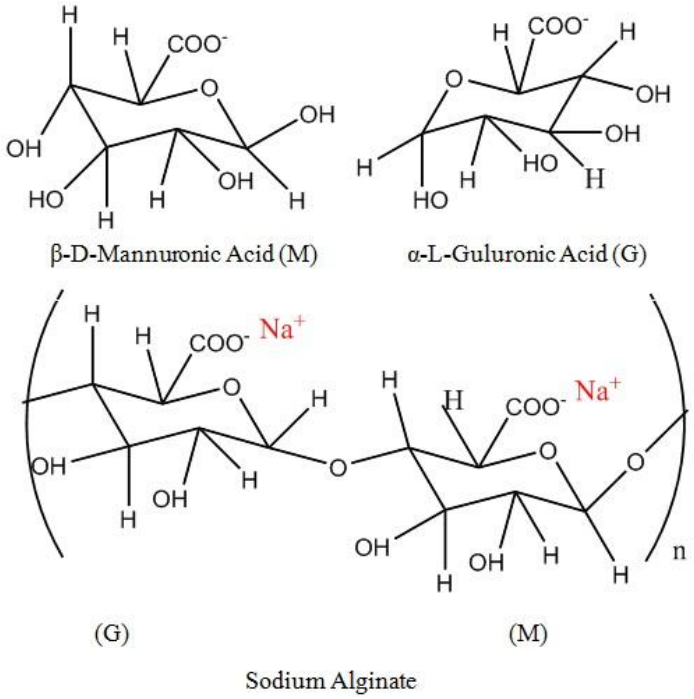

Figure 2.6: Schematic diagram of β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers, and a -(G-M) -structure sodium alginate (Cao, 2011)

The ability of alginate to form easy gelling is due to divalent cations (Ca^{2+}) presence that enables the interactions of these cations with the carboxyl groups on backbone of the polymer. Monovalent ions exchanged in the surrounding solution during the calcium ions crosslinking have caused the varied mechanical properties of ionic crosslinked alginate hydrogels in vitro over time (Cao, 2011). Hydrogels can maintain its form since excessive swelling and subsequent shrinking do not occurred during calcium cross-linking. Different shape of hydrogels can be manufactured since cross-linking can be carried out with absence of organic solvents, at low temperature and at very mild conditions (Dumitriu & Popa, 2013).

Alginate is attractive biopolymers which is high biodegradability, biocompatibility, non toxic and immunologically inert hydrogel. Due to its ease gelation, alginate is suitable for incorporation with biomacromolecules and living cells, therefore pharmaceutical applications such as dental impression materials, wound dressings, *in vitro* cell culture and tissue engineering applications are the examples of utilisation of alginate scaffold. Alginate also widely used in drug delivery applications, for instance pancreatic islet cells microencapsulation,

encapsulation techniques for cell matrix support and large scale manufacturing of alginate-based bioreactors for biological product (Cao, 2011).

Due to its compatibility with various substances, alginate can be prepared in either neutral or charged form. Two types of gel which is acid or ionotropic gel, which is affected by the media pH, the drug release activation will be provided by the physicochemical properties and the swelling ability of alginate itself. The physical thickness, rate of drug release, gel form type is influenced by the G units proportion and alginate concentration. Minimal invasive surgical procedures cell transplantation can be accomplished by *in-situ* gelation of alginate-cell suspensions (Cao, 2011).

However, alginate also has some drawbacks in its properties that limit its applications in tissue engineering (Cao, 2011). The subsequent dissolution of the gel as wells as loss of divalent ions caused the uncontrollable degradation kinetics of alginate. The degradation of alginate can be controlled by a few approaches such as isolation of polyguluronate blocks with molecular mass of 60 KDa from alginate chain, partially oxidation, and covalent crosslinking with adipic dihydrazide. Besides, the degradation and mechanical behavior of alginate also can be controlled by the gelation and cross-linking density. In addition, cell culture growth also inhibited by the use of a high calcium concentration to crosslink alginate (Cao, 2011).

Numerous applications in soft and hard tissue engineering include, cartilage, skin, liver, heart, ovarian follicle development, nerve, pancreas and bone are the applications of alginate gels, microcapsules, and porous scaffolds. Cell adhesion peptide sequence can be modified since the native alginate does not support significant cell attachment (Burdick & Mauck, 2010). It was proven that after alginate has been modified, it enabled and enhanced cell adhesion, proliferation and differentiation (Cao, 2011). Moreover, to further enhance the performance biologically and mechanical behavior of alginate scaffold, the blend of alginate with other materials also was investigated. Bone repair and drug release for instance are the applications of tissue engineering for alginate/chitosan scaffold (Cao, 2011).

Due to the drawbacks of chitosan to be used alone such as low strength, chitosan also has been used in the incorporation of various materials either natural or synthetic to improve its strength. For instance, the mixture of chitosan, carboxymethy chitosan (CMC) with magnesium gluconate (MgG) to form porous

scaffold was conducted with freezing-induced phase separation and following with lyophilisation step. The result showed that the scaffold produced improved mechanical strength and also showed that cells (osteoblast and fibroblast) are viable upon seeded on the scaffold up to 3 days incubation (Adhikari *et al*., 2016). In another study, the incorporation of copper with chitosan was conducted to form copper/chitosan scaffold and it was used to study the bone regeneration. The result showed that the copper/chitosan scaffold is able to enhance the bone regeneration process with feasibility and safety to be used for bone tissue engineering (D'Mello *et al*., 2015). Besides, the usage of chitosan to produce polycaprolactone (PCL)/chitosan and nanohydroxyapatite in the nHA/PCL/chitosan scaffold by freeze drying technique were also studied towards fibroblast cells. Other than having antibacterial properties, produced scaffold also found to be non toxic and further enhance the fibroblast cells proliferation in vitro up to 3 days incubation (Jin *et al*., 2015).

Chitosan and alginate biodegradable scaffolds have improved mechanical and biological properties as compared to its chitosan counterpart and the polymer blend shows high degree of tissue compatibility based on in vivo study. The attachment and proliferation of osteoblasts cells on chitosan alginate scaffold in a short time are one of the researches that involved chitosan alginate scaffold with cell culture (Baysal *et al*., 2013). Besides, chitosan alginate also has been used in the research for drug delivery applications. As an excellent therapeutic effects on wide spectrum of cancers, the incorporation of medical clay, Cloisite 30B with biodegradable chitosan alginate nanocomposites by using curcumin as a prototype drug has caused it to be commercial success as the best seller among various anticancer agents (Malesu *et al*., 2011).

Silver nanoparticles exhibited antibacterial properties due to there are high surface area are available, thus there are more nanoparticles that are able to incorporate inside the bacteria and promoting its efficacy in a sustained manner. In another study, due to the antibacterial properties of silver nanoparticles and chitosan, chitosan-silver/hydroxyapatite a composite coating on titanium dioxide (TiO2) nanotubes was developed. This biocomposite exhibited uniform porous structure thus will enhance the adhesion of cells (Yan *et al*., 2015). The chitosan/silver nanoparticles hydrogel composite also was produced for the application of wound healing applications. The produced composite was found with excellent antibacterial

REFERENCES

Abdelmonem, A. M. (2014). *Nanoparticles: Synthesis, Surface Modification and Functionalization for Biological and Environmental Applications.* Philipps-Universität Marburg University: Ph.D. Thesis.

Adhikari, U., Rijal, N. P., Khanal, S., Pai, D., Sankar, J., & Bhattarai, N. (2016). Magnesium incorporated chitosan based scaffolds for tissue engineering applications. *Bioactive Materials*, *1*(2), 132-139.

Agarwal, S., & Murthy, R. S. R. (2015). Effect of different polymer concentration on drug release rate and physicochemical properties of mucoadhesive gastroretentive tablets. *Indian journal of pharmaceutical sciences*, *77*(6), 705.

Alasalvar, C., Miyashita, K., Shahidi, F., & Wanasundara, U. (Eds.). (2011). *Handbook of seafood quality, safety and health applications*. John Wiley & Sons.

Ansell, J., & Rauscher, H. (2011). Report of the Joint Regulator-Industry Ad Hoc Working Group: Currently Available Methods for Characterization of Nanomaterials. Paris. *International Cooperation on Cosmetics Regulation (ICCR-5), from http://ec. europa. eu/consumers/sectors/cosmetics/files/pdf/iccr5_char_nano_en. pdf*.

Archana, D., Upadhyay, L., Tewari, R. P., Dutta, J., Huang, Y. B., & Dutta, P. K. (2013). Chitosan-pectin-alginate as a novel scaffold for tissue engineering applications.*Indian Jornal of Biotechnology, 12(4),* pp. 475-482.

Aryaei, A. (2014). *Mechanical properties of bio-nanocomposites and cellular behavior under mechanical stimulation*. University of Toledo: Ph.D. Thesis.

Badawy, M. E., & Rabea, E. I. (2011). A biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*,*2011*.

Barel, A. O., Courage, W., & Clarys, P. (1995). Suction method for measurement of skin mechanical properties: the cutometer. *Handbook for non-invasive methods and the skin, 106,* pp. 335-40.

Barnes, H. A., Hutton, J. F., & Walters, K. (1989). *An introduction to rheology* (Vol. 3). Elsevier.

Baysal, K., Aroguz, A. Z., Adiguzel, Z., & Baysal, B. M. (2013). Chitosan/alginate crosslinked hydrogels: Preparation, characterization and application for cell growth purposes. *International journal of biological macromolecules, 59*, 342-348.

Bhat, S., & Kumar, A. (2012). Biomaterials in Regenerative Medicine. *Journal of Postgraduate Medicine Education and Research*, *46*(2), 81-89.

Bolsover, S. R., Shephard, E. A., White, H. A., & Hyams, J. S. (2011). *Cell biology: a short course*: John Wiley & Sons.

Boy, R. (2010). *Generic films and fibers from polysaccharides: chitosan and alginate*. Auburn University: Ph.D. Thesis.

Brandl, K., & Beutler, B. (2013). Phenotypic Mutation 'phoebus'. Retrieved Jun 10, 2015, from https://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=359

Burdick, J. A., & Mauck, R. L. (2010). *Biomaterials for tissue engineering applications: a review of the past and future trends*: Springer Science & Business Media.

Cao, N. (2011). *Fabrication of alginate hydrogel scaffolds and cell viability in calcium-crosslinked alginate hydrogel*. University of Saskatchewan: Master's Thesis.

Callister, W. D., & Rethwisch, D. G. (2011). *Materials science and engineering* (Vol. 5). John Wiley & Sons.

Censi, R. (2010). Temperature sensitive hydrogels for protein delivery and tissue engineering. Chai, Q., Jiao, Y., & Yu, X. (2017). Hydrogels for biomedical applications: Their characteristics and the mechanisms behind them. *Gels*, *3*(1), 6.

Chalmers, J. M., & Meier, R. J. (Eds.). (2008). *Molecular characterization and analysis of polymers* (Vol. 53). Elsevier.

Chapman, J., Regan, F., & Sullivan, T. (2012). *Nanoparticles in anti-microbial materials: Use and characterisation*: Royal Society of Chemistry.

Chen, Q., & Thouas, G. (2014). *Biomaterials: A Basic Introduction*: CRC Press.

Cioffi, N., & Rai, M. (Eds.). (2012). *Nano-antimicrobials: progress and prospects*. Springer Science & Business Media.

Costa-Pinto, A. R., Martins, A. M., Castelhano-Carlos, M. J., Correlo, V. M., Sol, P. C., Longatto-Filho, A., & Neves, N. M. (2014). In vitro degradation and in vivo biocompatibility of chitosan–poly (butylene succinate) fiber mesh scaffolds. *Journal of Bioactive and Compatible Polymers*, *29*(2), 137-151.

D'Mello, S., Elangovan, S., Hong, L., Ross, R. D., Sumner, D. R., & Salem, A. K. (2015). Incorporation of copper into chitosan scaffolds promotes bone regeneration in rat calvarial defects. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *103*(5), 1044-1049.

Dai, X. (2017). *Electrospun curcumin gelatin blended nanofibrous mats accelerate wound healing by Dkk-1 mediated fibroblast mobilization and MCP-1 mediated anti-inflammation* Technical University of Munich: Ph.D. Thesis.

Dai, Z., Ronholm, J., Tian, Y., Sethi, B., & Cao, X. (2016). Sterilization techniques for biodegradable scaffolds in tissue engineering applications. *Journal of tissue engineering*, *7*, pp. 1-13.

Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011). Polymeric scaffolds in tissue engineering application: a review. *International Journal of Polymer Science, 2011*.

Diridollou, S., Patat, F., Gens, F., Vaillant, L., Black, D., Lagarde, J. M., ... & Berson, M. (2000). In vivo model of the mechanical properties of the human skin under suction. *Skin Research and technology*, *6*(4), pp. 214-221.

Dumitriu, S. (2004). *Polysaccharides: structural diversity and functional versatility*: CRC press.

Dumitriu, S., & Popa, V. I. (2013). *Polymeric Biomaterials* (Vol. 1): CRC Press.

Ebnesajjad, S. (Ed.). (2012). *Handbook of biopolymers and biodegradable plastics: properties, processing and applications*. William Andrew.

El-Sherbiny, I. M., & Yacoub, M. H. (2013). Hydrogel scaffolds for tissue engineering: Progress and challenges. *Global Cardiology Science and Practice*, 38.

Enderle, J. D., & Bronzino, J. D. (2012). *Introduction to biomedical engineering*: Academic press.

Fisher, J. P., Mikos, A. G., & Bronzino, J. D. (2007). *Tissue engineering*: CRC Press.

Francis, N. L. (2012). *Development of a Multi-Functional Biopolymer Scaffold for Neural Tissue Engineering.* Drexel University: Ph.D. Thesis.

Garg, N., & Garg, A. (2010). *Textbook of endodontics*. Boydell & Brewer Ltd.

Gazori, T., Khoshayand, M. R., Azizi, E., Yazdizade, P., Nomani, A., & Haririan, I. (2009). Evaluation of Alginate/Chitosan nanoparticles as antisense delivery vector: formulation, optimization and in vitro characterization. *Carbohydrate Polymers*, *77*(3), pp. 599-606.

Giri, N., Natarajan, R., Gunasekaran, S., & Shreemathi, S. (2011). 13C NMR and FTIR spectroscopic study of blend behavior of PVP and nano silver particles. *Archives of Applied Science Research*, *3*(5), pp. 624-630.

Gentile, P., Chiono, V., Carmagnola, I., & Hatton, P. V. (2014). An overview of poly (lactic-co-glycolic) acid (PLGA)-based biomaterials for bone tissue engineering. *International journal of molecular sciences*, *15*(3), pp. 3640-3659.

Guo, B., & Ma, P. X. (2014). Synthetic biodegradable functional polymers for tissue engineering: a brief review. *Science China Chemistry*, *57*(4), pp. 490-500.

Habibi, Y., & Lucia, L. A. (2012). *Polysaccharide building blocks: a sustainable approach to the development of renewable biomaterials*. John Wiley & Sons.

Han, J., Zhou, Z., Yin, R., Yang, D., & Nie, J. (2010). Alginate–chitosan/hydroxyapatite polyelectrolyte complex porous scaffolds: Preparation and characterization. *International journal of biological macromolecules*, *46*(2), pp. 199-205.

Hendriks, F. M., Brokken, D., Oomens, C. W. J., Bader, D. L., & Baaijens, F. P. T. (2006). The relative contributions of different skin layers to the mechanical behavior of human skin in vivo using suction experiments. *Medical engineering & physics*, *28*(3), pp. 259-266.

Hiemstra, C. (2007). *In situ forming biodegradable hydrogels and their application for protein delivery*: University of Twente: Ph.D. Thesis.

Honary, S., Maleki, M., & Karami, M. (2009). The effect of chitosan molecular weight on the properties of alginate/chitosan microparticles containing prednisolone. *Tropical Journal of Pharmaceutical Research*, *8*(1), pp. 53-61.

Jayakumar, R., Prabaharan, M., & Muzzarelli, R. A. (Eds.). (2011). *Chitosan for biomaterials I* (Vol. 243). Springer.

Jin, R. M., Sultana, N., Baba, S., Hamdan, S., & Ismail, A. F. (2015). Porous pcl/chitosan and nha/pcl/chitosan scaffolds for tissue engineering applications: fabrication and evaluation. *Journal of Nanomaterials*, *16*(1), pp. 138.

Kementerian Kesihatan Malaysia.(2015). Kempen "YB Jom Ikrar! Derma Organ" Sempena Persidangan Parlimen 2015[Press release]. Retrieved July 8, 2017, from http://www.moh.gov.my/index.php/database_stores/attach_download/337/658

Khan, M. A. M., Kumar, S., Ahamed, M., Alrokayan, S. A., & AlSalhi, M. S. (2011). Structural and thermal studies of silver nanoparticles and electrical transport study of their thin films. *Nanoscale research letters*, *6*(1), pp. 1-8.

Kim, S. K. (Ed.). (2013). *Chitin and chitosan derivatives: Advances in drug discovery and developments*. CRC Press.

Kim, Y. B., & Kim, G. H. (2015). PCL/alginate composite scaffolds for hard tissue engineering: fabrication, characterization, and cellular activities. *ACS combinatorial science*, *17*(2), pp. 87-99.

Kulig, D., Zimoch-Korzycka, A., Jarmoluk, A., & Marycz, K. (2016). Study on Alginate–Chitosan Complex Formed with Different Polymers Ratio. *Polymers*, *8*(5), pp. 167.

Kumar, H., & Rani, R. (2013). Structural characterization of silver nanoparticles synthesized by micro emulsion route. *International Journal of Engineering and Innovative Technology (IJEIT), 3.*

Kumbar, S., Laurencin, C., & Deng, M. (2014). *Natural and Synthetic Biomedical Polymers*: Newnes.

Kumbhar, S. G., & Pawar, S. H. (2016). Facile synthesis, characterization and antimicrobial activity of chitosan-Alginate scaffolds. *Indian Journal of Applied Research*, *5*(6).

Kuo, C. K., & Ma, P. X. (2008). Maintaining dimensions and mechanical properties of ionically crosslinked alginate hydrogel scaffolds in vitro. *Journal of Biomedical Materials Research Part A*, *84*(4), pp. 899-907.

Kyzas, G. Z., Kostoglou, M., Lazaridis, N. K., & Bikiaris, D. N. (2013). Decolorization of Dyeing Wastewater Using Polymeric Absorbents-An Overview. In *Eco-Friendly Textile Dyeing and Finishing*. InTech.

Lanza, R., Langer, R., & Vacanti, J. P. (Eds.). (2011). *Principles of tissue engineering*. Academic press

Lee, K. Y., & Mooney, D. J. (2012). Alginate: properties and biomedical applications. *Progress in polymer science*,*37*(1), pp. 106-126.

Li, Z. (2007). *Hybrid chitosan-alginate scaffolds for bone and cartilage tissue engineering*. University of Washington: Ph.D. Thesis.

Loh, X. J. (2015). *In-Situ Gelling Polymers*. Springer Singapore.

Madaghiele, M., Demitri, C., Sannino, A., & Ambrosio, L. (2014). Polymeric hydrogels for burn wound care: Advanced skin wound dressings and regenerative templates. *Burns & trauma*, *2*(4), pp. 153.

Maji, K., Dasgupta, S., Pramanik, K., & Bissoyi, A. (2016). Preparation and evaluation of gelatin-chitosan-nanobioglass 3D porous scaffold for bone tissue engineering. *International journal of biomaterials*, *2016*.

McHugh, K. J., Tao, S. L., & Saint-Geniez, M. (2013). A novel porous scaffold fabrication technique for epithelial and endothelial tissue engineering. *Journal of Materials Science: Materials in Medicine*, *24*(7), pp. 1659-1670.

Malesu, V. K., Sahoo, D., & Nayak, P. (2011). Chitosan–sodium alginate nanocomposites blended with Cloisite 30b as a novel drug delivery system for anticancer Drug curcumin.

Malina;, D., Sobczak-Kupiec;, A., Wzorek;, Z., & Kowalski, Z. (2012). Silver Nanoparticles Synthesis With Different Concentrations Of Polyvinylpyrrolidone. *Digest Journal of Nanomaterials and Biostructures*, *7*, *pp.*. 1527-1534.

Matsumoto, T. (2007). Skin biomechanics from microscopic viewpoint: Mechanical properties and their measurement of horny layer, living epidermis, and dermis. *Fragrance Journal*, *35*(2), pp. 36.

Meyer, U., Handschel, J., Meyer, T., & Wiesmann, H. P. (2009). *Fundamentals of tissue engineering and regenerative medicine*: Springer.

Mirzaei, A., Janghorban, K., Hashemi, B., Bonyani, M., Leonardi, S. G., & Neri, G. (2017). Characterization and optical studies of PVP-capped silver nanoparticles. *Journal of Nanostructure in Chemistry*, *7*(1), pp. 37-46.

Mitra, T., Sailakshmi, G., Gnanamani, A., & Mandal, A. B. (2013). Studies on cross-linking of succinic acid with chitosan/collagen. *Materials Research*, *16*(4), pp. 755-765.

Mushi, N. E. (2014). *Chitin nanofibers, networks and composites: Preparation, structure and mechanical properties*. KTH Royal Institute of Technology: Ph.D. Thesis.

Nielsen, S. S. (Ed.). (2010). *Food analysis*. Springer.

O'brien, F. J. (2011). Biomaterials & scaffolds for tissue engineering. *Materials today, 14*(3), pp. 88-95.

Orlando, G., Soker, S., Stratta, R. J., & Atala, A. (2013). Will regenerative medicine replace transplantation?.*Cold Spring Harbor perspectives in medicine*, *3*(8).

Park, J. B. (2011). The use of hydrogels in bone-tissue engineering. *Med Oral Patol Oral Cir Bucal, 16*(1), pp. 115-118.

Patel, H., Bonde, M., & Srinivasan, G. (2011). Biodegradable polymer scaffold for tissue engineering. *Trends Biomater Artif Organs*, *25*(1), pp. 20-29.

Pieróg, M., Gierszewska-Drużyńska, M., & Ostrowska-Czubenko, J. (2009). Effect of ionic crosslinking agents on swelling behavior of chitosan hydrogel membranes. *Progress on Chemistry and Application of Chitin and its Derivatives. Polish Chitin Society, 14*.

Rajan, M., Raj, V., Al-Arfaj, A. A., & Murugan, A. M. (2013). Hyaluronidase enzyme core-5-fluorouracil-loaded chitosan-PEG-gelatin polymer nanocomposites as targeted and controlled drug delivery vehicles. *International journal of pharmaceutics*, *453*(2), pp. 514-522.

Ramli, R. H., Soon, C. F., & Rus, A. Z. M. (2016). Synthesis of Chitosan/Alginate/Silver Nanoparticles Hydrogel Scaffold. In *MATEC Web of Conferences* 78, pp 01031.

Rasa, A. (2014). Applying dynamic mechanical analysis to research & development for viscoelastic damping materials. In *INTER-NOISE and NOISE-CON Congress and Conference Proceedings*, 249 (5), pp. 2959-2968.

Rashid, M. U., Bhuiyan, M. K. H., & Quayum, M. E. (2013). Synthesis of silver nano particles (Ag-NPs) and their uses for quantitative analysis of vitamin C tablets. *Dhaka University Journal of Pharmaceutical Sciences, 12(1),* pp. 29-33.

Robinson, J. W., Frame, E. S., & Frame II, G. M. (2014). *Undergraduate instrumental analysis*. CRC Press.

Rodríguez-Vázquez, M., Vega-Ruiz, B., Ramos-Zúñiga, R., Saldaña-Koppel, D. A., & Quiñones-Olvera, L. F. (2015). Chitosan and its potential use as a scaffold

for tissue engineering in regenerative medicine. *BioMed research international*, *2015*.

Rouchi, A. H., & Mahdavi-Mazdeh, M. (2015). Regenerative medicine in organ and tissue transplantation: shortly and practically achievable?. *International journal of organ transplantation medicine*, *6*(3), pp. 93.

Roy, P., & Sailaja, R. R. N. (2015). Chitosan–nanohydroxyapatite composites: Mechanical, thermal and bio-compatibility studies. *International journal of biological macromolecules*, *73*, pp. 170-181.

Saarai, A., Kasparkova, V., Sedlacek, T., & Saha, P. (2011). A comparative study of crosslinked sodium alginate/gelatin hydrogels for wound dressing. *Recent Researches in Geography, Geology, Energy, Environment and Biomedicine*, pp. 384-389.

Saikia, C., Gogoi, P., & Maji, T. K. (2015). Chitosan: A Promising Biopolymer in Drug Delivery Applications. *Journal of Molecular and Genetic Medicine*, *2015*.

Salehi, M., Naseri Nosar, M., Amani, A., Azami, M., Tavakol, S., & Ghanbari, H. (2015). Preparation of pure PLLA, pure chitosan, and PLLA/chitosan blend porous tissue engineering scaffolds by thermally induced phase separation method and evaluation of the corresponding mechanical and biological properties. *International Journal of Polymeric Materials and Polymeric Biomaterials*, *64*(13), pp. 675-682.

Sámano-Valencia, C., Martínez-Castañón, G. A., Martínez-Gutiérrez, F., Ruiz, F., Toro-Vázquez, J. F., Morales-Rueda, J. A., & Martínez, N. (2014). Characterization and biocompatibility of chitosan gels with silver and gold nanoparticles. *Journal of Nanomaterials*, *2014*, pp. 142.

Sawyer, L., Grubb, D. (2012). *Polymer microscopy*. Springer Science & Business Media.

Schwartz, M. (2008). *Smart materials*: CRC Press.

Sethuraman, S., Krishnan, U. M., & Subramanian, A. (Eds.). (2016). *Biomaterials and Nanotechnology for Tissue Engineering*.CRC Press.

Sharma, R., Bisen, D., Shukla, U., & Sharma, B. (2012). X-ray diffraction: a powerful method of characterizing nanomaterials. *Recent Research in Science and Technology*, *4*(8).

Singh, S., Kumar, M., Singh, T., & Tyagi, L. (2011). Hydrogels used as a potential drug delivery system: A review. *International Journal of Pharmaceutical & Biological Archives, 2*(4), pp. 1068-1076.

Song, Y. J., Wang, M., Zhang, X. Y., Wu, J. Y., & Zhang, T. (2014). Investigation on the role of the molecular weight of polyvinyl pyrrolidone in the shape control of high-yield silver nanospheres and nanowires. *Nanoscale research letters*, *9*(1), pp. 17.

Steinhoff G., (2016). *Regenerative medicine- from protocol to patient: 5. Regenerative therapies II*. Springer Science.

Straccia, M. C., d'Ayala, G. G., Romano, I., Oliva, A., & Laurienzo, P. (2015). Alginate hydrogels coated with chitosan for wound dressing. *Marine drugs*, *13*(5), pp. 2890-2908.

Stuart, B. H. (2008). *Polymer analysis* (Vol. 30): John Wiley & Sons.

Sun, J., & Tan, H. (2013). Alginate-based biomaterials for regenerative medicine applications. *Materials*, *6*(4), pp. 1285-1309.

Sunatkari, A. L., Talwatkar, S. S., Tamgadge, Y. S., & Muley, G. G. (2015). Influence of Surface Passivation by L-Arginine on Linear and Nonlinear Optical Properties of Ag-PVP Nanocomposite. *American Journal of Materials Science*, *5*(3A), pp. 21-30.

Szymańska, E., & Winnicka, K. (2015). Stability of Chitosan—A Challenge for Pharmaceutical and Biomedical Applications. *Marine drugs, 13*(4), pp. 1819-1846.

Tan, H., & Marra, K. G. (2010). Injectable, biodegradable hydrogels for tissue engineering applications. *Materials*, *3*(3), pp. 1746-1767.

Tang, L., Thevenot, P., & Hu, W. (2008). Surface chemistry influences implant biocompatibility. *Current topics in medicinal chemistry*, *8*(4), pp. 270-280.

Tanigawa, J., Miyoshi, N., & Sakurai, K. (2008). Characterization of chitosan/citrate and chitosan/acetate films and applications for wound healing. *Journal of applied polymer science, 110*(1), pp. 608-615.

Tateishi, T. (2008). *Biomaterials in Asia: In Commemoration of the 1st Asian Biomaterials Congress, Tsukuba, Japan, 6-8 December 2007*. World Scientific.

Theivasanthi, T., & Alagar, M. (2011). Electrolytic synthesis and characterizations of silver nanopowder. *Nano Biomedicine and Engineering*, 4(2), pp. 58-65..

Theocharis, A. D., Skandalis, S. S., Gialeli, C., & Karamanos, N. K. (2016). Extracellular matrix structure. *Advanced drug delivery reviews*, *97*, pp. 4-27.

Thi Hiep, N., Chan Khon, H., Dai Hai, N., Byong-Taek, L., Van Toi, V., & Thanh Hung, L. (2017). Biocompatibility of PCL/PLGA-BCP porous scaffold for bone tissue engineering applications. *Journal of Biomaterials Science, Polymer Edition*, *28*(9), pp. 864-878.

Thomas, S., Grohens, Y., & Ninan, N. (2015). *Nanotechnology Applications for Tissue Engineering*: William Andrew.

Thomas, S., Durand, D., Chassenieux, C., & Jyotishkumar, P. (Eds.). (2013). *Handbook of biopolymer-based materials: from blends and composites to gels and complex networks*. John Wiley & Sons.

Thwala, L. N. (2012). *Preparation and characterization of alginate-chitosan nanoparticles as a drug delivery system for lipophilic compounds.*University of Johannesburg: Ph.D. Thesis.

Trachtenberg, J. E., Placone, J. K., Smith, B. T., Fisher, J. P., & Mikos, A. G. (2017). Extrusion-based 3D printing of poly (propylene fumarate) scaffolds with hydroxyapatite gradients. *Journal of Biomaterials Science, Polymer Edition*, *28*(6), pp. 532-554.

Trivedi, M. (2015). Characterization of physicochemical and thermal properties of chitosan and sodium alginate after biofield treatment. *Pharmaceutica Analytica Acta-Open Access*, *6*(10).

Upadhyay, R. K. (2015). Role of regeneration in tissue repairing and therapies. *Journal of Regenerative Medicine and Tissue Engineering*, *4*(1), pp. 1.

U.S. Department of Health & Human Services (2016). U.S. Department of Health & Services. Retrieved on May 20, 2016, from https://optn.transplant.hrsa.gov/.

Varkey, M., Ding, J., & Tredget, E. E. (2015). Advances in skin substitutes—potential of tissue engineered skin for facilitating anti-fibrotic healing. *Journal of functional biomaterials*, *6*(3), pp. 547-563.

Venkatesan, J., Bhatnagar, I., & Kim, S.-K. (2014). Chitosan-alginate biocomposite containing fucoidan for bone tissue engineering. *Marine drugs*, *12*(1), pp. 300-316.

Vishwakarma, A., Sharpe, P., Shi, S., & Ramalingam, M. (Eds.). (2014).*Stem cell biology and tissue engineering in dental sciences*. Academic Press.

Walters, K. A., & Roberts, M. S. (2002). The structure and function of skin. *Drugs and the pharmaceutical Sciences, 119*, pp. 1-40.

Wilburn, F. W. (1989). Introduction to thermal analysis, techniques and applications: Michael E. Brown. Chapman and Hall, London.

Wong, J. (2013). *Synthesis and characterisation of chitosan from shrimp shells*. Universiti Tunku Abdul Rahman: Degree's Project Report.

Yahia, L., Chirani, N., Gritsch, L., Motta, F. L., & Fare, S. (2015). History and applications of hydrogels. *Journal of biomedical sciences*, *4*(2).

Yan, Y., Zhang, X., Li, C., Huang, Y., Ding, Q., & Pang, X. (2015). Preparation and characterization of chitosan-silver/hydroxyapatite composite coatings onTiO 2 nanotube for biomedical applications. *Applied Surface Science, 332*, pp. 62-69.

Yao, H., & Kimura, K. (2007). Field emission scanning electron microscopy for structural characterization of 3D gold nanoparticle superlattices. *Modern research and educational topics in microscopy.*

Yue, B. (2014). Biology of the extracellular matrix: an overview. *Journal of glaucoma*, 2014, pp. 20-23.

Zhang, X. C. (Ed.). (2016). *Science and Principles of Biodegradable and Bioresorbable Medical Polymers: Materials and Properties*. Woodhead Publishing.

Zhao, F., Yao, D., Guo, R., Deng, L., Dong, A., & Zhang, J. (2015). Composites of polymer hydrogels and nanoparticulate systems for biomedical and pharmaceutical applications. *Nanomaterials*, *5*(4), pp. 2054-2130.

Zhao, J., Han, W., Chen, H., Tu, M., Zeng, R., Shi, Y., Zhou, C. (2011). Preparation, structure and crystallinity of chitosan nano-fibers by a solid–liquid phase separation technique. *Carbohydrate Polymers, 83*(4), pp 1541-1546.

Zhu, J. (2010). Bioactive modification of poly (ethylene glycol) hydrogels for tissue engineering. *Biomaterials, 31*(17), pp. 4639-4656.

Zhu, J., & Marchant, R. E. (2011). Design properties of hydrogel tissue-engineering scaffolds. *Expert review of medical devices*, *8*(5), pp. 607-626.

Zucolotto, V. (2013). *Nanotoxicology: materials, methodologies, and assessments*. Springer Science & Business Media.