PREPARATION OF BIOMATERIALS USING FISH COLLAGEN AND SEAWEED ALGINATE TO PROMOTE IN-VIVO CELL GROWTH AND PROLIFERATION ACTIVITY

By

WOO MUN JENG

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Specially dedicated to

my beloved mother and father

ABSTRACT

PREPARATION OF BIOMATERIALS USING FISH COLLAGEN AND SEAWEED ALGINATE TO PROMOTE IN-VIVO CELL GROWTH AND PROLIFERATION ACTIVITY

Woo Mun Jeng

Biomaterials have long been used as medical implants in humans to increase the rate of healing and prolong patient lifespan. The material of choice for the fabrication of biomaterials mimic the ECM (extracellular matrix) of humans, such as animal sourced collagen. The main source of collagen from animals are usually from bovine and porcine skin and bone wastes. However, due to local religious and cultural sensitivities in Malaysia, therefore limited research has been carried out on bovine and porcine sourced collagen. Little research has been directed towards using fish waste as a source of collagen, specifically from local fish sources. Therefore, this study was carried out to investigate the feasibility of using collagen extracted from fish waste of the local fish processing industry, specifically the skin waste of Nile tilapia (*Oreochromis niloticus* sp) and hybridising it with sodium alginate from the *Sargassum Polycystum* sp seaweed which is found in abundance along the coastal areas of

Port Dickson, Malaysia for the fabrication of biomaterials. Samples of Nile Tilapia skin waste were obtained from the wet market in Bandar Sungai Long, and collagen was extracted using pH 3.25 acetic acid at 0.5 M. Sodium alginate has been used in the fabrication of biomaterials due to it's biocompatibility with the ECM of humans and ease of gelation. The biomaterials were fabricated with different ratios of collagen to sodium alginate. FTIR (Fourier Transform Infrared Spectroscopy) analysis proved that collagen was successfully extracted while HPLC (High Performance Liquid Chromatography) showed that the collagen from Nile Tilapia is rich in imino acids hydroxyproline, proline and glycine. Polyacrylamide gel electrophoresis, in the presence of 10 % SDS using 7.5 % stacking gel illustrated a doublet pattern for $\alpha1$ and $\alpha2$ chains (at approximately 120 kDa to 125 kDa) and a β chain (approximately 220 kDa). Swelling ratio test demonstrated the hydrophilic nature of the biomaterials fabricated with higher ratio of sodium alginate to collagen, namely the 30% C : 70% SA with 12.75 % swelling ratio as compared to 90% C : 10% SA with 0.04% swelling ratio. SEM (Scanning Electron Microscope) showed the highly porous surface morphology of the fabricated biomaterials, particularly the pure sodium alginate and 50%C : 50% SA biomaterial. Results of the Trypan Blue Staining, MTT assay ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) and direct contact test demonstrated that the fabricated biomaterials had a positive impact on cell growth and proliferation activity.

From the MTT assay results, it is shown that cell growth rates and proliferation activity were higher when cultured with fabricated biomaterials as compared to the control. The 80%C : 20%SA had the highest percentage viability around 30 % on Day 2, while control only had around 15 %. On Day 4, the 80 % C: 20 % SA biomaterial reached almost 95 % viability as compared to the control which only achieved around 50% viability. In conclusion, the hybridised biomaterials maybe used to enhance cell growth and proliferation activity. Biomaterials fabricated with higher ratios of sodium alginate to collagen demonstrated optimum cell growth and proliferation activity.

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Thank you.

Woo Mun Jeng

APPROVAL SHEET

This dissertation titled **"PREPARATION OF BIOMATERIALS USING FISH COLLAGEN AND SEAWEED ALGINATE TO PROMOTE IN-VIVO CELL GROWTH AND PROLIFERATION ACTIVITY"** was prepared by WOO MUN JENG and submitted as partial fulfillment for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr.Woo Kwan Kit)   Date:

Assistant Professor / Supervisor

Department of Chemical Engineering

Lee Kong Chian Faculty of Engineering and Science

Universiti Tunku Abdul Rahman

_____

(Dr. Leong Loong Kong)   Date:

Assistant Professor / Co-supervisor

Head of Department of Chemical Engineering

Lee Kong Chian Faculty of Engineering and Science

Universiti Tunku Abdul Rahman

SUBMISSION SHEET

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date:

SUBMISSION OF DISSERTATION / THESIS

It is hereby certified that **WOO MUN JENG (ID No: 14UEM70884)** has completed this thesis titled "Preparation Of Biomaterials Using Fish Collagen And Seaweed Alginate To Promote In-Vivo Cell Growth and Proliferation Activity" under the supervision of Dr. Woo Kwan Kit (Supervisor) from the Department of Chemical Engineering, Lee Kong Chian Faculty of Engineering and Science, and Dr. Leong Loong Kong, (Co-supervisor) head of the Department of Chemical Engineering, Lee Kong Chian Faculty of Engineering and Science.

I understand that the University will upload a softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(Woo Mun Jeng)

DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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(WOO MUN JENG)

Date

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
cm^{-1}	Reciprocal Wavelength
Em	Emission Wavelength
kDa	Kilo Daltons
kv	Kilovolt
M	Molarity
N	Normality
nm	Newton Meter
nmol	Nanomole
pH	Potential Hydrogen
rpm	Rotations per Minute
sp	Species
μl	Microliter
μm	Micro Meter
μmol	Micromole
μmol/ml	Micromole / Mililitre

CHAPTER 1

INTRODUCTION

1.1   Background

Biomaterials are any natural or synthetic material implanted into the body in order to enhance or restore the natural capacity of a living tissue. Biomaterials may form a part of, or in certain cases, replace the entirety of living tissues in the body (Tathe, Ghodke, and Nikalje., 2010). They are constantly in direct contact with bodily fluids like the blood, lymph and immune system (Agrawal, 1998).

Examples of biomaterials include passively functioning biomaterials like heart valves and bioactive biomaterials like hydroxy-apatite coated hip implants or knee joints, and dental implants (Tathe, Ghodke, and Nikalje, 2010). Fossilised skeletons give rise to evidence that biomaterials had been in use since the earliest of civilisations. The earliest discovered biomaterial by early civilisations date back to roughly 600 A.D., when the Mayan people used nacre from seashells to fashion dental implants known as nacre teeth.

Analysis of the fossilised skulls showed that the nacre teeth had become fully integrated with the jawbone of the Mayan fossil, effectively achieving what is now known as osseointegration (Ratner, et al., 1997). Another example was a fossil in Europe from 200 A.D., whose iron dental implants achieved full osteointegration with the jawbone (Crubezy et al., 1998).

It is interesting to note that this was achieved despite early human societies lacking modern day science, knowledge and technology with regards to material science, toxicity and immunogenicity. In actual fact, biomaterials were commonly used in many early civilisations. Large external bodily wounds were closed by suturing and linen sutures had been used in ancient Egypt while Galen of Pergamon (circa 130-200 A.D.) wrote about using gold wire as ligatures in Greek ancient literature (Ratner, et al., 1997).

In modern times, biomaterials have been significantly improved and been made into heart valves, knee and hip joints and employed within drug delivery systems. Prior to the 1950's, it was not uncommon for biomaterials to be rejected or to cause fatalities due to inflammation, both of which due to the host's immune response. Unlike during modern times, back then there was limited knowledge and research about biocompatibility and immunogenicity (Ratner, et al., 1997).

The latest advancements of the biomaterial engineering field include the production of polymer hydrogels and biomaterials made from natural polymers, that are synthesized with physical or chemical crosslinking. Intensive research regarding the feasibility of these natural polymer based biomaterials for tissue regeneration have been carried out with promising results (O'Brien., 2011). An example of natural polymer based biomaterials would be collagen based biomaterials.

The advantages of collagen based biomaterials include biocompatibility with the extracellular matrix (ECM), biodegradability and non-toxicity towards living tissue. However, as expected, collagen based biomaterials posses several weaknesses as compared to synthetic polymer based biomaterials, namely lower mechanical strength, lower stability and higher degradation rate (Vaissiere et al., 2000).

Despite their disadvantages, however collagen based biomaterials encourage the growth and proliferation activity of cells due to their bioactive properties and coincidentally this gives rise to their biocompatibility with living tissue. Therefore, the biomaterials in this research project were fabricated using different ratios of collagen and alginate.

This study was carried out to determine the effect of biomaterials fabricated from a higher collagen ratio to sodium alginate and vice versa. The biomaterials were fabricated with different rates of collagen to sodium alginate in order to study their influence on cellular proliferation activity.

Fish waste collagen was used as marine based collagen can be easily obtained in abundance as byproducts of the fish processing industry. Byproducts of the fish processing industry, such as the manufacturing of canned fish, whereby the skin and bones are discarded, is a huge source of fish waste (Nagarajan and Shakila., 2013).

There are several reasons which make collagen sourced from fish waste more preferable over collagen from other wastes, namely less susceptibility to diseases that are transmittable from animals such as cows to humans like bovine spongiform encephalopathy and FMD (bovine foot and mouth disease) (Lodish, Berk and Zipursky, 2000).

Fish collagen also has high biological value, as it is a rich source of essential amino acids such as hydroxyproline, the main component of collagen (Nagarajan and Shakila., 2013). Marine collagen is also accepted by all religions and ethnicities, unlike porcine and bovine sourced collagen.

1.2   Aims and Objective

The principle objectives of this research study is the feasibility of utilising fish skin, which is a major waste product of the fish processing industry, as a source of collagen. This marine based source of collagen would then be hybridized with brown seaweed alginate to be fabricated into biomaterials. The next objective would be the diversification of underutilised brown seaweed alginate which is naturally found in abundance along the coastal areas of Port Dickson, Malaysia.

Collagen will be extracted from the discarded skin of fish, while alginate will be extracted from brown seaweed. Alginate will play a role as the cross-linker in the fabricated biomaterials, while collagen would be used for its' intrinsic properties which supports cell growth and proliferation. The fabricated biomaterials would be examined in terms of their physio-chemical properties, surface morphology as well as their ability to support cellular proliferation activity.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Polymers

Natural polymers, either from protein or polysaccharides are derived from nature and have been used extensively in tissue engineering studies. Examples of protein based polymers include collagen and silk fibroin, while polysaccharide based polymers include alginate, chitin, agarose and hyaluronic acid. Natural polymers as suitable for tissue engineering due to their capacity to support the growth and proliferation activity of living cells.

Natural polymers used for tissue engineering usually have high biocompatibility and poses low antigenicity towards the host's cells (Boccaccini and Blaker., 2005). The drawbacks of natural based polymers include lower load-bearing capacity and mechanical stability (Chan and Leong, 2008). Therefore, two or more natural polymers are commonly crosslinked together with a crosslinking agent to improve the load-bearing capacity and mechanical stability (Boccaccini and Blaker., 2005).

2.1.1   Collagen

Collagen is the most commonly available and important protein in animals. Interestingly, collagen shares a similar responsibility in animals as polysaccharides and cellulose in plants. Collagen consists of more than 25 % of the total protein in living animals, especially mammals, and it can be found everywhere in the body, such as in the skin, organs, muscle, bones, cartilage, tendons, eyes and even the teeth (Muller, 2003; Miyata et al., 1992).

Collagen plays an important role in protecting the skin from microbial and chemical infiltration, promotes cellular proliferation and growth of cells, and maintains the structural integrity of the bones, tissues, muscles and organs (Buehler, 2006; Zhang et al., 2011). The first recorded use of collagen as a biomaterial was by Joseph Lister and William Macewen in 1881. They successfully extracted "catgut", a collagen rich material from the small intestine of sheep to be used as sutures (Chattopadhyay and Raines, 2014).

Since then, collagen has become the material of choice for the fabrication of biomaterials due to their similarity with the ECM of humans (Ma, 2008). Extracted soluble collagen is also a poor immunogen (Werkmeister, Ramshaw and Ellender, 1990), making it safe for fabricating biomaterials.

This is especially true for Type I collagen which would be the least likely to provoke an immune response compared to the other types of collagen (Werkmeister, Ramshaw and Ellender, 1990). Collagen based biomaterials have been proven to support the growth of cells (Ehrmann and Gey, 1965), increase cell adhesion on biomaterial surfaces, promote mitotic activity, as well as encourage cellular differentiation and proliferation activity (Doillon, Silver and Berg, 1987). It is interesting to note that such advantages are exclusive to natural collagen as similar benefits are non-replicable in synthetic collagen polymers.

Therefore, many composites have been fabricated with natural collagen or surface coated with natural collagen to increase their cell growing properties (Civerchia-Perez et al., 1980). Another factor for selecting collagen as a natural polymer in the fabrication of biomaterials is that collagen is the main fibrous structural protein of living organisms (Zhang et al., 2011), therefore natural collagen may have bioactive properties that promote intrinsic cell interaction properties (Hutmacher, et al., 2001; Zuber et al., 2015; Wang et al., 2012). Collagen also provides tensile and mechanical strength to biomaterials by adapting their morphology according to their function and location in the body.

For example, collagen in the tendons are all mostly linearly arranged along the length of the tendon, while collagen in the skin are arranged in all directions (Werkmeister, Peters and Ramshaw, 1989). This unique arrangement of the collagen fibrils is intended to give the necessary mechanical strength and elasticity needed depending on it's function. For example, the tendons are needed to support heavy weights, while the skin has to be elastic and stretchable in all directions.

Extracted collagen is able to be reformed *in vitro* into the ordered fibrillar structures of the host's native collagen fibrils. This unique feature is a benefit of using collagen in the fabrication of biomaterials (Hutmacher et al., 2001). The extracted collagen easily assimilates with host's collagen and ECM (Miyata, Taira and Noishiki, 1992).

2.1.2   Structure Of Collagen

Collagen appears as elongated fibrils and are mostly found in fibrous tissues such as the tendons, ligaments, skin and cartilage (Belbachir et al, 2009). Collagen fibres have high tensile strength, are mostly whittish, opaque and viscoelastic (Gupta and Nayak., 2015). According to Fligiel et al., (2003), collagen fibrils consist of a chain of amino acid subunits.

Numerous individual subunits of amino acids combine to form amino acid chains which are also known as α-chains. These α-chains twist around each other in a triple helical structure to form the collagen fibrils or tropocollagen which maybe up to 300 nm in length and 1.4 nm in diameter (Figure 2.1; Figure 2.2).

Triple Helix	1.4 nm
Alpha Chain	
Amino Acid Sequence	- Gly – X -Y – Gly – X- Y – Gly – X – Y -
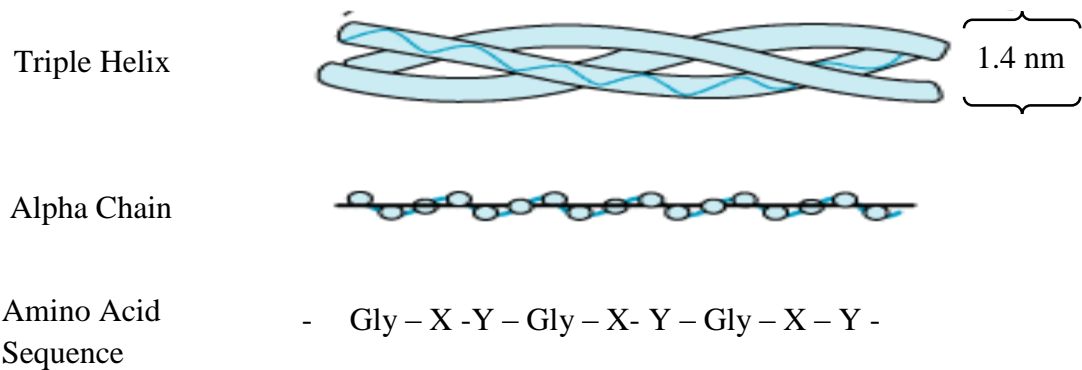
Figure 2.1: Structure of collagen by Dr. Namrata Chhabra, 2017

Amino acids (1 nm)

Tropocollagen (300 nm)

Fibrils (1 μm)

Fibers (10 μm)
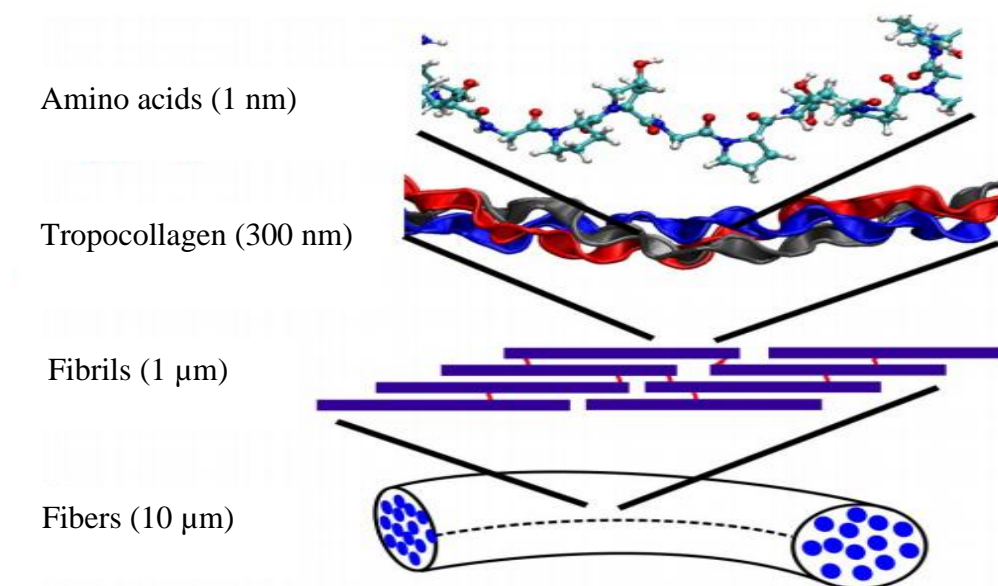
Figure 2.2: The design of collagen from amino acid polypeptide chain to fibres by Buehler, 2006

The five main Types of collagen are Type I, Type II, Type III, Type IV and Type V (Belbachir et al., 2009). Type I is mostly found in the skin, tendons, blood vessels, organs and bones. Type II is found in cartilage. Type III in the reticular fibres of connective tissues. Type IV forms the basal membranes. Type I collagen, Type II, Type III and Type IV collagen are fibrillar in shape.

Type I collagen is found in abundance in most of the connective tissues, making them the most commonly available collagen (Parenteau-Bareil et al., 2010). Type I collagen consists of a chain of alternating hydroxyproline, glycine and proline amino acids (Belbachir et al., 2009).

Type I collagen is considered as the material of choice for biomaterial fabrication due to their high biocompatibility (Minary-Jolandan and Yu., 2009). Type II collagen consists of dense connective tissue like the articular cartilage and hyaline cartilage. Type II collagen forms a dense network of collagen fibres and provides tensile strength to soft tissues. Examples of Type II collagen are the collagen in the nose, ears, trachea, larynx, and smaller respiratory tubes (Silvipriya et al., 2015).

Type III collagen is a fibrous scleroprotein which is found in cartilaginous tissues. Type III collagen comprises a large percentage of the reticular fibres in bones, cartilage, tendon, bone marrow and organ of the lymphatic system like the thymus and spleen (Cheah, 1985).

Type IV collagen has a basal membrane structure. This is caused by the absence of the amino acid glycine in it's backbone structure, thus instead of twisting and coiling, it forms a sheet, which is known as the basal lamina. Patients with Alport Syndrome, an inherited genetic defect which inhibits the production and assembly of Type IV collagen, affects the formation of normal basal membranes in the kidneys, ear and eye.

Type V collagen plays a lesser role in connective tissue activity hence it is found in smaller quantities in the human body. However, Type V collagen is found in every tissue and is usually found together with Type I and Type III collagen, usually around the borders of cells and serve as a cellular cytoskeleton. There seems to be a higher percentage of Type V collagen in the intestines compared to other tissues such as the hair and placenta (Graham et al., 1988).

2.1.3   Fish Waste As Source Of Collagen

Type I collagen is mostly sourced from the waste of large mammals such as bovine byproducts from the meat processing industry. Bovine byproducts of the meat processing industry, such as the skin and bones are an easy source for large yields of collagen. Collagen from bovine, porcine, and chicken wastes have been commonly used in medical and scientific research fields. However, it would be beneficial to study other collagen sources, as nearly 3 % of the world's population is allergic to bovine collagen (Stegman, Chu, and Armstrong, 1988).

It is known that consumption of or usage of bovine based products is forbidden in Hinduism, which is one of the largest religions in the world. Furthermore, usage of bovine sourced collagen increases the risk of transmitting foot-and-mouth disease (FMD) and transmissible spongiform encelopathy (TSE) in humans (Parenteau-Bareil et al., 2010; Ahuja et al., 2012).

The second largest mammalian source of collagen are from porcine wastes. Waste byproducts of the porcine meat processing industry, such as porcine skin and bones is a rich source of collagen. In modern times, the dermis of porcine has been used for skin grafting and reconstructive surgery.

However, the usage and consumption of porcine based collagen faces several roadblocks. The consumption of, or skin contact with porcine is forbidden in Islam, which is one of the largest religions in the world (Cortial et al., 2006). Collagen can also be extracted from marine sources such as fishes, starfish, jellyfish, sea sponge, sea urchin, octopus, squid and prawn (Song et al., 2006). Marine sourced collagen is preferable over mammalian sourced collagen because of almost non-existent fish-borne diseases which are transmittable to humans (Lodish, Berk and Zipursky, 2000).

As of now, there have not been any major epidemics which are caused by the use of marine collagen (Addad et al., 2011; Exposito et al., 1999, 2002). Examples of fish species whereby Type I collagen has been extracted and widely studied includes the silver carp, Japanese sea-bass, chub mackerel, bullhead shark, skipjack tuna, Japanese sea-bass, yellow sea bream, horse mackerel and Nile tilapia *Oreochromis niloticus* sp (Sugiura et al., 2009; Song et al., 2006).

Moreover, fish collagen enhances the matrix mineralization of bone synthesizing cells *in vitro* by helping to promote bone healing and osteoblast cell regeneration (Yamada et al., 2013). Recently, an antibacterial peptide from fish collagen, known as collagencin was discovered to completely stop the growth of *Staphylococcus aureus* sp bacteria, which is responsible for skin infections, respiratory infections and septicaemia (Ennaas et al., 2016).

The osteogenic and microbial properties of fish collagen can be exploited in the field of bone and tissue engineering. Fish collagen is also acceptable by all ethnicities and religions, and is not a religious or dietary prohibition in any culture or religion.

In the fishing industry, the skin of fishes are usually discarded, thereby providing an abundant source of collagen (Tronci et al., 2000). The type of collagen used in this experiment was Type I collagen as more than 90 % of the collagen in the human body consists of Type I collagen (Tronci et al., 2000).

2.1.4   Applications Of Collagen Based Products

Collagen based products have been used for numerous applications in the pharmaceutical, biomedical, healthcare and food technology fields (Hemanth, Spandana, and Poonam, 2011). Collagen has been used as a main ingredient in various health and beauty products like facial creams, health supplements and cosmetic products.

Collagen hydrogels have also been applied in numerous fields and studies as scaffolds for cartilage tissue engineering, for wound healing and as a pro-angiogenic site for the transplantation of the islets of Langerhans cells (Hesse et al., 2010) in the pancreas of humans.

Collagen demonstrates good tolerance when placed in a foreign ECM and has even been shown to support cell proliferation activity of foreign living tissues (Sun and Tan, 2013). For example, collagen based implants have been used in heart valves, for plastic surgery and in dental implants (Sandhu et al, 2012) with success. The bioactive properties of collagen makes them suitable for reconstructive plastic surgery, such as skin grafting in burn victims (Peng et al, 2004).

2.1.5   Extraction of Collagen

Two most commonly used methods for extracting collagen from marine sources are the acid-soluble collagen method (ASC) (Nagai and Suzuki, 2000) and the pepsin-soluble method (PSC) (Pati et al., 2010). Both methods use similar chemicals, which are acetic acid and sodium hydroxide, with the only difference being the addition of pepsin as a digestive enzyme in the pepsin-soluble method. The pepsin-soluble method uses the enzyme pepsin for dissolving the collagen. Pepsin is an enzyme that breaksdown proteins into much smaller peptides (Fruton., 2002). Pepsin can be found naturally in the digestive systems of humans where it plays a role in breaking down protein (Fruton., 2002).

Both methods consist of soaking fish skin in sodium hydroxide to breakdown the skin, defatting of the skin with butanol, treating and extracting collagen with acetic acid, salting out the skin in Tris, forming precipitates from the dissolved collagen with sodium chloride and dialysing the precipitated collagen agaits acetic acid and distilled water (Zhang et al., 2011; Sujithra et al., 2013; Wahl et al., 2006.). Acid soluble collagen had been extracted from the skin and scales of several fish and aquatic animals.

Examples of aquatic animals from which collagen had been extracted using both the ASC and PSC methods include the lizard fish (*Saurida* sp*),* horse mackerel (*Trachurus japonicas* sp), grey mullet (*Mugil cephalis* sp), flying fish (*Cypselurus melanurus* sp) and yellowback seabream (*Dentex tumifrons* sp) by Thuy et al., (2014).

Other examples include the african catfish (*Clarias gariepinus* sp), salmon (*Salmo salar* sp) and baltic cod (*Gadus morhua* sp) by Tylingo et al., (2016); surf smelt (*Hypomesus pretiosus japonicus brevoort* sp), bluefin tuna (*Thunnus Orientails* sp) by Han et al., (2011), carp fish (*Cyprinus carpio* sp) by Zhang et al., (2011); crown-of-thorns starfish (*Acanthaster planci* sp) by Tan et al., (2013) and nile tilapia (*Oreochromis niloticus* sp) by Sujithra et al., (2013).

Based on a study by Sujithra et al., (2013) who reported that the lyophilised dry weight of *Oreochromis niloticus* sp ASC at 22 % was lower compared to PSC at 60 %. The yield obtained by Sujithra et al., 2013 was greater than that of Jelly fish at 25 % − 35 % by Nagai and Suzuki (2000), Baltic cod (*Gadus morhua*) at 21.5%, Japanese sea bass at 51.4% (*Lates calcarifer*) by Sinthusamran et al., (2013), and chub mackerel (*scomber japonicus*) at 49.8% by Nagai and Suzuki (2000). Therefore, fish waste from *Oreochromis niloticus* sp is a potentially potent source of collagen.

The spectral analyses for collagen extracted from both the ASC and PSC methods, using the Fourier Transform Infrared Spectroscopy (FTIR) method showed that the collagen samples were undenatured, conformed with standard collagen spectral range and retained their original triple helical structure (Sujithra et al., 2013). Sodium dodecyl sulphate electrophoresis (SDS-PAGE) tests showed that collagen extracted using both methods results maintained their doublet pattern for upper α_1 and lower α_2 chains and had a single β chain (Sujithra et al., 2013).

Scanning electron microscopy (SEM) analyses showed that collagen extracted using both the ASC and PSC methods were fibrous (Sun et al., 2017). These results indicate that there are negligible differences between the effectiveness of either the ASC or PSC method. However, Kittiphattanabawon et al., (2010), mentioned that the triple helical structure of collagen maybe denatured by pepsin. Cleavage of the telopeptide regions of tropocollagen would result in the secondary structure of PSC becoming affected, thus cause the collagen protein to unravel and lose their native characteristics.

2.2　Silk Fibroin

The other protein of natural origin is silk fibroin. Silk is a naturally occurring fibre consisting of hydrophobic fibroin and hydrophilic sericin. Since the end of the 19th century, silk fibroins from silkworms of *Bombyx mori* sp have been utilised as sutures and biomaterials.

The most common sources of silk fibroins are from the cocoons of *Antheraea mylitta* sp and *Bombyx mori* sp silkworms (Bhardwaj and Kundu, 2010). Silk fibroins have extremely strong tensile strength and stretching capacity. In actual fact, silk fibres have greater resistance to failure in compression (Cunniff et al., 1994) compared to other natural polymers.

Generally, the advantages of silk fibroins include high mechanical strength, stability, biocompatibility, they do not provoke host immunity response and their biodegradability. However, only degummed silk fibroin have these properties as macrophage activating sericin of silk fibroins require removal (Chan and Leong, 2008). Sericin from silk fibroin evoked intense immunogenic reactions in cell culture studies (Soon and Kenyon, 1984).

Numerous *in vitro* experiments involving silk fibroins demonstrated that once macrophage activating sericin was removed, the silk fibroin no longer provoked host immune response (Gupta et al., 2007). An example is the Seri Fascia surgical scaffold used for plastic and reconstructive surgery (Horan et al., 2009).

Unfortunately, silk fibroins have similar characteristics as amyloid (Panilaitis et al., 2003). The occurrence of neurodegenerative diseases such as Parkinson's and Alzheimer's in humans have been attributed to high amyloid levels (Kenney et al., 2002). In an independent study, implanted biomaterials made from silk fibroin was found to be responsible for high levels of accumulated amyloid in mice (Lundmark et al., 2005).

2.3 Polysaccharides

Another category of natural based polymers are polysaccharides. The four common types of polysaccharides are alginate, agarose, chitosan and hyaluronic acid (Hutmacher, Goh and Teoh., 2001). One of the main properties of polysaccharides are their ability to be fabricated into hydrogels. Polysaccharide hydrogel's physiological characteristics are influenced by the ratio and arrangement of their monosaccharides.

2.3.1 Alginate

Alginate, which constitutes the cell walls of brown algae and seaweeds (Vismanauthan and Nillamuthu, 2014) are comprised of a diverse family of polysaccharides that are traditionally considered as natural binders. This is due to their biocompatibility with other organic materials and biodegradability. Alginate is one of the three phycocolloids that form the cell walls of seaweeds, with the other two phycocolloids being agars and carrageenans. Phycocolloids form the primary components of both the cell walls and extracellular matrix of seaweeds.

Phycocolloids act as a backbone which increases the mechanical strength and flexibility of the cell wall (Smidsrod and Skjak-Braek, 1990; Rehm and Valla, 1997). In it's natural state, extracted alginate can invoke inflammation and host immune response (Skaugrud., Borgersen., and Dornish., 1999). Therefore, extracted alginate has to be purified to prevent systemic toxicity in humans (Takka and Acarturk, 1999). The purification methods remove residual impurities which may provoke the host's immune response, but does not affect the physiochemical structure of alginates (Oerther et al., 1999; Suzuki et al., 1998).

Alginate readily combines with sodium ions to form viscous sodium alginate. Alginate crosslinks with divalent cations such as calcium or sodium to form calcium alginate or sodium alginate, due to the unique ability of phycocolloids to accumulate divalent metal ions and form gels. (Smidsrod and Skjak-Braek, 1990; Rehm and Valla, 1997). Alginate is a polysaccharide which is widely used in biomedical science research (Zhang et al., 2011). Biomaterials fabricated from calcium alginate and sodium alginate have been utilised for developing medical foams, gauzes and surgical dressings (Skaugrud., Borgersen., and Dornish., 1999).

The advantages of alginates include their non-toxicity towards living tissue, biodegradability, excellent water absorbtion capacity, large pore size, natural antiseptic properties and non-immunogenicity (Sun et al., 2013; Andersen et al., 2012), making alginates a suitable polymer for the fabrication of hydrogels and biomaterials. Unfortunately, alginate based biomaterials posses several drawbacks as compared to synthetic based biomaterials such as lower mechanical strength and poor cell adhesion and proliferation capabilities. Fortunately, according to Chung et al., 2002, the structural strength of alginate based biomaterials can be improved via crosslinking them with other polymers.

Alginate hydrogels which are used in the field of cartilage regeneration share similar characteristics with the extracellular matrix (ECM) (Li et al., 2012). Injectable alginate based hydrogels and gel microspheres have been utilised in cartilage regeneration and tissue engineering fro medical purposes (Lubiatowski et al., 2006). Alginate based products have been used in dental impression moulds, prosthetic impressions, as taxidermy moulds, treating gastroeosophageal acid reflux, plant fertiliser, food emulsifier, and thickening agent for ice-cream and paint (Szekalska et al., 2016).

2.3.2   Sources of Raw Alginate

Alginate can be found in all seaweed, however it is particularly available in abundance from brown algae of the Genus *Macrocystis, Laminaria, Ascophyllum, Alario, Ecklonia, Eisenia, Nercocystis, Sargassum, Cystoseira,* and *Fucus* (Wallis, 1985). Seaweed of Genus *Sargassum* from Port Dickson beach, Malaysia was used in the present study.

2.3.3   Structure Of Alginate

Alginates are a naturally occurring structural component in the cell walls of marine brown seaweed. Alginates form long chains of heteropolysaccharides which consists of M-blocks (1-4)-linked β-D-mannuronic acid and G-blocks (α-L-guluronic acid) with variations in their ratios and structural arrangements (Mushollaeni, 2011) (Fig 2.3). The M-blocks and G-blocks are sequentially assembled in either repeating (-M-M- or -G-G-) or alternating (-M-G-M-G-) polysaccharide chains (Ma, 2008).

Alginates can either be categorised as high-G alginate, intermediate-G alginate, or low-G alginate (Fig 2.3; Fig 2.4) based on their respective ratios of M-blocks and G-blocks (Oostgaard et al., 1993; Mushollaeni, 2011; F., 2011). Alginates with lower ratios of M-blocks to G-blocks form stronger and stiffer gels (Mushollaeni, 2011).

M G **M M M M G G G G M G M G**

M – block   G – block   MG - block
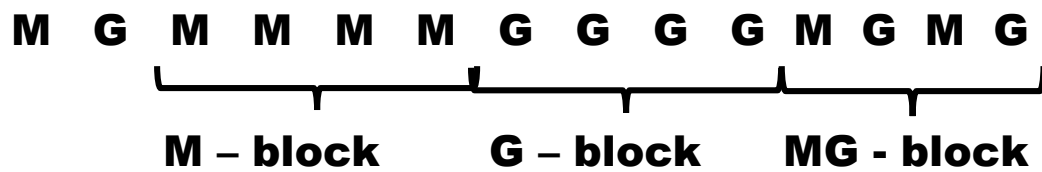
Figure 2.3: Chemical structure of alginate with M-blocks and G-blocks (Parades-Juarez et al., 2014)

GGMGMMMGGGGMMGGGGG High G alginate

M blocks   G blocks

MMMMGMGMMMMGGGGGMMMM Low G alginate

M blocks   GM blocks   G blocks
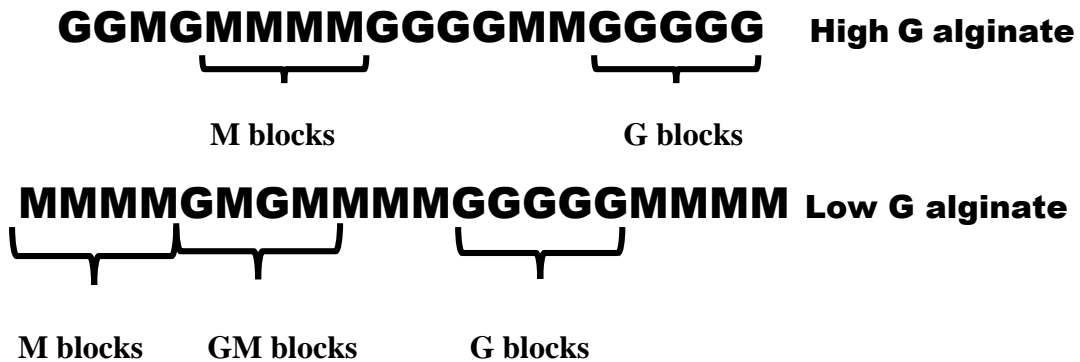
Figure 2.4: The arrangement of M-blocks and G-blocks of High G and Low G alginate (Ma and Feng, 2011)

The different ratios of M-blocks (1-4)-linked β-D-mannuronate to G-blocks (α-L-guluronate) of different seaweed species influences the physical properties of it's extracted alginate (Andriamanantoanina and Rinaudo., 2010). The different ratios of M-blocks (1-4)-linked β-D-mannuronate to G-blocks (α-L-guluronate) of different seaweed species influences the physical properties of it's extracted alginate (Andriamanantoanina and Rinaudo., 2010) and subsequently alters the structural properties and viscoelasticity (Draget et al., 2000) of their hydrogels.

Only the G-blocks of alginate participate in intermolecular crosslinking with divalent cations such as Ca^{2+} and Na^{2+} to form hydrogels. Hydrogels fabricated from high G-block alginates are stiffer and have higher stability. Generally, seaweeds from the Genus *Sargassum* (Saraswathi, Babu, and Rengasamy., 2003) have higher G-block (α-L-guluronate) to M-block (1-4)-linked β-D-mannuronate ratios, thereby producing less viscoelastic alginate which are suitable for the fabrication of hydrogels and biomaterials.

27

Less viscoelastic alginate which produces stronger biomaterials are preferable in cell culture and biomedical applications, whereas alginates with higher M-blocks (1-4)-linked β-D-mannuronate to G-blocks (α-L-guluronate) ratios from seaweeds like *Eisenia arborea* sp and *Macrocystis pyrifera* sp are preferable for applications in the food industry owing to their higher viscoelasticity (Murillo-Álvarez and Hernández-Carmona, 2007).

Generally, seaweeds from the Genus *Sargassum* have a rather low M-block (1-4)-linked β-D-mannuronate to G-block (α-L-guluronate) ratio, which falls within the range of 0.8 to 1.5, as compared to other seaweed species with high ratios like *Ascophyllum nodosum* sp, at ratio of 1.2, and *Laminaria japonica* sp at ratio of 2.2 (Minghou et al., 1984). Therefore, sodium alginate extracted *Sargassum polycystum* sp seaweed was suitable to be used for the fabrication of biomaterials for cell culture studies.

2.3.4   Extraction of Sodium Alginate

Two commonly used methods for extracting alginate are the alkaline extraction method and the high temperature extraction method. The alkaline extraction method has been proven to be preferable among various researchers. Various authors have used the room temperature alkaline extraction method for the extraction of alginate from various seaweed species to obtain a significant yield of sodium alginate.

Authors who have extracted sodium alginate using the alkaline extraction method include Mushollaeni et al., 2011 who extracted sodium alginate from *Sargassum crassifolium* sp, *Sargassum polycystum* sp, *Padina* sp and *Sargassum echinocarpum* sp, Jayasankar et al., 1993 who extracted sodium alginate from *Sargassum Wightii* sp, Viswanathan and Nallamuthu, 2014 who extracted sodium alginate from *Colpomenia sinuosa* sp*, Lobophora variegate* sp*, Chnoospora implexa* sp*, Padina gymnospora* sp*, Sargassum tenerrium* sp and *Dictyota dichotoma* sp and Basha et al., 2011 who extracted sodium alginate from *Sargassum Subrepandum* sp.

Basha et al., 2011 extracted sodium alginate from *Sargassum Subrepandum* sp using both the alkaline extraction method and high temperature extraction method at 80 ℃ and compared the percentage yield. Basha et al., 2011 obtained about of 21 % wet weight using the high temperature extraction method, and 17.5 % wet weight using the alkaline extraction method. According to Basha et al., 2011, while the high temperature extraction method yielded a slightly higher amount of sodium alginate, about 4.5% more than the alkaline extraction method, however the difference is very slight.

The high temperature extraction method uses a similar method of extraction however extraction will be done at 80°C instead of 50°C and uses formalin for pre-treatment of the seaweed (Basha et al., 2011). When formalin was used for pre-treatment of alginate, the viscosity of the sodium alginate increased by up to 60 %, extract yield increased by up to 10 % and transmittance increased by up to 30 % (Jayasankar, 1993). As expected, not using formalin for pretreatment of sodium alginate leads to a slightly lower extract yield and viscosity. However, formalin was not used in this experiment due to it being a known carcinogen.

2.3.5   Uses Of Alginate Based Products

Alginate based wound dressings like hydrogels, sponges and electrospun mats provide benefits like haemostatic capability and gel forming ability upon absorbtion of wound exudates (Sun and Tan, 2013; Límová, 2010). Alginate based dressings show increased rate of water absorbtion, better water vapour transmission rate and natural antiseptic properties (Sun and Tan., 2013). Not surprisingly, many oral and topical pharmaceutical formulations contain sodium alginate.

Sodium alginate has also been used both as a binder and disintegrator in sustained-release oral tablets and in oil-in-water emulsions such as creams and pastes, as well as a thickener, emulsifier and stabiliser in oil-in-water emulsions like facial creams (Mithal., 2007). Sodium alginate is also effective as an adjuvant in immunisation of two types of influenza virus and for treating esophagitis (Mokarram et al., 2006).

The properties of sodium alginate which are their biodegradability, ease of manipulation into microspheres and nanospheres, mucoadhesion to cells and high protein loading capacity (Jiang et al., 2007) makes them ideal for protein drug delivery. Alban et al., (2002) reported good blood-compatibility and anti-coagulation of both natural and synthetic sulfated polysaccharides.

Laboratory rats with hypertension which were fed low molecular mass potassium alginate from brown algae reported decreased systolic blood pressure (Ji et al., 2009), while calcium cross-linked sodium alginate biomaterials have been applied as haemostatic wound healing dressing (Matthew et al., 1995) with promising results. There have been studies which showed the capacity of injectable alginate-based scaffolds in bone regeneration models.

Tang et al., (2012) conducted a study by culturing mesenchymal stem cells on an alginate scaffold to determine how it effects the growth and formation of damaged bone tissue. Tang et al., (2012) found that the alginate scaffold had both osteogenic and angiogenic properties as it promoted the growth and proliferation activity of the cells.

Many researchers have also studied the suitability of using alginate-based hydrogels, alginate based porous scaffolds and alginate microspheres for controlled drug delivery in tissue engineering (Mi et al., 2003) with promising results.

In summary, alginate has been widely used in numerous fields and for various medical purposes. This is promising because usage of alginate based materials will reduce the reliance on non-organic materials like metal or plastic implants and help to reduce environmental pollution as alginate based products are biodegradable and does the environment.

2.4   **Other Polysaccharides For Fabricating Biomaterial**

Natural polymer based polysaccharides include alginate, agarose, chitosan and hyaluronic acid. Agarose, like alginate is also extracted from seaweed. Agarose is a linear polysaccharide consisting of repeating units of agarobiose, which is a disaccharide that is made up from both D-galactose and 3,6-anhydro-L-galactopyranose. Cell culture studies of agarose gels with stem cells like bovine mesenchymal stem cells, human mesenchymal stem cells, and adipose derived stem cells have shown promising results (Renier et al., 2007).

However, agarose has a lower melting temperature, is less stable and is more suitable to be used in the fabrication of hydrogel, instead of biomaterials, which are meant to last longer and have greater durability. Chitosan is a major waste product of the crab and shrimp canning industry, which is suitable to be used as a biomaterial due to it's biocompatibility with the human extracellular matrix, biodegradable, non-toxicity, good binding ability with mammalian cells, enhances formation of osteoblast cells in bones, haemostatic and fungistatic properties (Dutta et al., 2004).

Chitosan is a linear semi-crystalline polysaccharide that is comprised of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan (*N*-acetyl D-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-D-glucan (D-glucosamine) units (Rinaudo, 2006). The chitosan amino groups are positively charged and therefore they become strongly attracted to the mucin layer (Dhawan, Singla and Sinha., 2004) of the epithelial tissues.

This mucoadhesive property makes chitosan suitable for use as a polymer in biomaterials for *in vitro* purposes. In fact, this polycationic nature of chitosan gives chitosan analgesic effects, which gives it some pain relieving effect (Croisier and Jerome, 2013). The analgesic effect is achieved through protonation of amino groups of chitosan D-glucosamine in the presence of proton ions which are excreted at site of inflammation (Croisier and Jerome, 2013).

Therefore, chitosan has been used in various biomedical and pharmaceutical applications like sutures and dental implants (Rinaudo, 2006). Chitosan has shown biocompatibility with various other natural polymers and have been successfully fabricated with other biomaterials (Inmaculada et al., 2009). However, residual proteins from chitosan extraction could cause allergic reactions in patients (Chatelet, Damour and Domard., 2001).

Moreover, certain physiochemical alterations to the structure of chitosan could cause cytotoxicity (Chiellini et al., 2011). Hyaluronic acid originates from the ECM of all living animals and is obtainable in large quantities from cartilage, skin and tendons of chickens and cows. Being the main glycosaminoglycan (GAG), hyaluronic acid is responsible for rheological and physiological functions, namely lubricating joints, cartilages, regulation of substances into and out of cells and providing stability to the collagen fibres in the body (Hutmacher, Goh and Teoh, 2001).

Another polysaccharide worth noting is hyaluronic acid, which has been utilised for the fabrication of biomaterials by researchers due to their excellent cell and tissue compatibility (Galassi et al., 2000). In an experiment by Wohlrab et al., (2013), hyaluronic acid was cross-linked with 1,4-butanediol-diglycidylether, in order to form a stable biomaterial and cultured rat dermal fibroblasts on it's surface. They found that the cross-linked hyaluronic acid biomaterial showed little difference in cell proliferation activity compared to a non cross-linked hyaluronic acid biomaterial, therefore hyaluronic acid did not show much promise for the fabrication of biomaterials.

2.5 Cell Proliferation Activity on Fabricated Biomaterial

Studies regarding cancer cell proliferation activity were carried out by carrying out experiments on cancer cell models (Vargo-Gogola and Rosen, 2007). The most commonly used cancer cell model is the HeLa cancer cell line model, named after Henrietta Lacks who died in 1951 due to aggressive adenocarcinoma of the cervix. Henrietta Lacks' cancer cells were harvested and cultured as the first ever human cancer cell line in the world.

HeLa cancer cells continue to be cultured and used in various research laboratories around the world until today (Lucey et al., 2009) and is one of the most valuable tools in cancer research (Gazdar et al., 2010). Using cancer cell lines as experimental model confers advantages over normal cell lines (Vargo-Gogola and Rosen, 2007) because cancer cells lines can be easily handled and manipulated (Vargo-Gogola and Rosen, 2007).

There is a large pool of cancer cell lines to choose from (Gazdar et al., 2010), and HeLa cancer cell lines can be used as a source of unlimited cell lines (Burdall et al., 2003) since they will replicate indefinitely. Contaminated cancer cell cultures are easily retrievable, as most cancer cell lines are tough and hardy (Burdall et al., 2003).

37

Experimental results with HeLa cancer cell lines are easily reproducible and HeLa cancer cells adapt to their environment easily (Van Staveren et al., 2009). Therefore, HeLa cancer cell lines were used in this study to determine the cytotoxicity of the collagen-sodium alginate biomaterials. Cell proliferation activity and viability of the HeLa cells can be determined using the MTT assay method and trypan blue staining (Lucey et al., 2009).

2.5.1   Cytotoxicity Tests

In vitro biocompatibility assays are used to determine the suitability of fabricated biomaterials to be used in humans and animal model studies. These biocompatibility assays are performed using cell cultures to determine their cytotoxicity towards living cells and their influence on cell proliferation and growth activity (Silver and Doillon, 1989). Cytotoxicity assays usually require microscopic evaluation to observe the morphology of cells and the use of special stains like Trypan blue dye to stain the viable cells and a haemocytometer to calculate the percentage of viable cells. Quantification of cells can also be performed using a colorimetric assay such as the MTT assay.

2.5.2   MTT Assay

MTT assay, also known as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, is considered the gold standard to determine cell viability and proliferation activity since it's development by Mosmann in 1983 (Tonder, Joubert and Cromarty., 2015). The MTT assay has been used for measuring cell viability in terms of reductive activity due to enzymatic conversion of the water soluble tetrazolium compound into insoluble formazan crystals (Stockert et al., 2012). Only viable cells will bind with the yellow tetrazolium salt, MTT and become cleaved into purple formazan crystals, which is detected by a reader. The absorbance of the cells in a 96-well microplate is read using a microplate reader (Chung et al., 2015).

The conversion from tetrazolium to formazan crystals is caused by dehydrogenases which are naturally found within the mitochondria of living cells. Other organelles of the living cell such as the endoplasmic reticulum also produces dehydrogenases in lower quantities (Stockert et al., 2012). This gold-standard method has several advantages and disadvantages. The advantages of the MTT assay is that it is a well-known method, therefore the results of the experiment is easily reproducible. It can also be used for high-throughput screening and miniaturisation (Hamid et al., 2004).

However, the disadvantages of the MTT assay method are that conversion of tetrazolium to formazan crystals depends on the metabolic rate and number of mitochondria in the sample. Therefore, dead cells with metabolically active mitochondria may contribute to the conversion of tetrazolium to formazan (Sieuwerts et al., 1995; Mosmann., 1983), and thus give false positives.

Besides, multiple steps of washing with DMSO or isopropanol are also required (Mosmann., 1983). Some disadvantages of the MTT assay include innacurate cell counts because the conversion to formazan crystals in influenced by metabolic rate and the quantity of mitochondria in the cells, which may lead to false positives (Jabbar, Twentyman and Watson., 1989; Hayon et al., 2003).

According to Hayon et al., (2003), the rate of conversion of tetrazolium to formazan is directly linked to cellular metabolic activity. However, the working principle of the conversion of tetrazolium to formazan crystals by mitochondrial succinic dehydrogenases can also be caused by cytosolic enzymes like nicotinamide adenine dinucleotide (NADH) reductase and flavin oxidase which may also convert tetrazolium to formazan (Lü et al., 2012).

In other words, increased cellular metabolic activity of each cell and increased intracellular glucose concentrations may cause an increase in the conversion activity of tetrazolium to formazan and vice versa. Since conversion to formazan is mainly caused by mitochondrial activity, therefore larger living cells with more mitochondria would cause a greater conversion of formazan crystals than smaller cells or cells with less mitochondria (Jabbar, Twentyman and Watson., 1989). These factors are the known weaknesses of this "gold-standard" method.

2.5.3 Trypan Blue Exclusion Test

The trypan blue dye exclusion test is commonly used for counting the number of viable cells which would not be stained blue and the dead cells which would be stained blue using a haemocytometer. This method gives a rough count of the number of viable and dead cells. The principle of the trypan blue dye exclusion test is that the trypan blue diazo dye is negatively charged and will not interact with cells unless it's membrane is damaged.

Therefore, only dead cells would be stained blue (Tran et al., 2011; Priya and Rao, 2015; Chung et al., 2015). The advantage of the trypan blue dye is that only non-viable or dead cells would appear stained as blue while viable cells would remain unstained when viewed under a microscope (Stoddart., 2011).

Due to the molecular size of trypan blue molecules which is around 960 Daltons (Tran et al., 2011) which makes it impermeable to the cellular membrane of living cells which have a functioning cellular membrane. This is because viable cells have an intact cell membrane therefore they do not absorb dye from the surrounding growth medium, while non-viable cells easily absorb dye from the surrounding growth medium due to the lack of a cellular membrane (Stoddart., 2011).

Under a light microscope, the blue stained non-viable cells would appear swollen and bluish, while viable cells would appear bright and transparent. The trypan blue exclusion test is a cheap and fast method. However, it has to be done manually using a haemocytometer. It is therefore prone to human error such as oversight or miscalculation of cell population (Stoddart., 2011).

2.5.4   Direct Contact Test

The direct contact test is a method of material cytotoxicity testing which is carried out to evaluate the cytotoxicity of the material towards cells cultured in the presence of a material. This method is suitable for testing the cytotoxicity of low density polymers like biomaterials (Sun and Tan., 2013).

While being incubated, chemicals from the biomaterial will leach out and come into direct contact with the cells in the culture medium. Thereafter, the reaction of the cultured cells towards the biomaterial will be examined using the scanning electron microscope (SEM) to determine the rate of attachment and proliferation, deformations or lysis of the cells which come in contact with the biomaterial (Lim., 2016.)

2.6 Potential Of Collagen-Sodium Alginate Biomaterial

One example of using collagen-sodium alginate biomaterials is for the manufacturing of tissue engineered cartilage. According to Sun and Tan, (2013), this artificially engineered cartilage would be useful for patients suffering from cartilage injuries resulting from sports activities, accidents or those suffering from osteoarthritis. This can be done by injecting stem cells from healthy bones into a biomaterial which is then placed at the site of injury inside the patient's body (Sun and Tan, 2013). Collagen-alginate based hydrogels have displayed osteogenic, and angionenic properties (Lee et al., 2012).

However, this method still requires much researching before being implemented. Among the challenges facing researchers are cell seeding inefficiency, since the current method of seeding human mesenchymal stem cells causes great loss of human mesenchymal stem cells (Andersen, Auk-Emblem, and Dornish, 2015). Another difficulty is cell distribution, because the pores of scaffolds are usually either too small to allow cells inside the scaffold or too large to retain the cells inside them (Andersen, Auk-Emblem, and Dornish, 2015).

CHAPTER 3

METHODOLOGY

3.1   Raw Materials

The list of chemicals used in this research project for the extraction of sodium alginate from *Sargassum polycystum* sp seaweed include industrial grade calcium chloride, hydrochloric acid, sodium hydroxide, butanol and ethanol, while the chemicals used for extracting collagen from the skin of *Oreochromis niloticus* sp fish are industrial grade sodium hydroxide, butanol, acetic acid and Tris. The collagen-sodium alginate biomaterials were fabricated using sodium hydroxide, butanol, acetic acid, sodium chloride and Tris. Cultures of live HeLa cancer cells were cultured in $75cm^2$ cell culture flasks (SPL Life Sciences, South Korea) with DMEM (Dulbeccos' Modified Eagle's Medium, USA) – high glucose and FBS (Fetal Bovine Serum) (ScienCell Research Laboratories, USA) as the growth medium, and trypsinisation agent, Trypsin-EDTA (ChemSoln, USA).

3.2 Methods

Several tests were carried out on the extracted collagen and sodium alginate, as well as the fabricated collagen-sodium alginate biomaterials to determine their physiochemical characteristics. The tests carried out include the Fourier Transform Infrared Spectroscopy (FTIR), swelling behaviour test, sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), surface morphology analysis and reverse phase-high performance liquid chromatography (RP-HPLC). The cell proliferation tests include the colorimetric assay (MTT), Trypan blue staining and the direct contact test which was carried out using the scanning electron microscope (SEM).

3.2.1 Preparation of Sodium Alginate

The seaweed *Sargassum polycystum* sp was obtained from the shallow areas of Port Dickson beach, Malaysia. After collection, the seaweed was washed then stored in a freezer. Prior to use, the frozen seaweed was thawed and cleaned with water. Then, epiphytes were removed with a toothbrush. Seaweed was dried out on an aluminum foil for a week prior to acidification and extraction (Visweswara and Mody,1964).

Firstly, 20 g of seaweed was soaked in 300 ml of 1% (w/v) $CaCl_2$ for 18 hours at room temperature to form calcium alginate. Next, the seaweed was rinsed with 300 ml of distilled water thrice, after which it was soaked in 5% (v/v) hydrochloric acid for an hour to form alginic acid.

Following that, the seaweed was again rinsed thrice with 300 ml of distilled water to remove excess hydrochloric acid. The seaweed was then soaked with 300 ml of 3 % (w/v) $NaCO_3$ for an hour in order to convert alginic acid into soluble sodium alginate. Three hundred ml of distilled water was then added to the soluble sodium alginate solution before it was left to dry in an oven at 50 °C for 3 hours. Next, the seaweed solution was centrifuged at $3'000 \times g$ (Sigma Sartorius, USA) for 5 minutes to collect the sticky gel-like supernatant.

The sticky gel-like supernatant was collected and mixed with 300 ml of 100 % (v/v) ethanol and stirred until a solid precipitate was obtained from the mixture. The precipitate was collected and dried in an oven at 50 °C for 24 hours. The dried precipitate was blended (Warring, USA) into powder, labelled as sodium alginate powder and kept dry in a dessicator until use.

3.2.2   Preparation of Collagen

The method for extracting collagen from fish skin was based on the acid soluble collagen (ASC) method (Nagai and Suzuki, 2000) with slight modifications. Firstly, the discarded skin of *Oreochromis niloticus* sp fish was obtained from a wet market in Sungai Long, Kuala Lumpur, then leftover flesh, fins and scales were scraped away with a knife. The skin was sliced into smaller pieces before chemical pre-treatment, which begin by soaking 100 g of fish skin in 800 ml of 0.1 M sodium hydroxide for 6 hours to soften the fish skin, with the sodium hydroxide solution being changed every 3 hours.

Next, the fish skin was thoroughly washed with distilled water until the pH of the fish skin was between a neutral pH range of $6.8 - 7.2$. The final step the of chemical pre-treatment process was the soaking of fish skin in 1 L of 10 % butanol for 72 hours to defat the skin. Excess butanol was rinsed off with distilled water. Collagen was extracted by soaking the treated fish skin in a solution of 0.63 M acetic acid pH 3.25 at ratio acetic acid to fish skin of 2.5 : 1 fish skin at 4 °C for 72 hours.

Then, fish skin was salted out by sodium chloride precipitation by adding 2.5 M NaCl in 0.05 M Tris pH 7.0 to acetic acid solution at ratio of 5 : 1 acetic acid. Finally, the solution was centrifuged at 3'000 × g for 15 minutes at 4 ºC, then precipitated collagen was collected and dissolved with minimum amount of 0.5M acetic acid.

The dissolved collagen was loaded into a visking tube and dialysed against 0.1 M acetic acid at ×200 from the total dissolved volume of precipitate at 4 ºC for 48 hours. Dialysis continued with the change of same amount of distilled water for another 48 hours below 4 ºC. The dialysate was collected and freeze dried for 48 hours (Labconco, USA) at -20 ^{0} C before use.

3.2.3   Fabrication Of Biomaterials

The method of fabricating the collagen-sodium alginate biomaterials were based on the methodology by Sang et al., (2011) with slight modifications. Firstly, lyophilised collagen was dissolved in 0.25 ml of 0.5 M acetic acid to obtain a 0.7 % (w/v) collagen solution and stirred with a magnetic stirrer until complete dissolution of collagen. Two hours later, 2 M sodium hydroxide was added dropwise until the collagen solution achieved pH between 6.8 to 7.2.

Pure collagen biomaterial was prepared from 10 ml of 0.7 % (w/v) pH adjusted collagen, poured into a petri dish and allowed to gelate at room temperature for 48 hours. It was then frozen for 48 hours at - 20 ºC, prior to lyophilisation for another 48 hours.

The preparation of pure sodium alginate biomaterial was carried out by dissolving 10 g of sodium alginate powder in 100 ml of phosphate buffer (CaissonLabs, USA) in order to obtain a 10 % (w/v) sodium alginate solution, which was mixed well with a magnetic stirrer for 2 hours. Ten ml of the 10 % (w/v) sodium alginate solution was poured into a separate petri dish, briefly degassed in a sonicator and allowed to gelate at room temperature for 48 hours, prior to lyophilisation for 48 hours.

Collagen-sodium alginate biomaterials were prepared in a similar manner. The fabrication of 90 % collagen : 10 % sodium alginate biomaterial was carried out by mixing 9 ml of 0.7 % (w/v) collagen and 1 ml of 10 % (w/v) sodium alginate solution. The mixture was stirred with a magnetic stirrer for 30 minutes until homogenised before being poured into a petri dish and allowed to gelate for 48 hours at room temperature. The gelated mixture was frozen at - 20 ℃ for 48 hours prior to lyophilisation for 48 hours and labelled as collagen-sodium alginate biomaterial 90 % C : 10 % SA.

Subsequent collagen-sodium alginate biomaterials were prepared in similar fashion with ratios of 80 %:20 %, 70 %:30 %, 60 %:40 %, 50 %:50 %, 40 %:60 %, 30 %:70 %, 20 %:80 % and 10 %:90 %. Both 100 % collagen and sodium alginate biomaterials were used as controls.

3.2.4   Fourier Transform Infrared Spectroscopy (FTIR)

All spectral readings of the fabricated biomaterials were obtained using FTIR (Nicolet iS10) spectrophotometer (Thermo Scientific, USA). Biomaterials prepared from Section 3.5 were analysed by placing them between the crystal and knob of the FTIR apparatus. At an interval of 1.0 cm^{-1}, each sample was subjected to 32 scans from 4000 cm^{-1} to 400 cm^{-1} at a resolution of 2.0 cm^{-1}. Background spectral analysis was performed before the spectral analysis of each sample. The results were recorded as percentage transmission (%) against absorbance (cm^{-1}). Samples of the biomaterials fabricated in Section 3.5 were analysed in triplicates (Belbachir et al., 2009).

3.2.5   Scanning Electron Microscopy (SEM)

Surface morphology and micro structures of the fabricated biomaterials were analysed with a SEM (Hitachi, Japan). Based on the method by Liu, Ma and Gao, (2012) square pieces measuring 1 cm x 1 cm were cut and fixed on the specimen holder prior to sputter coating with gold and palladium for 5 minutes. High resolution images were captured with accelerating voltage of 15 kv and $\times$ 50 to $\times$ 1.0 k magnification. Both SE (scattered electrons) and BSE (back scattered electrons) were used for scanning morphological analysis.

3.2.6   Swelling Behaviour

Equilibrium swelling ratio test was carried out to determine the degree of swelling for each of the collagen-sodium alginate biomaterials. Standard sized pieces ($\pm$ 0.5 mm) in diameter of the collagen-sodium alginate biomaterials were prepared with single-hole paper puncher. The pieces were prepared in triplicates, submerged in 15 ml of 10 % (w/v) phosphate buffer solution pH 7.2, then incubated at 37 °C. After blotting out excess phosphate buffer solution with filter papers, they were weighed at time intervals of 6, 24 and 48 hours to determine their final wet weight. Equilibrium swelling ratio was calculated using Equation 3.1 (Özeroglu and Birdal, 2009).

$$\text{Swelling Ratio} = \frac{W_s - W_d}{W_d}$$

<div align="right">(Eq 3.1)</div>

(Where W_s = final weight, W_d = initial dry weight)

3.2.7   Gel Electrophoresis

Gel electrophoresis was carried out by Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemlli, 1970) for estimating the molecular weight of extracted collagen. Five mg of collagen was dissolved in 250 µl of 0.1 M acetic acid prior to mixing with 4 µl of 5× Laemlli buffer at a ratio of 4 : 1. The 5× Laemlli buffer was prepared by mixing 1 ml of 10 % (w/v) bromophenol blue with 10 ml of deionised water.

Type I Bovine Achilles Tendon (SigmaAldrich, USA) was similarly prepared as the positive control. The SDS-PAGE High Range Molecular Weight Standard (45.0 kDa to 200.0 kDa) (Biorad, USA), Type I Bovine Achilles Tendon, and collagen were boiled for 10 minutes with 5 times sample buffer (distilled water, 0.5 M Tris-HCL (pH 6.8), glycerol, 10 % (w/v) SDS solution and 0.1% Bromophenol Blue) at a sample buffer to sample ratio of 1:4 (v/v) before being separated on a 4.5% polyacrylamide stacking gel (deionised water,

30 % bis-acrylamide, 0.5 M Tris - HCL (pH 6.8), 10 % (w/v) SDS solution, TEMED and 10 % (w/v) ammonium persulfate and 7.5 % polyacrylamide separating gel (deionised water, 30 % bis-acrylamide, 1.5 M Tris - HCL (pH 8.8), 10 % (w/v) SDS solution, TEMED and 10 % (w/v) ammonium persulfate.

Following electrophoresis at a constant current of 70 V/gel (140 V for 2 gels), 0.05 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and 5 % (v/v) acetic acid were used to stain the polyacrylamide gel, after which 30 % (v/v) methanol and 10 % (v/v) acetic acid were used for destaining the polyacrylamide gel.

3.2.8   Amino Acid Analysis

Amino acid analysis was carried out using reverse phase High Performance Liquid Chromatography (RP-HPLC) to determine the amino acids present in collagen extracted from skin of the *Oreochromis niloticus* sp fish. Ten mg of lyophilised collagen was mixed with 6 N Hcl to a ratio of 1:10 for hydrolysis prior to carrying out RP-HPLC analysis using a microwave digestor (Berghof, Germany) for 6 hours at 150 °C.

After hydrolysis, the hydrolysate was cool for 2 minutes in an ice bath, filtered with a 0.4 µm membrane and derivatised. Derivatisation reagents OPA (o-phthalaldehyde) (Sigma-Aldrich, USA) and FMOC (9-Fluorenylmethyl chloroformate) (Sigma-Aldrich, USA) were used to increase the hydrolysate's detection sensitivity by the UV detector (Shimadzu, Japan) with 265 nm to detect primary amino acids and 330 nm to detect secondary amino acids.

The derivatised hydrolysate was left to stand for 1 to 2 minutes in a fridge below 4 ℃ before 10 µl of derivatised hydrolysate was loaded into a Zorbax Eclipse Plus C18 Column (4.6 x 150 mm) (Agilent, USA) in a RP-HPLC system (Shimadzu, Japan). These steps except hydrolysis were repeated for the Amino Acid Standard (Sigma-Aldrich, USA).

Two mobile phases were prepared for this study. Mobile phase A was 0.1 M Potassium Phosphate pH 6.9, while Mobile Phase B was acetonitrile, methanol and distilled water at a ratio of 9:8:3, with oven temperature (Shimadzu, Japan) set 35 ℃ and flow rate of 0.40 ml/minute. The elution programme is shown in Table 3.1.

Table 3.1: Mobile Phase Elution Programme

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 5	100	0
5 – 6	40	60
6 – 15	40	60
15 – 20	60	40
20 – 20	100	0

The amount of amino acid present in the collagen hydrolysate was estimated based on the area under the RP-HPLC chromatogram for the Amino Acid Standard. The concentration of amino acids present in the collagen hydrolysate was determined by comparing the concentration of amino acids present in the standard from Equation 3.2 (Kupiec, 2014) while the hydroxylation ratio (%) of Proline was determined from Equation 3.3 (Nagarajan et al., 2013).

$$\text{Concentration Amino Acid (µmol/ml)} = \left[\frac{\text{Sample Peak Area}}{\text{Standard Peak Area}} \right] \times \left[\frac{\text{Concentration}}{\text{of Standard}} \right]$$

(Eq 3.2)

$$\text{Hydroxylation ratio of Proline (\%)} = \left[\frac{\text{Hydroxyproline}}{\text{Hydroxyproline + Proline}} \right] \times 100$$

(Eq 3.3)

57

3.3   Cell Proliferation Tests

Cell proliferation tests were carried out to determine the cytotoxicity of the collagen-sodium alginate biomaterials towards live HeLa cancer cells which were subcultured from a laboratory officer's parent culture. The HeLa cancer cells were subcultured until 100 % confluency before seeding on the collagen-sodium alginate biomaterials. The HeLa cancer cells were cultured in 75 cm^2 cell culture flasks (SPL Life Sciences, South Korea) with 10 % Fetal Bovine Serum complete growth medium which consists of Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (ScienCell Research Laboratories, USA) and Fetal Bovine Serum (ScienCell Research Laboratories, USA) in an incubator at 37 ºC, 5 % CO_2.

The complete growth medium was changed every 2 days and the subcultured HeLa cancer cells were periodically observed until they achieved 90 % - 100 % confluency. Then, triplicate pieces of the 50 % C : 50 % SA and 80 % C : 20 % SA biomaterials were prepared using single-hole paper puncher and incubated with HeLa cancer cells in a 6-well microtitre plate over a period of 8 days.

3.3.1 Colorimetric Assay (MTT)

In *vitro* cytotoxicity tests are carried out to determine the cytotoxicity of substances or materials that come into direct contact with living cells and to study how they affect the growth and proliferation activity of cells. *In Vitro* cytotoxicity tests includes the MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay, Trypan blue staining and direct contact test. The MTT assay was used to study metabolic rate and proliferation activity of the HeLa cancer cells.

The HeLa cancer cells which were seeded and cultured after Day 2, 4, 6 and 8 were trypsinised using EDTA-trypsin (ChemSoln, USA), treated with *In Vitro* Toxicology Assay Kit MTT (Sigma-Aldrich, USA) then incubated for 4 hours at 37 °C, 5 % CO_2. Four hours later, the residual MTT was removed, and MTT solubilization solution (Sigma-Aldrich, USA) was added and incubated for 15 minutes at 37 °C, 5 % CO_2. Finally, the absorbance (A_{570}) of purple formazan in the viable cells were detected by measuring their absorbance at 570 nm with a microplate reader (Tecan, Switzerland).

3.3.2   Trypan Blue Exclusion Dye Test

Trypan blue staining test uses a special 0.2 % (w/v) Trypan blue dye (HyClone Thermo Fischer, USA) which is unable to penetrate the cell membranes of living cells. Therefore, the number of viable and dead cells can be counted as the viable cells remain unstained, while the dead cells are stained blue. On days 1, 3, 5 and 8, a piece of biomaterial that had been incubated with HeLa cancer cells were trypsinised with trypsin/EDTA.

Five minutes later, growth media was added at a ratio of 1:10 of trypsin/EDTA to the trypsinised cells to neutralise trypsin activity. Next, 0.2 % (w/v) Trypan blue (Thermo-Fischer Scientific, USA) solution was added at a ratio of Trypan blue solution to growth media 1:1. Then, 10 µl of the solution was dropped into a haemocytometer and viewed under a light microscope.

The number of unstained viable cells and stained dead cells were counted and the percentage viability of the HeLa cancer cells for each biomaterial were calculated based on the formula in Eq 3.4 by Yevgeniy, (2014).

Total viable cells/ml = Viable cells x $\frac{dilution\ factor}{no.of\ squares}$ x $10'000$ (Eq 3.4)

Total viable cells/ml = Viable cells x $\frac{2}{5}$ x $10'000$ (Eq 3.5)

Percentage of viable cells (%) = $\frac{Total\ viable\ cells}{Total\ cell\ count}$ x 100 (Eq 3.6)

3.3.3　Direct Contact Test

The direct contact test was carried out to observe growth and proliferation activity of the HeLa cancer cells on the fabricated biomaterials. On Day 8 of cell culture, the collagen-sodium alginate biomaterials were rinsed with $10 \times$ Phosphate Buffer Saline pH 6.8 (CaissonLabs, USA), and fixed with 2.5 % (v/v) glutaraldehyde (Sigma-Aldrich, USA). An hour later, the biomaterials were dehydrated with a graded ethanol (Sigma-Aldrich, USA) series (30 %, 50 %, 70 %, 90 %, 99.5 %) for a total period of 15 minutes.

The dehydrated collagen-sodium alginate biomaterials were left to dry, then sputter coated (Quarum, United Kingdom) with gold and palladium. Next, adhesion of the HeLa cancer cells on the surface of the collagen-sodium alginate biomaterials were observed using scanning electron microscopy (Hitachi, Japan) at accelerating voltage of 15 kv between $\times$ 500 to $\times$ 1.0 k magnification.

CHAPTER 4

RESULTS

4.1   Fabrication of Biomaterials

The current study focuses on the usage of fish waste from wet markets in order to produce value added products. Collagen from discarded fish skin was extracted and blended with sodium alginate from seaweed to fabricate biomaterials. The collagen-sodium alginate biomaterials were characterised in terms of cytotoxicity tests, swelling behaviour, spectral analysis and surface morphology evaluation with SEM. Collagen-sodium alginate biomaterials were prepared with a combination of different collagen to sodium alginate ratios which includes 100 % C : 0 % SA, 90 % C : 10 % SA, 80 % C : 20 % SA, 70 % C : 30 % SA, 60 % C : 40 % SA, 50 % C : 50 % SA, 40 % C : 60 % SA, 30 % C : 70 % SA, 20 % C : 80 % SA, 10 % C : 90 % SA, and 0 % C : 100 % SA respectively.

4.1.1   Comparison of Fabricated Biomaterials

The biomaterials fabricated from each combination are shown in Figure 4.1. A noticeable difference between the biomaterials was their colour. Generally, the appearances of the biomaterials were influenced by their respective composition of sodium alginate and collagen.

The pure collagen biomaterial (Figure 4.1 (a)) was pure white, while the pure sodium alginate biomaterial (Figure 4.1 (c)) was a deep yellow. The biomaterials fabricated with higher concentrations of collagen to sodium alginate (60 % C : 40 % SA 70 % C : 30 % SA, 80 % C : 20 % SA, and 90 % C : 10 % SA) showed a similar appearance as the pure collagen biomaterial (Figure 4.1 (a)).

The biomaterials fabricated with higher concentrations of sodium alginate to collagen (40 % C : 60 % SA, 30 % C : 70 % SA, 20 % C : 80 % SA, and 10 % C : 90 % SA) showed a similar appearance as the pure sodium alginate biomaterial (Figure 4.1 (c)).
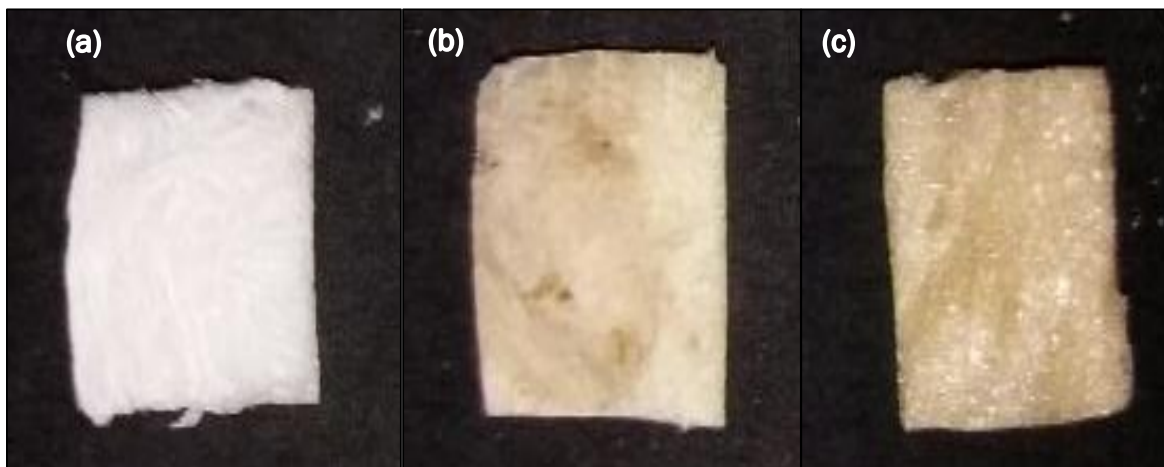
Figure 4.1: Noticeable colour differences between the biomaterials with different ratios of collagen to sodium alginate; a.) pure collagen biomaterial; b.) 50 % C : 50 % SA; c.) sodium alginate biomaterial.

4.2   Fourier Transform Infrared Spectroscopy (FTIR) of Collagen –

Sodium Alginate Biomaterial

FTIR analysis was conducted on all the collagen-sodium alginate biomaterials. The purpose of carrying out spectral analysis was to reveal the functional groups of the fabricated biomaterials. The pure collagen biomaterial (Figure 4.2 a) showed the five characteristic spectral intervals of collagen which are the amide A band at 3419.31 cm^{-1} , amide B band at 2924.20 cm^{-1}, amide I band at 1636.42 cm^{-1}, amide II band at 1540.78 cm^{-1} and amide III band at 1235.78 cm^{-1} (Belbachir et al., 2009; Sujithra et al., 2013).

The pure sodium alginate biomaterial (Figure 4.2 b) showed the characteristic spectral intervals of polysaccharides which correspond to the functional groups, OH group at 3231.34 cm^{-1}, carboxylate group (C-O-O) at 1597.29 cm^{-1} and 1406.43 cm^{-1}, pyranose ring at 1025.27 cm^{-1}, and uronic acid group at 946.11 cm^{-1} (Daemi et al., 2012; Narra et al., 2012).

Biomaterials fabricated with different collagen-sodium alginate ratios demonstrated the distinctive spectrums of both the pure collagen biomaterial and the pure sodium alginate biomaterial, for example the 50 % C : 50 % SA biomaterial (Figure 4.3) shows the presence of OH group, amide B, amide II, C-O-O group, pyranose ring and uronic acid group, indicating thorough mixing of collagen with sodium alginate. This indicates that collagen and sodium alginate were successfully crosslinked in the collagen-sodium alginate biomaterials. The other fabricated biomaterials with various collagen – sodium alginate ratios showed similar FTIR results.
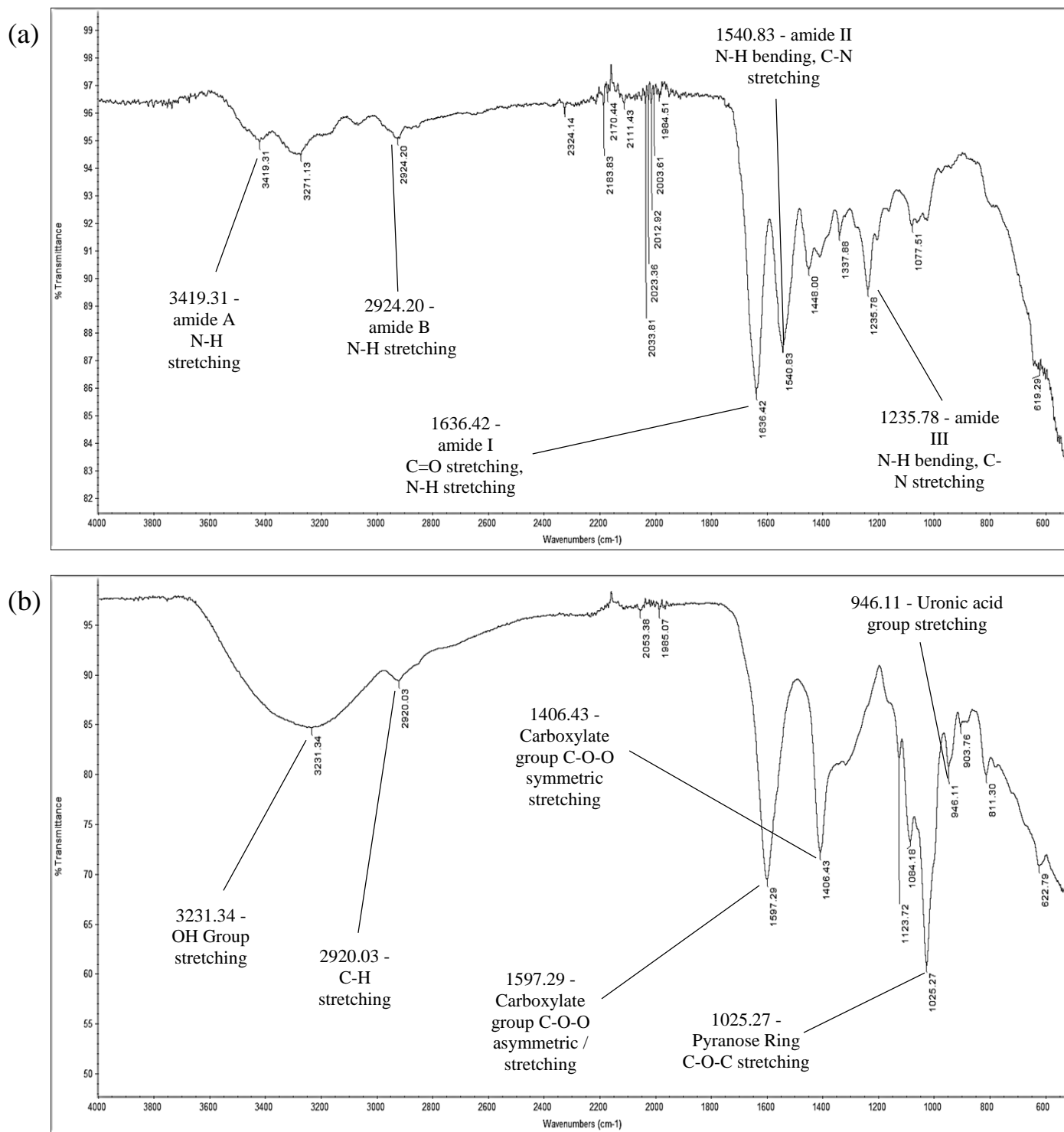
Figure 4.2: Comparison of FTIR spectral analyses of (a), pure collagen biomaterial; (b), pure sodium alginate biomaterial.
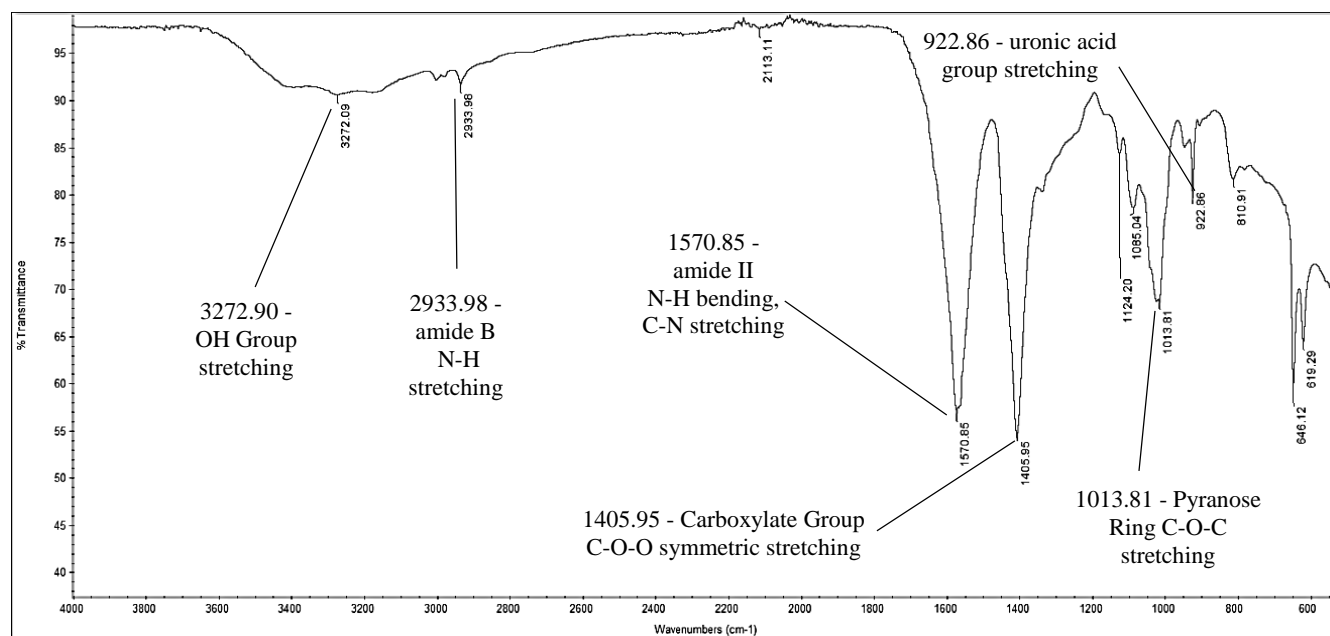
Figure 4.3: FTIR spectral analysis of 50 % C : 50 % SA biomaterial.

4.3   **Surface Morphology Analysis (SEM) of Collagen – Sodium Alginate Biomaterial**

The surface morphology of the collagen-sodium alginate biomaterials were observed using SEM (scanning electron microscopy). SEM analysis of the pure sodium alginate biomaterial (Figure 4.4 (a,b)) shows a highly interconnected open pore network. This is due to the property of sodium alginate which is made up of alternating buckle shaped G-blocks (L-guluronic acid) and ribbon shaped M-blocks ((1→4)-β-D-mannuronic acid), which are covalently bonded in sequences of blocks. The pure collagen biomaterial (Figure 4.5) showed a poreless and veinous surface, whereby the collagen fibrils tightly overlapped each other, forming a compact poreless surface.

The distinct surface morphology of the 20 % C : 80 % SA biomaterial (Figure 4.6) whereby the individual collagen fibrils are clearly seen to be branching out in random directions in linear fashion. Many of the collagen fibrils overlap each other in a cursive manner. Collagen form elongated fibrils due to their molecular structure which consists of numerous polypeptide chains of amino acids which are wound together into a stiff triple-helical structure. The triple-helical structures are then wound together in bundles to form each individual collagen fibril.

The individual collagen fibrils of the 20 % C : 80 % SA biomaterial (Figure 4.6) can be clearly seen and identified due to the low concentration of collagen in the biomaterial, unlike the pure collagen biomaterial (Figure 4.5). Surface morphology of the 50 % C : 50 % SA biomaterial (Figure 4.7) clearly shows that it has many pores on it's surface. Collagen fibrils can be seen beneath the surface of the 50 % C : 50 % SA biomaterial (Figure 4.7), indicating that the biomaterial was three dimensional in shape and has structural depth.

The other biomaterials with different ratios of collagen to sodium alginate had similar surface morphology. In general, the collagen – sodium alginate biomaterials which were fabricated from both sodium alginate and collagen had an ideal balance of pore interconnectivity and structural depth to support the growth and proliferation of cells.

(a)
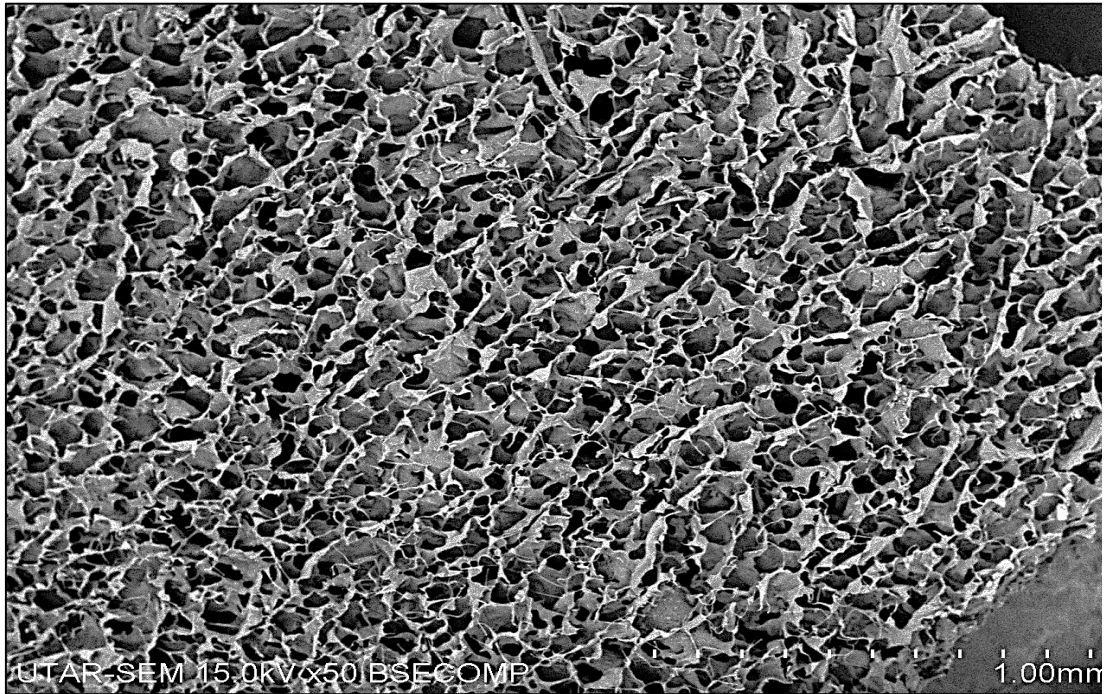
(b)
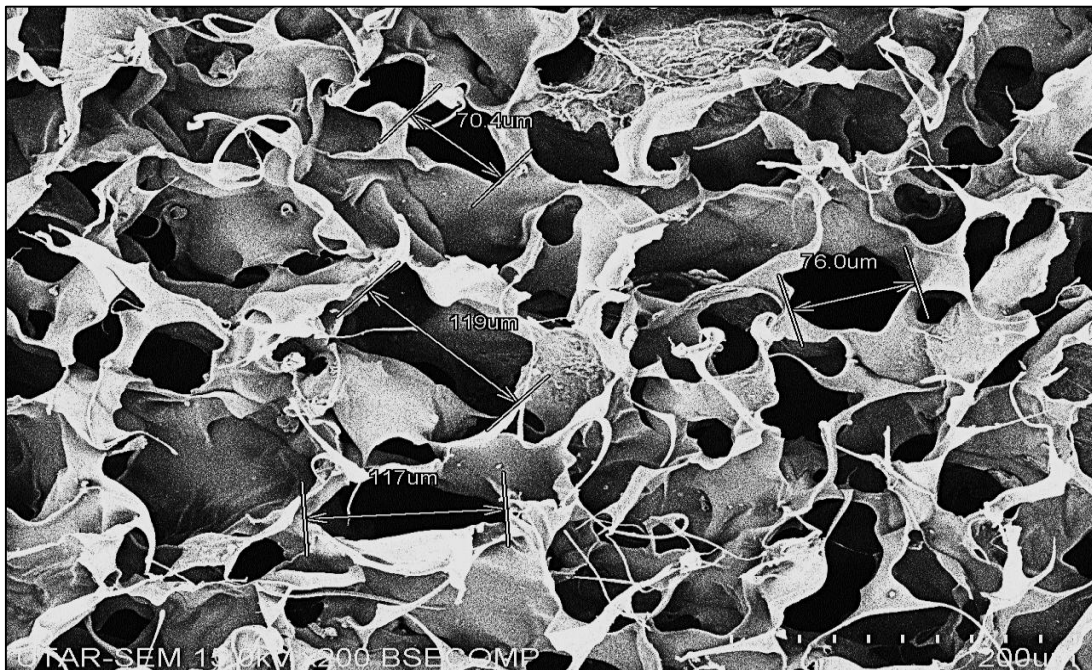
Figure 4.4: SEM analysis of pure sodium alginate biomaterial at (a), × 50 magnification. (b), × 200 magnification.

(a)

UTAR-SEM 15.0kV x150 SE   300um

(b)

63.2um

53.6um

58.0um

UTAR-SEM 15.0kV x500 BSECOMP   100um
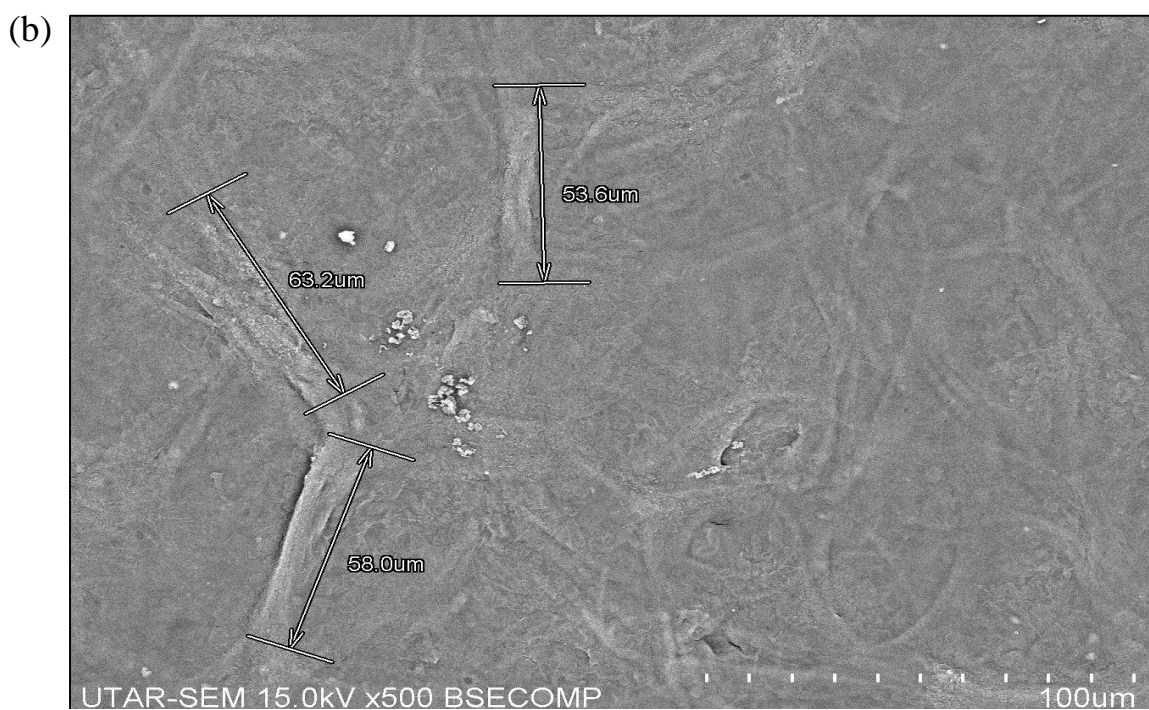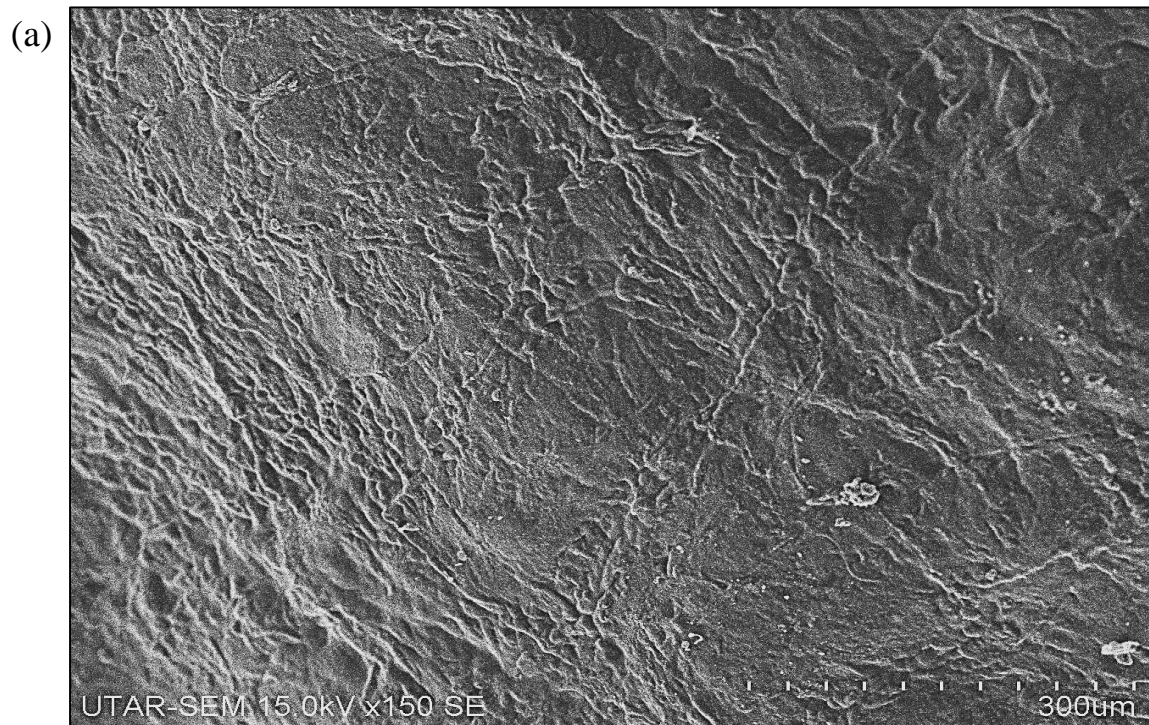
Figure 4.5: SEM analysis of pure collagen biomaterial at, (a), × 150 magnification; (b) × 500 magnification.

(a)

UTAR-SEM 15.0kV x200 BSECOMP   200um

(b)

57.8um

60.9um

UTAR-SEM 15.0kV x1.00k BSECOMP   50.0um
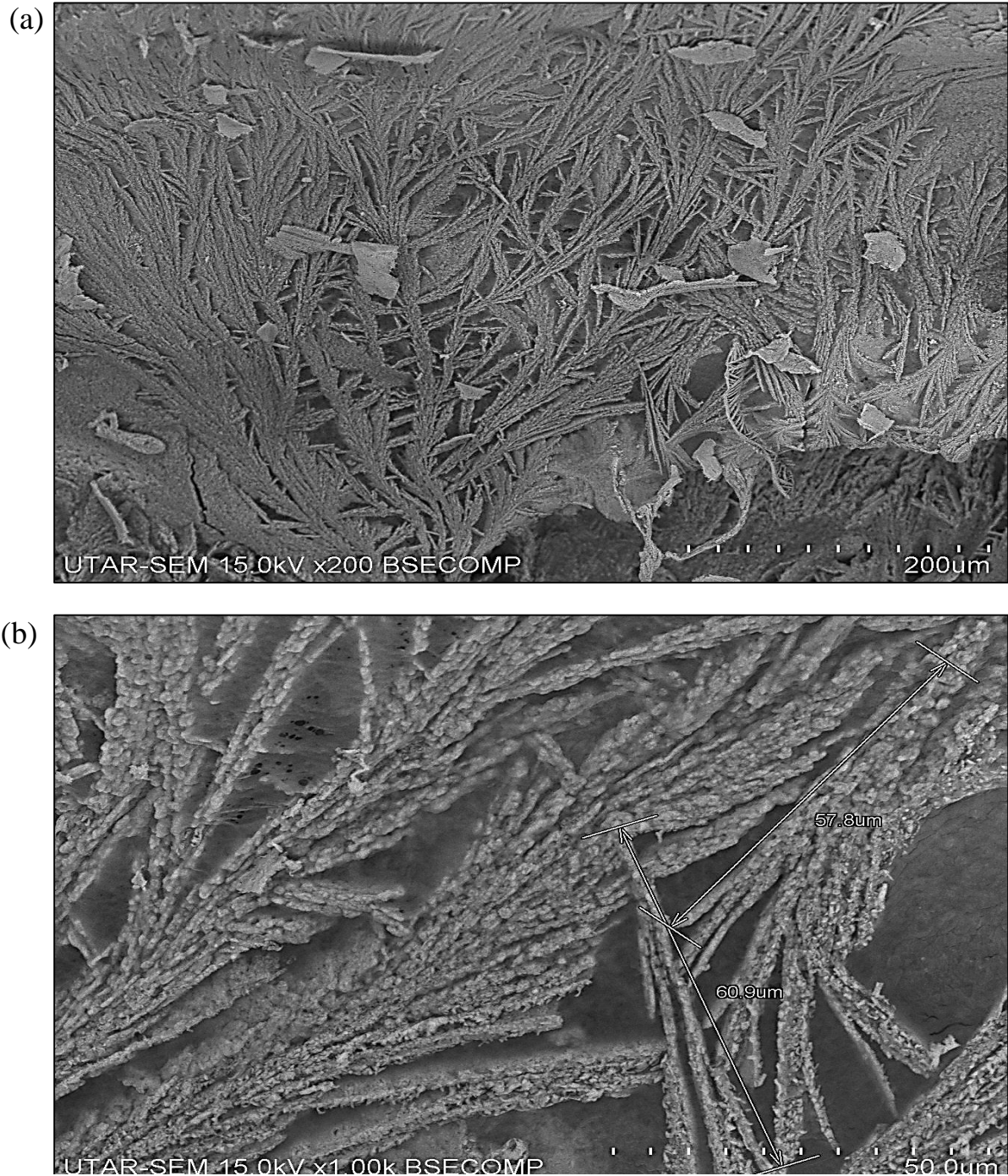
Figure 4.6: SEM analysis of 20 % C : 80 % SA biomaterial at (a), × 200 magnification; (b), × 1.0 k magnification with observable collagen fibrils.

(a)

387um

407um

300um

UTAR-SEM 20.0kV 8.3mm x50 SE   1.00mm

(b)

UTAR-SEM 20.0kV 8.3mm x50 SE   1.00mm
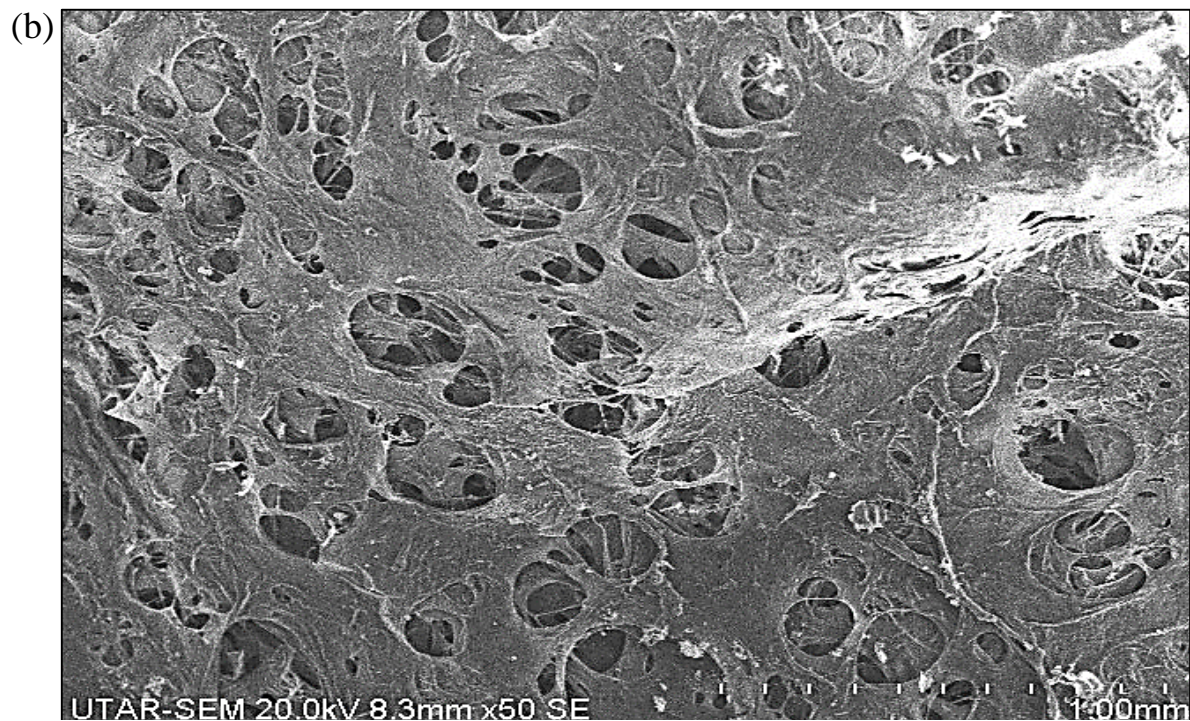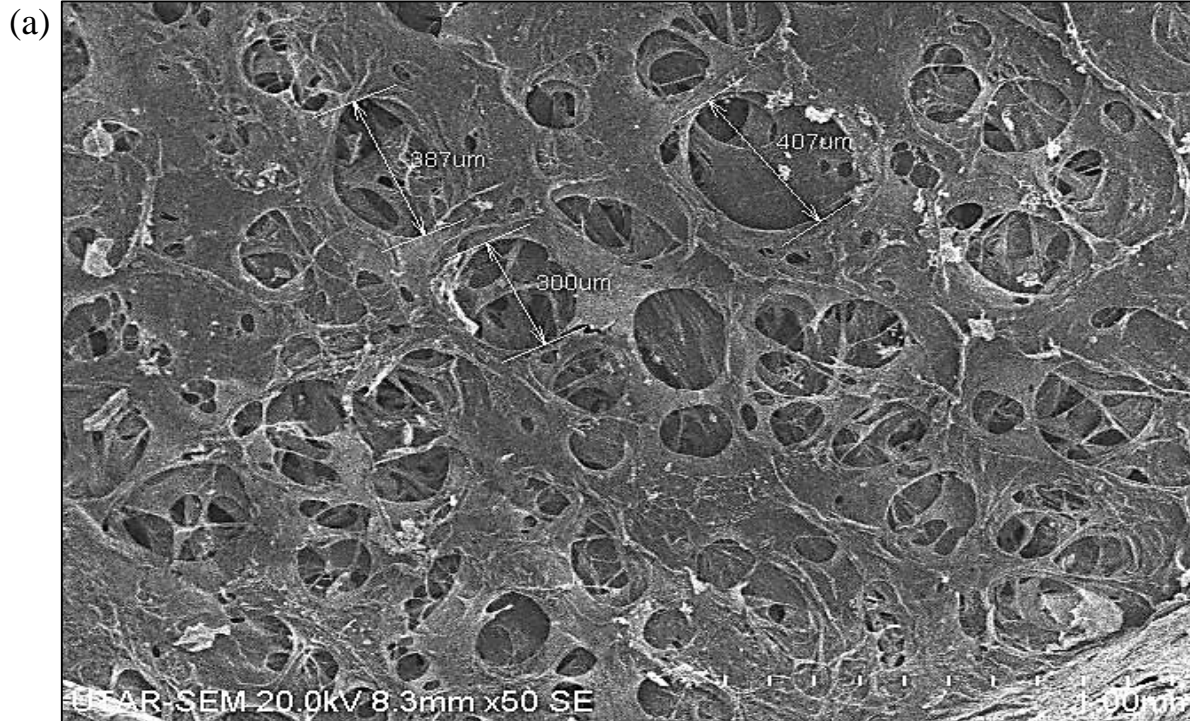
Figure 4.7: SEM analysis of 50 % C : 50 % SA biomaterial at, (a), $\times$ 50 magnification with pore diameters ranging from 300µm to 407µm; (b), $\times$ 50 magnification.

4.4   Swelling Behaviour

Polymers expand in volume and gain weight as they absorb liquid from their environments. The degree of swelling of polymers can be calculated through their changes in weight. The purpose of studying the swelling behaviour of the fabricated biomaterials was to determine their degree of swelling. The swelling behaviour of the collagen-sodium alginate biomaterials were studied by calculating the difference between their initial and final wet weights within a 48 hour period.

It is interesting to note that the collagen-sodium alginate biomaterials fabricated from higher sodium alginate to collagen ratios such as the 10 % C : 90 % SA; 20 % C : 80 % SA; 30 % C : 70 % SA and 40 % C : 60 % SA recorded a higher degree of swelling compared to collagen-sodium alginate biomaterials fabricated with lower sodium alginate to collagen ratios such as the 60 % C : 40 % SA; 70 % C : 30 % SA; 80 % C : 20 % SA and 90 % C : 10 % SA.

The 30 % C : 70 % SA biomaterial recorded the highest equilibrium swelling ratio at 12.75 % while the 90 % C : 10 % SA biomaterial recorded the lowest equilibrium swelling ratio at 0.04% (Figure 4.8). This is due to the hydrophilic nature of polysaccharides such as sodium alginate.

Generally, equilibrium swelling ratios increased with immersion time and reached a plateau after 24 hours for all the collagen-sodium alginate biomaterials. The 50 % C : 50 % SA biomaterial only reached a plateau after 48 hours, while the pure collagen and pure sodium alginate biomaterials became dissolved after 24 hours, therefore their equilibrium swelling ratios could not be determined.
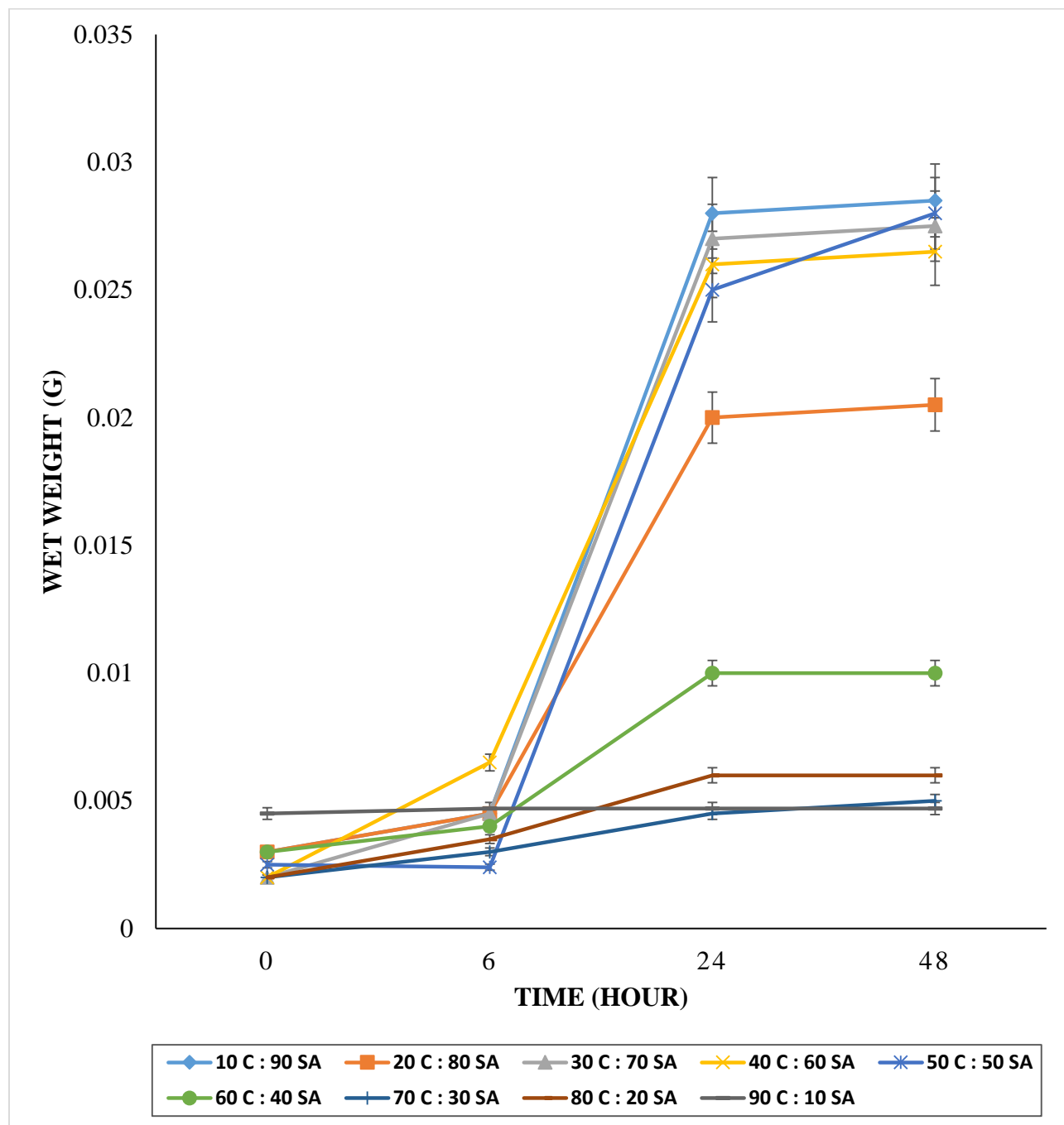
Figure 4.8: Swelling rates of the fabricated biomaterials in wet weight (g) against time (hour)

4.5   Gel Electrophoresis

Identification of type of collagen was carried out using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). Collagen from skin of *Oreochromis niloticus* sp, was electrophorised Type I Collagen of Bovine Achilles Tendon (Sigma-Aldrich, USA) as a positive control. The protein patterns of the extracted collagen sample matched Type I Collagen of Bovine Achilles Tendon (Figure 4.9) with a doublet pattern for the upper $\alpha 1$ (approximately 130 kDa to 135 kDa) and lower $\alpha 2$ chains (approximately 120 kDa to 125 kDa) and a single β chain (approximately 220 kDa to 225 kDa). The density for $\alpha 1$ was twice as much as α_2. The collagen extracted from the fish skin of *Oreochromis niloticus* sp has a composition of $(\alpha_1)_2\, \alpha_2$ heterotrimer, which confirmed it's identity as Type I collagen.
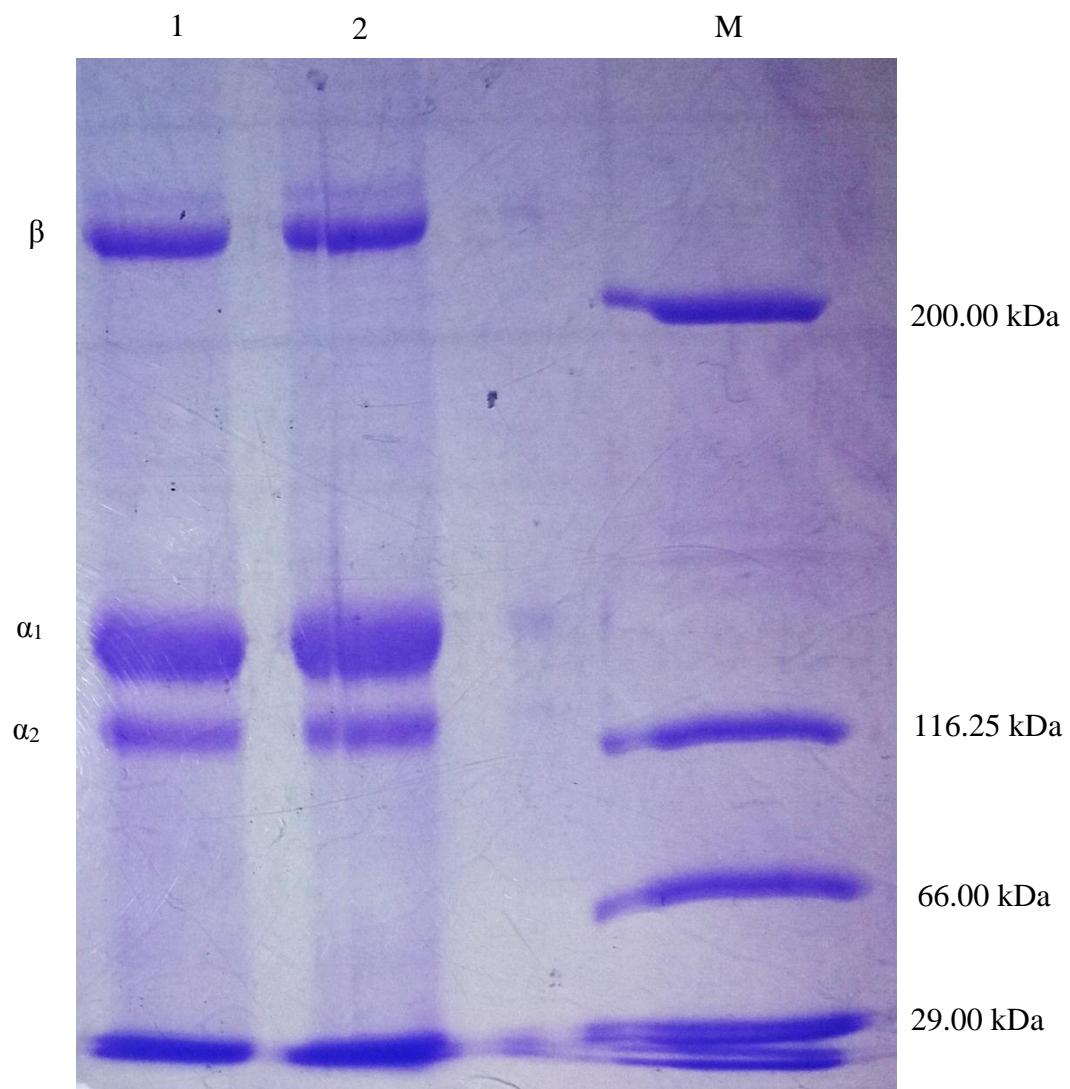
Figure 4.9: Gel electrophoresis results of, M, molecular weight marker; 1, Type

I collagen; 2, extracted collagen.

4.6   Amino Acid Analysis

      Reverse-phase HPLC was used to analyse the fish collagen samples. A total of 11 amino acids were detected in the extracted collagen. The amino acid composition of collagen (Figure 4.10) demonstrates that Imino acid (hydroxyproline and proline) and glycine were the amino acids with the highest concentrations. The concentration of the three most vital amino acids for forming the triple helical polypeptide structure of collagen which were hydroxyproline, proline and glycine were present at the highest concentrations whereby hydroxyproline was 2.477 µmol / ml, proline was 2.066 µmol / ml, while glycine was 1.422 µmol / ml. The degree of hydroxylation of proline for extracted collagen was calculated to be 54.52 %. Hydroxyproline is a non-essential amino acid derivitised during the post-translational protein modification of the essential amino acid proline. Hydroxyproline concentration in relation to proline concentration is used to measure the degree of collagen catabolism, therefore it is an invaluable indicator of collagen concentration (Sharma et al., 2008).
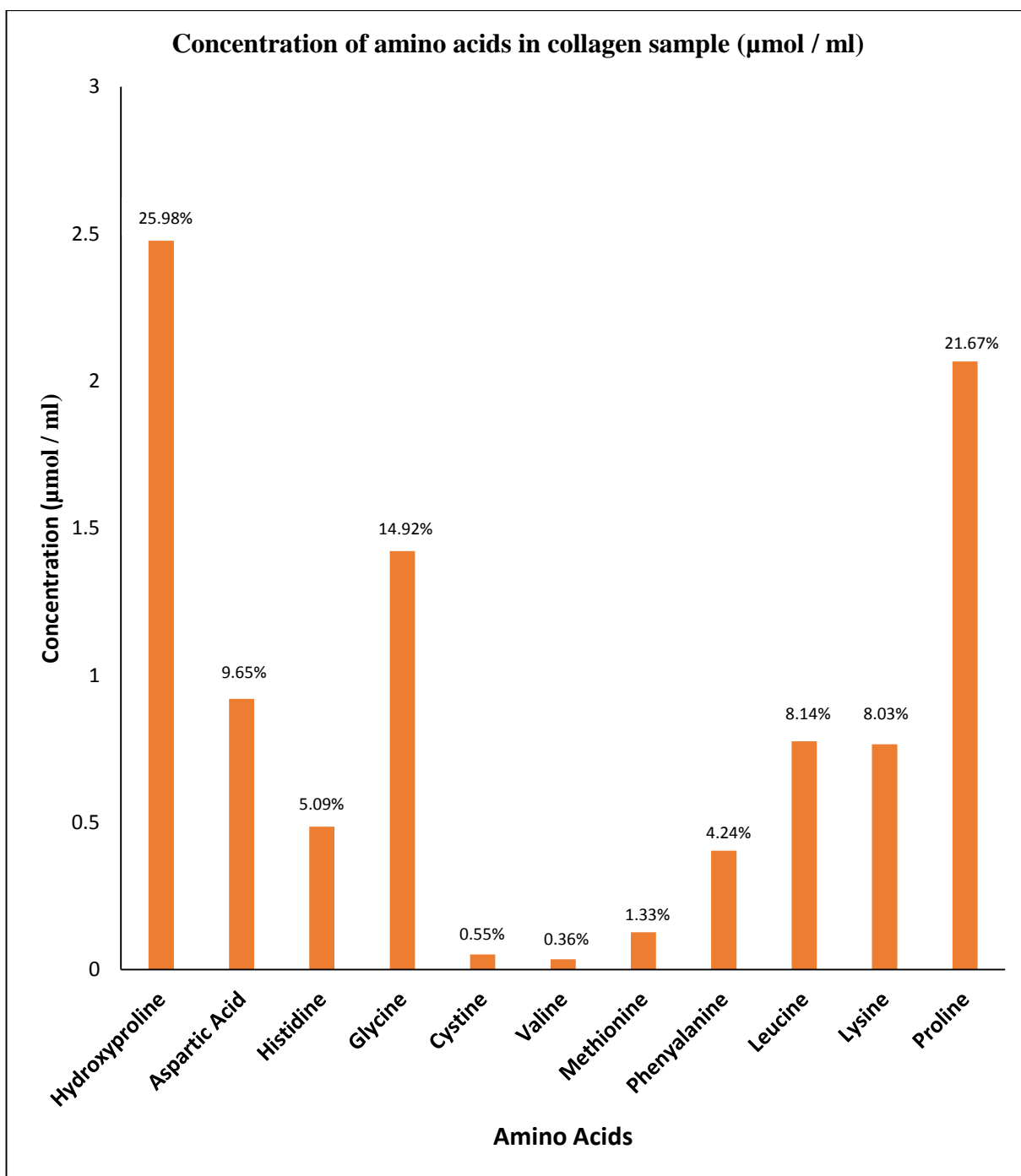
Figure 4.10: Concentration of amino acids in collagen sample (μmol / ml)

4.7　　　　Colorimetric Assay (MTT)

Cytotoxicity test using the fabricated biomaterials were carried out using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay seeded with HeLa cancer cells on Day 2, 4, 6 and 8 with absorbance of the viable cells measured at 570 nm using a microplate reader (Tecan, USA). Figure 4.11 showed the results of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]) assay on Days 2, 4, 6 and 8 of HeLa cancer cell seeding and culturing on the collagen-sodium alginate biomaterials.

On Day 2, the optical density (OD) readings of all the fabricated biomaterials was higher than the control with the 20 % C : 80 % SA biomaterial recording the highest OD, while the control had the lowest OD reading. On Day 4, all the biomaterials and control experienced more than double increase in OD readings. Again the 20 % C: 80 % SA biomaterial recorded the highest OD reading while the control recording the lowest OD reading. Maximum cell growth and proliferation activity occurred during this period.

On Day 6, all the biomaterials had a sharp decrease in OD readings but surprisingly, the OD readings were still higher than Day 2. Surprisingly on Day 8 all the biomaterials and control recorded an almost identical OD reading. This shows that the biomaterials positively influenced (Nazarpak et al., 2013) growth of the HeLa cancer cells. The fabricated biomaterials encouraged HeLa cancer cell proliferation as seen in Figure 4.13. It is also interesting to note that on Day 2, 4 and 6, almost all the biomaterials had consistently higher OD readings than the control, but on Day 8, all the biomaterials and control recorded almost similar OD readings.
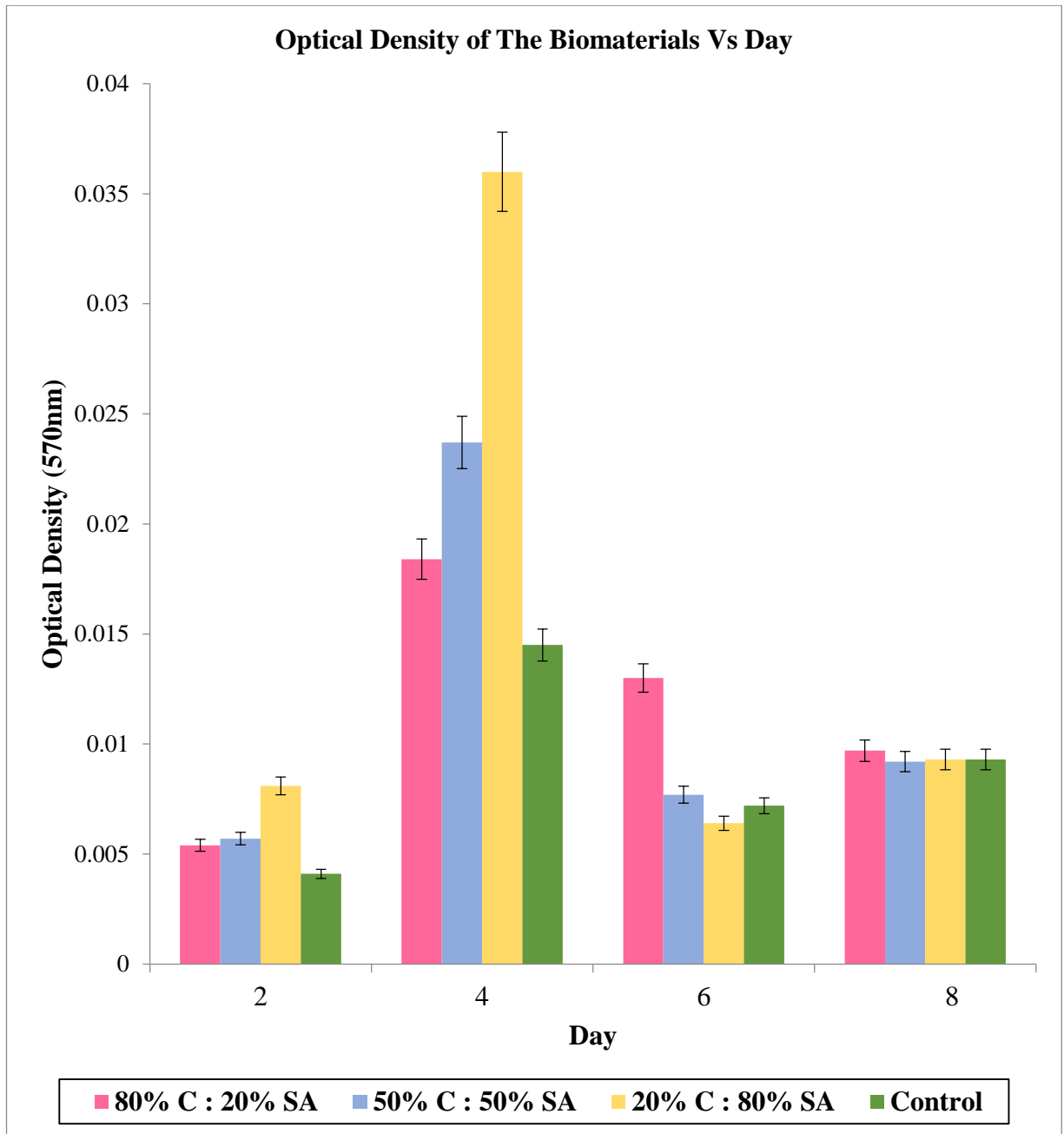
Figure 4.11: Mean Optical Density readings (570nm) of the collagen-

sodium alginate biomaterials versus day

4.8　　　　　Trypan Blue Staining

Cell counts of the HeLa cancer cell seeded biomaterials were done on Day 2, 4, 6 and 8 using the trypan blue staining technique, whereby the number of viable cells on the fabricated biomaterials were trypsinised, stained with trypsin blue (ThermoFischer Scientific, USA) and counted with a haemocytometer. The viable cells were counted and percentage of viable cells was calculated (Figure 4.12). The HeLa cancer cells were around 30% viability for the 80 % C : 20 % SA biomaterial, with the control recording the lowest viability, around 15 % (Figure 4.12) on Day 2 of the study. On Day 4, the percentage viability for the 80 % C : 20 % SA biomaterial reached almost 95%, while the control had about 50 % viability (Figure 4.12). However on Day 6, the number of viable cells of all fabricated biomaterials and control decreased drastically, with 80 % C : 20 % SA biomaterial recording about 40 % viability, while control recorded lowest percentage around 20 %. On Day 8, the fabricated biomaterials and control has almost viability (Figure 4.12).
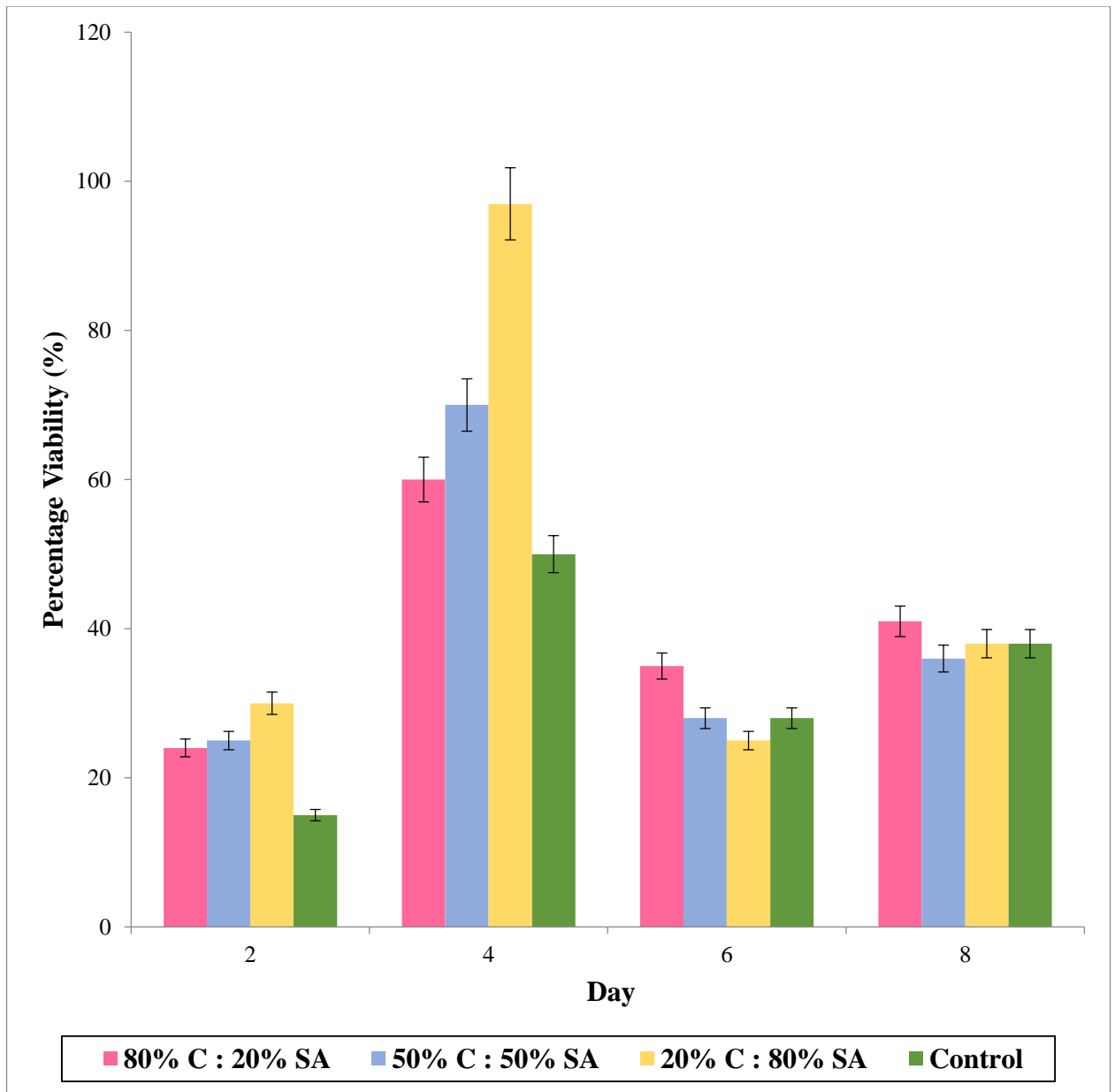
Figure 4.12: Percentage of viable HeLa cells from Day 2 to Day 8 of cell culture studies using different collagen – sodium alginate biomaterials

4.9   Direct Contact Test

The results of the direct contact test were observed with SEM. On Day 4, the direct contact test was carried out on the collagen - sodium alginate biomaterials which were used in the cell culture studies which were the 80 % C : 20 % SA; 50 % C : 50 % SA; and 20 % C : 80 % SA biomaterials. Direct contact test could not be carried out on Day 8 as by then all the biomaterials had been dissolved. The surface of the 50% C : 50 % SA biomaterial (Figure 4.13) had been fixed with HeLa cancer cells with ethanol and glutaraldehyde. The HeLa cancer cells in Figure 4.13 and had undergone lysis due to osmosis by ethanol and glutaraldehyde which was used as the fixative agent, with only the tough outer membranes of the Hela cancer cells remaining.

This gives the HeLa cancer cells a net-like appearance (Figure 4.13). The other collagen - sodium alginate biomaterials had a similar surface morphology. The cells seem to be able to fit snuggly into the many crevices and gaps on the uneven surface of the biomaterials. It is suggested that there were more HeLa cancer cells adhered on these biomaterials, however they had been lysed completely. In conclusion, the biomaterials did not pose any cytotoxicity towards the cells as they were able to support the attachment, growth and proliferation of the cells.

(a)

(b)

(c)
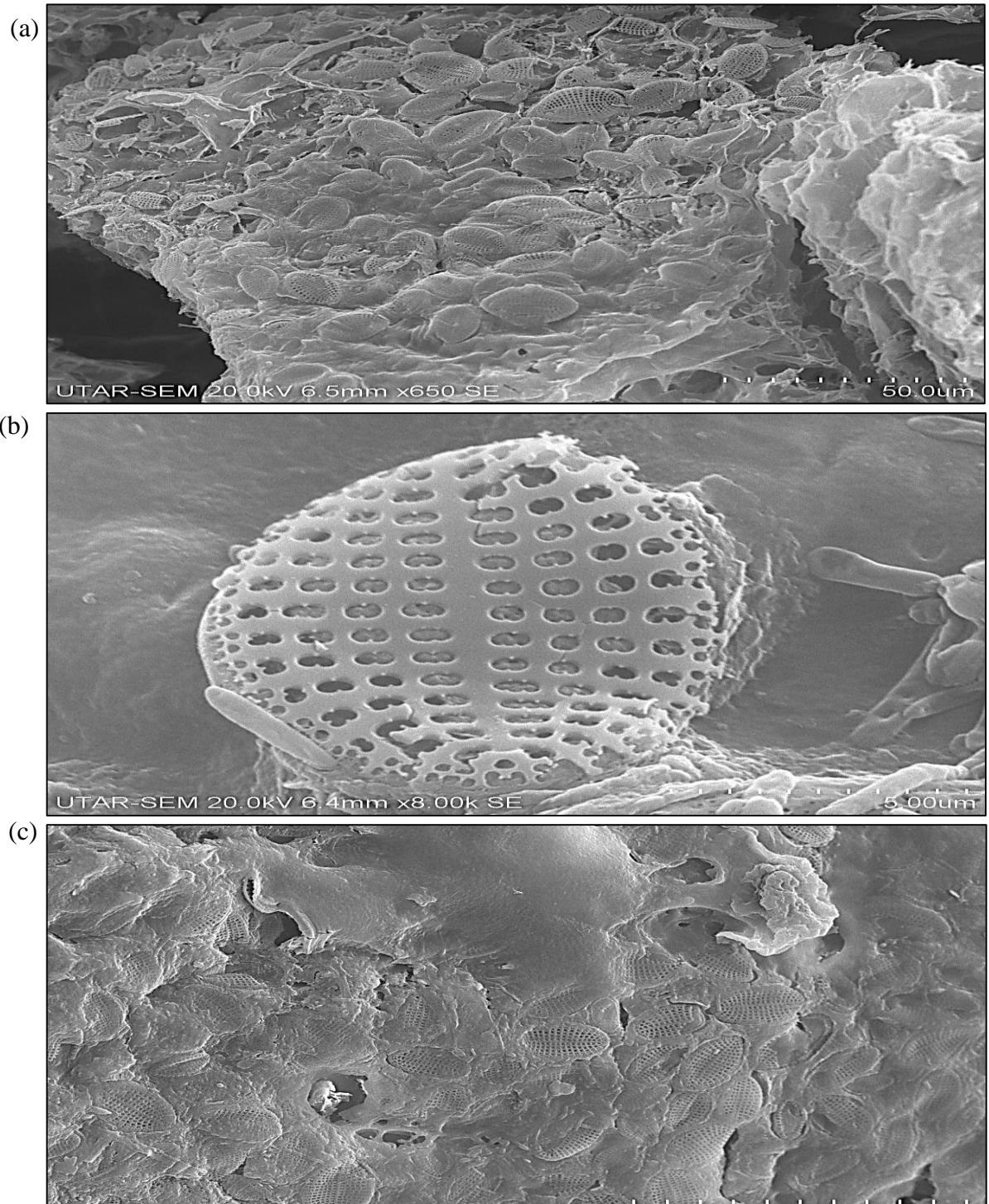
**Figure 4.13: SEM analysis of biomaterials with HeLa cancer cells adhered on
it's surface at, (a), × 650 magnification; (b), single HeLa cancer
cell at × 8.0k magnification; (c), × 900 magnification.**

CHAPTER 5

DISCUSSION

5.1   Yield of Collagen from Fish Waste

The dry weight of collagen extracted from fish skin of *Oreochromis niloticus* sp was about 16.5 % - 18.75 % of it's original weight. This amount is considered reasonable, as other authors like Potaros et al., (2009) extracted about 20.7 % of dry weight collagen and Sujithra et al., (2013) extracted 22.0 % of dry weight collagen from a similar fish species, while Sinthusamran et al., (2011) extracted about 15.8 % dry weight collagen from *Lates calcifer* sp.

This indicates that a relatively optimum amount of collagen was extracted from the fish skin of *Oreochromis niloticus* sp in the present study. Collagen, being a thermo-instable protein is influenced by changes in temperature and solvent pH and will become denatured even if left at room temperature. Therefore, the extraction process of collagen was carried out below 4 ℃ to avoid the denaturing of collagen.

Collagen has been traditionally extracted using neutral salt buffers, however (Gross and Kirk, 1958) various authors (Potaros et al., 2009; Sujithra at el., 2013; Sinthusamran et al., 2011; Nagai and Suzuki, 2000) have used organic acids such as dilute acetic acid to extract collagen from marine sources with Channel catfish skin yielding 24.0 % on dry weight basis (Nagai and Suzuki, 2000), Nile tilapia yielding 22.0 % on dry weight basis (Sujithra et al., 2013), Nile tilapia yielding 20.7 % on dry weight bases (Potaros et al., 2009), and Asian sea bass at 15.8 % on dry weight basis (Sinthusamran et al., 2011).

Extraction efficiency is influenced by the type of acid used for the extraction of collagen (Skierka and Sakowska, 2007). Organic acids like dilute 0.5 M acetic acid are used for collagen extraction from marine animal sources. Examples are Nagai and Suzuki, (2000) and Sujithra et al., (2013) who used dilute 0.5 M acetic acid to extract acid soluble collagen (ASC) from fish waste.

On the contrary, inorganic acids like 1.0 M hydrochloric acid will result in reduced collagen extraction yield (Skierka and Sakowska, 2007). Acetic acid concentration and pH also affects collagen extraction yield. According to Nagai et al., (2010), collagen yield reaches an optimum level when 0.63 M acetic acid pH 3.25 is used for extraction. According to Wang et al., (2008), the pH of the solvent used alters the charge density of protein molecules. By weakening their molecular structure and intermolecular electrostatic interaction, the rate of denaturation of collagen was thereby increased.

5.2 **Yield of Sodium Alginate from Seaweed**

The dry weight of sodium alginate extracted from the seaweed *Sargassum polycystum* sp in this experiment was about 12.5 % - 17.0 % of the dry weight of the seaweed. This amount is considered fair, as other researchers like Latifi et al., (2015) had extracted about 12.0 % - 16.5 % of dry weight sodium alginate, and Mushollaeni, (2011) extracted about 20.0 % of dry weight sodium alginate from the same seaweed species, while Basha et al., (2011) extracted about 17.5 % dry weight sodium alginate from *Sargassum subrepandum* sp.

It can be concluded that a relatively reasonable percentage of dry weight sodium alginate was extracted from *Sargassum polycystum* sp in this study. The addition of sodium hydroxide (NaOH) in excess produces a highly alkaline solution which causes rapid oxidative-reductive depolymerisation of the phenolic compounds of *Sargassum polycystum* sp, turning it brown in colour (Davis et al., 2004).

5.3   **FTIR Analysis of Biomaterial**

The biomaterial fabricated from pure collagen had the spectral intervals characteristic of Type I collagen such as the amide I band at 1636 cm^{-1}, amide II band at 1540 cm^{-1}, amide III band at 1235 cm^{-1}, amide A band at 3419 cm^{-1}, and amide B band at 2924 cm^{-1} (Belbachir et al., 2009; Sujithra et al., 2013).

The presence of amide I band enables the identification of the secondary structure of proteins such as collagen. This was because the amide I bands are related with the stretching vibrations of the carbonyl functional group on the polypeptide backbone of collagen. Therefore, detection of the amide I band spectrum indicated the presence of highly structured polypeptide chains such as the secondary structure of proteins (Campos Vidal and Mello., 2011).

The presence of amide II bands shows the N-H bending vibrations of the collagen polypeptide backbone. A shift to a lower frequency of 1540 cm^{-1} for the pure collagen biomaterial as seen in Figure 4.2 (a) is due to the presence of hydrogen bonds which causes hydroxylation. Amide III bands reflect the C-H stretching vibrations of the polypeptide backbone.

The results in Figure 4.2 (a) shows that collagen was successfully extracted from the skin of the *Oreochromis niloticus* sp fish using the acid soluble collagen (ASC) method by Nagai and Suzuki, (2000).

The pure sodium alginate biomaterial had the spectral intervals of functional groups which are characteristic of polysaccharides such as the hydroxyl (OH) functional group at 3231.34 cm^{-1}, C-H functional group at 2920.03 cm^{-1}, carboxylate (COO) group at 1597.29 cm^{-1} and 1406.43 cm^{-1}, pyranose ring at 1025.27 cm^{-1} and uronic acid group at 946.11 cm^{-1} (Narra et al., 2012).

The shift of the asymmetric and symmetric stretching of the carboxylate (COO) group to 1597.29 cm^{-1} from a range between 1620 cm^{-1} to 1598 cm^{-1} demonstrates the interaction of the homopolymeric chain of alginate with sodium ions to form insoluble sodium alginate (Daemi et al., 2012).

The shift of the infrared peaks of the asymmetric stretching vibration of the carboxylate (COO) functional groups was due to increases in the charge density, radius and atomic weight as alginate became bound with sodium ions. FTIR analysis of the carboxylate (COO) functional group shows analysis of alterations in the structure of alginate (Daemi et al., 2012).

The presence of the OH functional group at 3272.09 cm^{-1}, amide B band at 2933.98 cm^{-1}, amide II at 1570.85 cm^{-1}, carboxylate (C-O-O) group at 1405.95 cm^{-1}, pyranose ring at 1013.81 cm^{-1} and uronic acid functional group at 922.86 cm^{-1} in the FTIR spectral analysis of the 50 % C : 50 % SA biomaterial in Figure 4.3 shows that sodium alginate was present in the biomaterial. The other biomaterials fabricated with different ratios of collagen – sodium alginate demonstrated similar results.

The biomaterials fabricated with collagen and sodium alginate as the raw materials were cross-linked with calcium chloride. FTIR analysis demonstrated that calcium crosslinking had taken place, as evidenced by strong hydrogen bonding and electrostatic force of the fabricated biomaterials. Blending collagen and sodium alginate together enhanced the mechanical and physiochemical properties of the fabricated biomaterials (Thomas., 2013).

5.4   **Surface Morphology of Fabricated Biomaterials**

Pore morphology like the size, shape and interconnectivity of the pores influences cell growth rates and proliferation activity (Singh, Kumara and Ratner, 2004). In this study, the scanning electron microscope (SEM) was used to carry out surface analysis of the morphology of biomaterials (Abed et al., 2012) fabricated from pure collagen and pure sodium alginate as well as biomaterials fabricated from both collagen and sodium alginate.

Pore diameter size of the fabricated biomaterials should be larger than the critical size of 10 μm to effectively support cell proliferation and infiltration, as well as gas and nutrient diffusion deep within the biomaterial (Lyons et al., 2008; Phan et al., 2006). The pure sodium alginate biomaterial (Figure 4.4) showed a highly porous surface morphology with highly interconnected pores, some of which with diameters between 70.4 μm to 119 μm, making the biomaterial suitable for cell culture studies.

The reason for such a highly porous surface morphology is due to the structure of alginate which is comprised of alternating buckle shaped G-blocks (α-L-guluronic acid) and ribbon shaped M-blocks ((1→4)-β-D-mannuronic acid).

In the presence of bivalent cations like sodium ions, the sodium ions form hydrogen bonds with alginate to form gels with interconnected and highly porous surfaces through ionotropic gelation (Bodmeier and Wang., 1993). The pure sodium alginate biomaterial has a highly porous surface morphology suggesting good interconnectivity and plenty of room for cellular penetration into the biomaterial's surface.

Similar surface morphology was reported by Sang et al., (2011), Anderson et al., (2012), Baniasadi and Minary-Jolandan., (2015), Branco da Cunha et al., (2014) and Frampton et al., (2011). Surface morphology analysis of the 50 % C : 50 % SA biomaterial by SEM (scanning electron microscopy) (Figure 4.7) demonstrated the presence of pores which were enlarged as air bubbles exited the surface of the biomaterial during the freeze drying process.

The pores were much larger than the pure sodium alginate (Figure 4.4), with diameters of up to 300 μm and 407 μm. Upon further inspection, SEM analysis reveal that the biomaterial has several highly porous layers beneath its' surface, suggesting that the biomaterial is in a three dimensional structure. The pure collagen fabricated biomaterial (Figure 4.5) showed a flat and even surface without any pores or holes which is due to the tight overlapping of individual collagen fibrils until the collagen fibrillar meshwork could not be observed (Siddiqui et al., 2013).

Fabricating biomaterials with a lower concentration of collagen, such as 10 % − 30 % collagen enabled the collagen fibrils to be evenly spread out and observed clearly (Figure 4.6). Extracting collagen at lower temperatures rather than at higher temperatures decreases the rate of denaturation of collagen (Kadler et al., 1996). The collagen fibrillar meshwork (Figure 4.6) which consists of individual elongated collagen fibrils could be observed overlapping each other in a periodic cross-striating pattern.

Fibrillar collagen meshwork structures were also reported by researchers like Parenteau-Bareil et al., (2010), Belbachir et al., (2009), Fligiel et al., (2003), and Minary-Jolandan and Yu., (2009). This indicates that the fibrillar structure of collagen was preserved after extraction, in this study.

5.5   Swelling Behaviour

The growth and proliferation rate of anchorage dependent cells like HeLa cancer cells are limited by the surface area of the biomaterials to which they adhere to. Absorbtion of growth media by the collagen-sodium alginate biomaterials leads to their swelling and subsequent increase in total surface area. The degree of swelling of collagen-sodium alginate biomaterials is an important physical characteristic which can be calculated using the equilibrium swelling ratio test (Equation 3.1).

In general, the equilibrium swelling ratios of the collagen-sodium alginate biomaterials are influenced by their respective ratios of collagen to sodium alginate. Fabricated biomaterials with a lower ratio of collagen to sodium alginate for example the 30 % C : 70 % SA biomaterial (Figure 4.8) recorded the highest equilibrium swelling ratio at 12.75, while the 10 % C : 90 % SA biomaterial (Figure 4.8) recorded the lowest equilibrium swelling ratio at 0.04.

This is due to the hydrophilic nature of sodium alginate, which is a polysaccharide (Uzunalan et al., 2013). According to research by Lin et al., (2011) alginate is a hydrophilic polysaccharide which is made up of numerous carboxylic and hydroxyl groups.

This explains why biomaterials fabricated with lower collagen to sodium alginate concentrations have higher equilibrium swelling ratios, for example the 10 % C : 90 % SA biomaterial with equilibrium swelling ratio of 8.50, as compared to biomaterials fabricated with higher collagen to sodium alginate concentrations like the 90 % C : 10 % SA biomaterial, with an equilibrium swelling ratio value of 0.04.

Unfortunately, both the pure collagen biomaterial and pure sodium alginate biomaterials had been dissolved within 24 hours of incubation in phosphate buffer solution at 37 ℃. Interestingly, all the other collagen-sodium alginate biomaterials remained undissolved after 48 hours of incubation in phosphate buffer solution at 37 ℃ (Figure 4.8). This was probably due to the crosslinking achieved by the collagen-sodium alginate biomaterials, which made the biomaterials more stable.

In summary, it can be expected that biomaterials fabricated from lower collagen to sodium alginate concentrations have greater swelling behaviours. Their ability to expand makes them more suitable for supporting cell growth and proliferation activity.

As a comparison, the 20 % C : 80 % SA biomaterial in this study had an equilibrium swelling ratio of 8.17, while Sang et al., (2011) obtained an equilibrium swelling ratio value of 9.19 for a biomaterial of similar ratio. The 50 % C : 50 % SA biomaterial in this study recorded an equilibrium swelling ratio of 10.20 compared to 14.58 obtained by Sang et al., (2011), while the 80 % C : 20 % SA biomaterial in this study recorded a ratio of 2.00 compared to an equilibrium swelling ratio of 1.00 by Lee et al., (2012) for a biomaterial with the same ratio.

These comparisons show that the biomaterials in this study were homogenized and properly fabricated with adequate pore space for absorbing liquids and expanding. The slight difference in equilibrium swelling ratio values of the biomaterials obtained in this study as compared with authors like Sang et al., (2011) and Lee et al., (2012) were due to the different hydrophilicity of seaweed sources used by the other researchers. This is explained by Draget et al., (2000) whom states that different seaweed species consist of different quantities of G-blocks and M-blocks, which influence their psychochemical characteristics like water absorbtion (Draget et al., 2000).

5.6   Gel Electrophoresis

Gel electrophoresis (SDS-PAGE) was carried out on collagen from skin of *Oreochromis niloticus* sp, positive control Type I Bovine Achilles Tendon on a 7.5 % polyacrylamide gel in the presence of SDS. The electrophoretic patterns shows collagen having a double α_1 (approximately 130 kDa to 135 kDa) chain and α_2 chain (approximately 120 kDa to 125 kDa) and β chain (approximately 220 kDa to 225 kDa), with the density of the α_1 chain being more than double the density of the α_2 chain (Figure 4.9).

Muyonga et al., (2004) characterised Type I collagen from Nile Perch *(Lates niloticus* sp*)* and reported the presence of double α_1 (approximately 120 kDa) and α_2 chain (approximately 115 kDa) and β chain (approximately 200 kDa). Ogawa et al, (2004) characterised Type I collagen from the skin of Black Drum (*Pogonias cromis* sp) and Sheepshead (*Archosargus probatocephalus* sp) fish and found double α_1 (at approximately 130 kDa) and α_2 chain (at approximately 110 kDa) and β chain (at approximately 200 kDa). Skierka and Sadowska, 2007 extracted Type I collagen from Baltic cod (*Gadus morhua* sp).

It is concluded that collagen extracted from skin of *Oreochromis niloticus* sp fish has a $(\alpha_1)_2 \ \alpha_2$ heterotrimer triple helical molecular structure which is characteristic of Type I collagen (Chang, Shefelbine, and Buehler., 2012) as reported by the other researchers. A similar electrophoretic pattern was reported by Sujithra et al., (2013) who extracted collagen from *Oreochromis niloticus* sp. The two distinct α_1 and α_2 chains and single β chain of collagen confirms it's identity as Type I collagen, with reference to Type I Bovine Achilles Tendon (Sigma-Aldrich, USA).

5.7   Amino Acid Composition

Protein is comprised of homogenous amino acids in a polypeptide chain that coil into a triple helix structure that is comprised of three polypeptide chains (α-chains), with the amino acid glycine at every third residue along each polypeptide chain in a repeating X-Y-Gly sequence, whereby X and Y can be any amino acid. The iminio acids hydroxyproline, proline and glycine appear the most frequently in a repeating Pro-Hyp-Gly sequence in the polypeptide chains of extracted fish skin collagen (Kadler et al., 1996).

The amino acid proline in a Pro-Hyp-Gly sequence are hydroxylised into hydroxyproline by hydrogen bonding (Muyonga et al., 2014). The imino acids hydroxyproline and proline in Pro-Hyp-Gly sequence allows the sharp twisting and coiling of the triple-helical structure of collagen (Brinckmann, Notbohm., and Müller., 2005), which increases the stability of collagen (Inouye, Sakakibara., and Prockop., 1976).

Authors such as Ogawa et al, 2004; Muyonga et al., (2004) and Kittiphatanabawon et al., (2009) also reported the presence of hydroxyproline, proline and glycine in their amino acid analyses. The amino acid composition of the extracted collagen (Figure 4.10) shows that the concentration of hydroxyproline was 2.477 µmol / ml, proline was 2.066 µmol / ml and glycine was 1.422 µmol / ml. This relates to the triple-helical structure of collagen in a Gly-Pro-Hyp sequence of Type I collagen as reported by Yamamoto et al., (2016).

Glycine, hydroxyproline and proline plays the role as major amino acids of collagen because of the repeating sequence of the Gly-Hyp-Pro arrangement of amino acids on collagen's polypeptide chain. The high concentration of the amino acids glycine, hydroxyproline and proline (Figure 4.10) identifies the material extracted from the *Oreochromis niloticus* sp fish as collagen.

Amino acid tryptophan was not found in the extracted collagen sample. The absence of amino acid tryptophan is consistent with the findings by other authors whose extracted collagen samples were also devoid of tryptophan such as Muyonga et al., (2004), Kittiphattanabawon et al., (2010) and Nagarajan and Shakila., (2013).

The total Imino acid (hydropxyproline and proline) content of collagen is influenced by the fish's diet, living environment and particularly habitat temperature (Nagarajan et al., 2013). Generally, fish have lower imino acid content than mammals (Foegeding et al., 1996), which contributes to their lower collagen content.

Oreochromis niloticus sp fish are bred by aquaculture in Malaysia and fed food such as corn, soybean, rice bran, brewer's grain, wheat pollard and molasses which are rich in carbohydrates but lacks a diet of other smaller fishes which is a varied source of protein (Foegeding et al., 1996). Asian waters may also be responsible for the lower imino acid content (Nagarajan et al., 2013).

5.8   Colorimetric Assay (MTT)

Increasing OD (optical density) readings (Figure 4.11) when HeLa cancer cells were cultured with the collagen-sodium alginate biomaterials than the control indicates greater cell proliferation activity and growth. This proves that the collagen-sodium alginate biomaterials had positive effects on the HeLa cancer cells. Cell proliferation activity was measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide) assay. The optical density (OD) readings of all the collagen - sodium alginate biomaterials from Day 2 to Day 8 showed a similar pattern as the control.

In this study, none of the collagen-sodium alginate biomaterials studied posed any cytotoxicity towards the HeLa cancer cells (Figure 4.13). This is proven by the fact that the HeLa cancer cells were able to attach on the collagen-sodium biomaterials and proliferate. Antibiotics were not added into the growth medium as it may interfere with HeLa cancer cell growth. Good biocompatibility was reported by all the collagen-sodium alginate biomaterials studied as indicated by sharp increases in mean optical density (OD) on Day 4 (Figure 4.11).

The HeLa cancer cells were metabolically active and proliferating, with a doubling time between 12 hours to 24 hours (Nema and Khare, 2012) as shown by the more than double increase in OD readings by all the collagen-sodium alginate biomaterials on Day 4 (Figure 4.11). It is worth noting that the 20 % C : 80 % SA biomaterial recorded the highest OD reading, while the control recorded the lowest OD reading respectively.

This was probably due to their highly porous surface morphology (Figure 4.6), which facilitated the migration and adherence of the HeLa cancer cells inside the biomaterials and the exchange of gases and growth medium. The exposed collagen fibrils on the biomaterials' surface which mimics the extracellular matrix of human tissue probably encouraged the attachment of the HeLa cells.

On Day 6, there was a sharp decrease in optical density (OD) reading. However, similar findings were recorded by Nazarpak et al., 2011 had recorded similar findings. HeLa cancer cells are anchorage dependent (Robert, Cote, and Archambault., 1992), meaning they require a surface to attach to and proliferate. This explains why the collagen - sodium alginate biomaterials recorded higher OD readings than the control.

It is interesting to note that on Day 8, the OD levels of all the collagen – sodium alginate biomaterials studied including the control reached a similar level. Throughout the entire period of the study, the control well always had the lowest OD reading. By comparing the OD (optical density) readings (Figure 4.11), it can be concluded that all the HeLa cancer cells cultured with the collagen-sodium alginate biomaterials consistently recorded higher OD readings than the control well.

This was due to the biomaterial's effect (Nazarpak et al., 2011) of the collagen-sodium alginate biomaterials which promotes HeLa cancer cell growth and proliferation. Authors Nazarpak et al., (2011); Liu, Ma and Gao., (2012); and Sang et al., (2011) recorded having similar biomaterial's effect in their respective cell culture studies.

Unfortunately, the pure collagen and pure sodium alginate biomaterials had been dissolved, therefore cell culture studies could not be carried out using these biomaterials. This was due to their intrinsic nature, whereby sodium alginate is a hydrophilic polysaccharide, while collagen is a thermo-instable protein (Skierska and Skakowska, 2007) which easily denatures. Biomaterials can be crosslinked to prevent it from dissolving, as done by Liu, Ma and Gao, (2012), who successfully fabricated a glutaraldehyde crosslinked collagen-chitosan biomaterial.

## 5.9	Surface Morphology Analysis of Cell Cultured Biomaterials

The penetration and proliferation of HeLa cancer cells deep into the three dimensional structure of the collagen-sodium alginate biomaterials may help enhance its' efficiency and functional capacity in the field of tissue engineering. Based on the results of the direct contact test, it can be concluded that the collagen-sodium alginate biomaterials are noncytotoxic and have sufficient porosity and pore interconnectivity for cellular proliferation11.

SEM surface morphology shows that the cells may also proliferate deep within the pores of the biomaterials (Figure 4.5). Direct contact test results (Figure 4.13) confirm that HeLa cancer cells are anchorage dependent cells, and therefore require surface adhesion and attachment for growth and proliferation. The hollowed out, fixed tough outer coating of HeLa cancer cells was observed by SEM analysis (Figure 4.13). The HeLa cancer cells appear flattened and dried out with a netted surface.

The netted surface of the HeLa cancer cells appear to consist of uniformly sized and laterally and vertically arranged puncture holes on its' entire surface (Figure 4.13), due to lysis by the fixing agents glutaraldehyde and ethanol. Just imagine a balloon that has been filled to the brim with water and was punctured.

During lysis, all the intracellular fluids of the HeLa cancer cell was drained out. There were probably other HeLa cancer cells that had been embedded on the surface of the biomaterials, however these cells had undergone partial or complete lysis and therefore could not be observed. In order to prevent lysis, the fixing time with glutaraldehyde and ethanol should have been decreased.

CHAPTER 6

CONCLUSION

In conclusion, the FTIR results (Figure 4.2; Figure 4.3) showed that collagen was successfully extracted with a promising yield from the skin of the *Oreochromis niloticus* sp fish, and sodium alginate was successfully extracted from *Sargassum polycystum* sp seaweed. The extracted collagen and sodium alginate were successfully crosslinked and fabricated into biomaterials.

Electrophoretic patterns (Figure 4.9) of the extracted collagen from the skin of *Oreochromis niloticus* sp fish had a doublet pattern for the upper $\alpha 1$ (approximately 130 kDa to 135 kDa) and lower $\alpha 2$ chains (approximately 120 kDa to 125 kDa) and a single β chain (approximately 220 kDa to 225 kDa), which confirmed it's identity as Type I collagen.

The amino acid analysis (Figure 4.10) of collagen showed the presence of the imino acids hydroxyproline and proline as well as amino acid glycine. SEM analysis shows that the collagen structure of *Oreochromis niloticus* sp was fibrillar, while sodium alginate from *Sargassum polycystum* sp was highly porous.

Equilibrium swelling ratios results (Figure 4.8) showed that the collagen – sodium alginate crosslinked biomaterials were able to absorb fluids from their sorroundings and expand. Biomaterials crosslinked with higher sodium alginate to collagen ratios showed better swelling capacity.

Direct contact test (Figure 4.13) shows that the fabricated biomaterials did improve the growth and proliferation rates of the HeLa cancer cells. The fabricated biomaterials proved to have no cytotoxicity and are able to support the growth of cells attached on the surface of the fabricated biomaterials.

Results of the current study confirmed the suitability of using collagen from *Oreochromis niloticus* sp and sodium alginate from *Sargassum polycystum* sp to fabricate biomaterials which are non-cytotoxic and capable of supporting the growth and proliferation of cells. The fabricated biomaterials are suitable to be used for *in vitro* cell culture studies. Further *in vivo* research in the field of biomimetics could be conducted using the same methodology and raw materials to fabricate the collagen – sodium alginate biomaterials.

Collagen and sodium alginate biomaterials have good biocompatibility and low immunogenicity with the human ECM. Even today, collagen remains as the protein of choice for the preparation of biomaterials. This is important as cells require a hospitable three-dimensional environment in order to thrive and proliferate. From this study, it is proven that Type I collagen can be extracted from the skin waste of Nile Tilapia. The extracted collagen can be reassembled in combination with sodium alginate and be used for the fabrication of biomaterials.

However, the major hurdles in developing such biomaterials include their rate of biodegradation and mechanical strength. Future studies could include fabricating the collagen-sodium alginate biomaterials with a non-cytotoxic crosslinker, such as glutaraldehyde to increase it's mechanical strength and decrease it's rate of biodegradation. After increasing the duration that the fabricated biomaterials would take to biodegrade, it would then be possible to compare the effectiveness of each biomaterial in promoting cell growth and proliferation activity, in order to determine the optimum ratio of collagen to sodium alginate for biomaterial fabrication.

The next step would be testing the fabricated biomaterial in animal models such as rats. Animal testing is considered as the midway between *in vitro* studies and human clinical trials. Therefore, animal models have been used extensively for *in vivo* testing in the fields of cell biology, genetics, biochemistry, infection and immunity, cancer research and tissue engineering, among others.

Small animal models such as mice and rats are affordable, easy to breed and easy to maintain. Rodent models are suitable subcutaneous and intramuscular models for the study of the biodegradation rate and cytotoxicity of biomaterials (Moran et al., 2016). Therefore, they can function as a good bridge between *in vitro* and *in vivo* experiments. However, due to their small size, rodent models have limited potential in the study of the effectiveness of new biomaterials as the healing processes which maybe successful in aiding small wounds may have negligible effect on larger wounds on larger animal models (Moran et al., 2016).

Other limitations for using collagen-sodium alginate biomaterials *in vivo* are the host's own immune response system. Implanting biomaterials *in vivo* invokes the host's chronic inflammatory response system and causes the release of large amounts of antibodies and cytokines. The secreted antibodies degrades the collagen bundles of the fabricated biomaterials in a process called phagocytosis until it completely disintegrates. Therefore, it would also be necessary to study the host immunological response system towards the biomaterial implanted *in vivo* by measuring the level of pro-inflammatory cytokine and antibody secretions as well as the population changes of the host's immune cells.

LIST OF REFERENCES

Abed, S. E., Ibnsouda, S. K., Latrache, H. and Hamadi, F., 2008. Scanning electron microscopy (SEM) and environmental SEM : suitable tools for study of adhesion stage and biofilm formation. *Scanning Electron Microscopy*, 1 (1), pp. 717–730.

Addad, S., Exposito, J-Y., Faye, C., Richard-blum, S., and Lethias, C., 2011. Isolation, characterisation and biological evaluation of jellyfish collagen for use in biomedical applications. *Marine Drugs,* 9 (6), pp. 967-983.

Agrawal, C, M., 1998. Reconstructing the human body using biomaterials. *The Journal of The Minerals, Metals and Materials Society,* 50 (1), pp. 31-35.

Ahuja, T., Dhakray, V., Mittal, M., Khanna, P., Yadav, B., and Jain, M., 2012. Role of collagen in the periodontal ligament – a review. *The Internet Journal of Microbiology,* 10 (1), pp. 1-10.

Alban, S., Schauerte, A., and Franz, G., 2002. Anticoagulant sulphated polysaccharides: Part I. Synthesis and structure-activity relationships of new pullulan sulfates. *Carbohydrate Polymers,* 47 (3), pp.267-276.

Andersen, T., Auk-Emblem, P., and Dornish, M., 2015. 3D cell culture in alginate hydrogels. *Microarrays*, 4 (2), pp 133-161.

Andersen, T., Strand, B. L., Formo, K., Alsberg, E., and Christensen, B. E., 2012. Alginates as biomaterials in tissue engineering. *Carbohydrate Chemistry: Chemical and Biological Approaches*, 37 (1), pp. 227-258.

Andriamanantoanina, H., and Rinaudo, M., 2010. Relationship between the molecular structure of alginates and their gelation in acidic conditions. *Polymer International,* 59 (11), pp. 1531-1541.

Baniasadi, M., and Minary-Jolandan, M., 2015. Alginate-collagen fibril composite hydrogel. *Materials,* 8 (2), pp. 799-814.

Basha, N. S., Rekha, R., Letensie, A., and Mensura, S., 2011. Preliminary invertigation on sodium alginate extracted from *sargassum subrepandum* of red sea of eritrea as tablet binder. *Journal of Scientific Research,* 3 (3), pp. 609-618.

Belbachir, K., Noreen, R., Gouspillou, G., and Petibois, C., 2009. Collagen types analysis and differentiation by FTIR Spectroscopy. *Analytical and Bioanalytical Chemistry*, 395 (3), pp. 829-837.

Boccaccini, A. R., and Blaker, J.J., 2005. Bioactive composite materials for tissue engineering scaffolds. *Expert Review of Medical Devices,* 2 (3), pp. 303-317.

Bodmeier, R., and Wang, J., 1993. Microencapsulation of drugs with aqueous colloidal polymer dispersions. *Journal of Pharmaceutical Sciences,* 82 (2), pp. 191-194.

Buehler, M. J., 2006. Nature designs tough collagen: explaining the nanostructure of collagen fibrils. *Proceedings of The National Academy of Sciences of The United States of America,* 103 (33), pp. 12285-12290.

Burdall, S. E., Hanby, A. M., Lansdown, M. R., and Speirs, V., 2003. Breast cancer cell lines: friend or foe? *Breast Cancer Research: BCR*, 5 (2), pp. 89-95.

Campos Vidal. B. d., and Mello, M. L. S., 2011. Collagen type I amide I band infrared spectroscopy. *Micron*, 42 (3), pp. 283-289.

Chang, S-W., Shefelbine, S, J., and Buehler, M, J., 2012. Structural and mechanical differences between collagen homo- and heterotrimers: Relevance for the molecular origin of brittle bone disease. *Biophysical Journal,* 102 (3), pp. 640-648.

Chattopadhyay, S., and Raines, R. T., 2014. Review of collagen-based biomaterials for wound healing. *Biopolymers,* 101 (8), pp. 821 − 833.

Chiellini, M. Dash., Ottenbrite, R. M., and Chiellini, E., 2011. Chitosan- a versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, 36 (8), pp. 981-1014.

Chung, D. M., Kim, J. H., and Kim, J. K., 2015. Evaluation of MTT and Trypan Blue assays for radiation-induced cell viability test in HepG2 cells. *International Journal of Radiation Research,* 13 (4), pp. 331-336.

Chung, T. W., Yang, J., Akaike, T., Cho, K. Y., Nah, J, W., Kim, S.L., and Cho, C, S., 2002. Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. *Biomaterials,* 23 (14), pp. 2827 – 2834.

Civerchia-Perez, L., Faris, B., Lapointe, G., Beldekas, J., Leibowitz, H., and Franblau, C., 1980. Use of collagen hydroxyethyl methcrylate hydrogels for cell growth. *Proceedings of the National Academy of Sciences USA,* 77 (4), pp. 2064-2068.

Cortial, D., Gouttenoire, J., Rousseau, C. F., Ronziere, M. C., Piccardi, N., Msika, P., Herbage, D., Mallein-Gerin, F., and Freyria, A. M., 2006. Activation by Il-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An *in vitro* model to address the effect of compounds with therapeutic potential in osteoarthritis. *Osteoarthritis Cartilage,* 14 (7), pp. 631-640.

Croisier, F., and Jerome, C., 2013. Chitosan-based biomaterials for tissue engineering. *European Polymer Journal,* 49 (4), pp. 780-792.

Crubezy, E., Ludes, B., Poveda, J. D., Clayto, J., Croudau-Roy, B., and Montagnon, D., 1998. Identification of Mycobacterium DNA in an Egyptian Pott's disease of 5'400 years old. *Comptes rendus de l'Academie des Sciences. Serie III, Sciences de la vie,* 321 (11), pp. 941-951.

Cunniff, P. M., Fossey, S. A., Auerbach, M. A., Song, J. W., Kaplan, D. L., Adams, W. W., Eby, R. K., Mahoney, D., and Vezie, D. L., 1994. Mechanical and thermal properties of dragline silk from the spider, *Nephila clavipes. Polymers for Advanced Technologies*, 5 (8), pp. 401-410.

Daemi, H., and Barikani, M., 2012. Synthesis and characterization of calcium alginate nanoparticles, sodium homopolymannuronate salt and its calcium nanoparticles. *Scientia Iranica*, 19 (6), pp. 2023–2028.

Davis, T. A., Ramirez, M., Mucci, A., and Larsen, B., 2004. Extraction, isolation and cadmium binding of alginate from *Sargassum* spp. *Journal of Applied Phycology,* 16, pp. 275-284.

Dhawan, S., Singla, A. K., and Sinha, V. R., 2004. Evaluation of muchoadhesive properties of chitosan microspheres prepared by different methods. *AAPS PharmSciTech,* 5 (4), pp. 122-128.

Doillon, C. J., Silver, F. H., and Berg, R. A.,1987. Fibroblast growth on a parous collagen sponge containing hyaluronic acid and fibronectin. *Biomaterials,* 8 (3), pp. 195-200.

Draget, K. I, 2000. *"Alginates" in handbook of hydrocolloids*. 1st ed. Cambridge, UK: Woodhead Publishing Limited.

Dr. Namrata Chhabra. 2017. *Structure of collagen.* [ONLINE] Available at: http://www.namrata.co/structure-of-collagen-solution-to-problem-based-on-osteogenesis-imperfecta/. [Accessed 1 September 2017].

Dutta, P. K., Dutta, J., and Tripathi, V. S., 2004. Chitin and chitosan: Chemistry, properties and applications. *Journal of Scientific and Industrial Research,* 63 (1), pp. 20 – 31.

Ennaas, N., Hammami, R., Gomaa, A., Bedard, F., Biron, E., Subirade, M., Beaulieu, L., and Fliss, I., 2016. Collagencin, an antibacterial peptide from fish collagen. Activity, structure and interaction dynamics with membrane. *Biochemical and Biophysical Research Communications,* 473 (2), pp. 642 – 647.

Fligiel, S. E. G. et al., 2003. Collagen degradation in aged/photodamaged skin in vivo and after exposure to matrix metalloproteinase-1 *in vitro*. *Journal of Investigative Dermatology*, 120 (5), pp. 842–848.

Foegeding, E, A., Lanier, T. C., and Hultin, H. O., 1996. Characteristics of edible muscle tissue. *Food Chemistry,* 15 (3)*,* pp. 879 – 942.

Frampton, J. P., Hynd, M. R., Shuler, M. L., and Shain, W., 2011. Fabrication and optimization of alginate hydrogel constructs for use in 3D neural cell culture. *Biomedical Materials,* 6 (1), pp. 1-18.

Fruton, J. S., 2002. A history of pepsin and related enzymes. *The Quarterly Review of Biology*, 77 (2), pp. 127-147.

Galassi, G., Brun, P., Radice, M., Cortivo, R., Zanon, G. F., Genovese, P., and Abatangelo, G., 2000. *In vitro* reconstituted dermis implanted in human wounds: degradation studies of the HA \-based supporting scaffold. *Biomaterials,* 21 (21), pp. 2183-2189.

Gazdar, A. F., Girard, L., Lockwood, W. W., Lam, W. L., Minna, J. D., 2010. Lung cancer cell lines as tools for biomedical discovery and research. *Journal of The National Cancer Institute,* 102 (7), pp. 1310-1321.

Graham, M. F., Diegelmann, R. F., Elson, C. O., Linbald, W. J., Gotschalk, N., Gay, S., and Gay, R., 1988. Collagen content and types in the intestinal strictures of Crohn's disease. *Gastroenterology,* 94 (2), pp. 257 – 265.

Gross, J., and Kirk, D., 1958. The heat precipitation of collagen from neutral salt solutions. *Journal of Biological Chemistry,* 233 (2), pp. 355-360.

Gupta, M. K., Khokhar, S. K., Phillips, D. M., Sowards, L. A., Drummy, L. F., Kadakia, M. P., and Naik, R. R., 2007. Patterned silk films cast from ionin liquid solublised fibroin as scaffolds for cell growth. *Langmuir: The ACS Journal of Surfaces and Colloids,* 23 (3), pp. 1315-1319.

Gupta, P., and Nayak, K. K., 2015. Characteristics of protein-based biopolymer and its application. *Polymer Engineering and Science,* 55 (3), pp. 485-498.

Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R., and Bullock, P., 2004. Comparison of alamar blue and MTT assays for high through-put screening. *Toxicology in Vitro: an International Journal Published in Association with BIBRA*, 18 (5), pp. 703-710.

Han, S-H., Uzawa, Y., Moriyama, T., and Kawamura, Y., 2011. Effect of collagen and collagen peptides from bluefin tuna abdominal skin on cancer cells. *Health*, 3 (3), pp. 129–134.

Hayon, T., Dvilansky, A., Shpilberg, O., and Nathan, I., 2003. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. *Leukemia and Lymphoma,* 44 (11), pp. 1957-1962.

Henderson, Jr. J. W., and Brooks, A., (2016). Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals. Wilmington, USA: Agilent Technologies, pp. 1-16.

Hemanth, M., Spandana, V., and Poonam, T., 2011. Extraction and determination of collagen peptide and it's clinical importance from tilapia fish scales (*oreochromis niloticus*). *International Research Journal of Pharmacy*, 2 (10), pp. 97–99.

Hesse, E., Hefferan, T. E., Tarara, J. E., Haasper, C., Meller, R., Krettek, C., Lu, L., and Yaszemski, M. J., 2010. Collagen type I hydrogel allows migration, proliferation, and osteogenic differentiation of rat bone marrow stromal cells. *Journal of Biomedical Materials Research, Part A,* 94 (2), pp. 442-449.

Horan, R., Bramono, D., Stanley, J., Simmons, Q., Chen, J., Boepple, H., and Altman, G., 2009. Biological and biomechanical assessment of a long-term bioresorbable silk-derived surgical mesh in an abdominal body wall defect model. *Hernia,* 13 (2), pp. 189 – 199.

Hutmacher, D. W., Goh, J. C. H., and Teoh, S. H., 2001. An introduction to biodegradable materials for tissue engineering applications. *Annals Academy of Medicine Singapore,* 30 (2), pp. 183-191.

Inmaculada, A., Marian, M., Ruth, H., Ines, P., Acosta, B., Galed, N., and Angeles, G. H., 2009. Functional characterisation of chitin and chitosan. *Current Chemical Biology*, 3 (2), pp. 203-230.

Inouye, K, Sakakibara, S., and Prockop, D. J., 1976. Effects of the stero-configuration of the hydroxyl group in 4-hydroxyproline on the triple-helical structures formed by homogenous peptides resembling collagen. *Biochimica et Biophysica Acta,* 420 (1), pp. 133-141.

Jabbar, S. A., Twentyman, P. R., and Watson, J. V., 19889. The MTT assay underestimates the growth inhibitory effects of interferons. *British Journal of Cancer,* 60 (4), pp. 523-528.

Jayasankar, R., 1993. On the yield and quality of sodium alginate from *sargassum wightii* (greville) by pre-treatment with chemicals. *Indian Journal of Applied Research,* 16 (1), pp. 63-66.

Ji, W., Chen. Y. Y., Du, J. R., Zheng, X. Y., Yang, C. X., Li, D. S., Zhao, C. Y., and Qiao, K. Y., 2009. Antihypertensive effect and pharmacokinetics of low molecular mass potassium alginate. *Journal of Sichuan University. Medical Science Edition,* 40 (4), pp. 694-696.

Jiang, G., Min, S., Oh, E. J., and Hahn, S., 2007. DNA/PEI/alginate polyplex as an efficient *in vivo* gene delivery system. *Biotechnology and Bioprocess Engineering,* 12 (1), 684-689.

Kadler, K. E., Holmes, D. F., Trotter, J. A., and Chapman, J. A., 1996. Collagen fibril formation. *Biochemical Journal,* 316 (1), pp. 1-11.

Kenney, J. M., Knight, D., Wise, M. J., and Vollrath, F., 2002. Amyloidogenic nature of spider silk. *European Journal of Biochemistry*, 269 (16), pp. 4159-4163.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., and Shahidi, F., 2010. Isolation and characterisaiton of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Charcharhinus limbatus*). *LWT – Food Science and Technolopgy,* 43 (5), pp. 792 – 800.

Kupiec, T., 2004. Quality-control analytical methods: High-performance liquid chromatography. *International Journal of Pharmaceutical Compounding,* 8 (3), pp. 223-227.

Latifi, A, M., Nejad, E,S., and Babavalian, H., 2015.Comparison of extraction methods of sodium alginate from brown alga *sargassum* sp. located in the southern of Iran. *Journal of Applied Biotechnology Reports,* 2 (2), pp. 251-255.

Lee, H-J., Ahn, S-H. and Kim, G. H., 2012. Three-dimensional collagen/alginate hybrid scaffolds functionalised with a drug delivery system (DDS) for bone tissue regeneration. *Chemistry of Materials*, 24 (1), pp. 881-891.

Li, X., Chen, S., Zhang, B., Li, M., Diao, K., Li, J., Xu, Y., Wang, X., and Chen, H., 2012. In situ injectable nano-caomposite hydrogel composed of curcumin, N,O-carboxymethyl chitosan and oxidised alginate for wound healing application. *International Journal of Pharmaceutics,* 437 (2), pp. 110 -119.

Límová, M., 2010. Active wound coverings: bioengineered skin and dermal substitutes. *The Surgical Clinics of North America,* 90 (6), pp. 1237-125.

Liu, Y., Ma, L., and Gao, C., 2012. Facile fabrication of the glutaraldehyde cross-linked collagen/chitosan porous scaffold for skin tissue engineering. *Materials Science and Engineering C*, 32 (8), pp. 2361–2366.

Lü, L., Zhang, L., Wai, M. S., Yew, D. T., and Xu, J., 2012. Exocytosis of MTT formazan could exacerbate cell injury. *Toxicology in Vitro : an International Journal Published in Association with BIBRA,* 26 (4), pp. 636-644.

Lubiatowski, P., Kruczynski, J., Grady's, A., Trzeciak, and T., Jaroszewski, J., 2006. Articular cartilage repair by means of biodegradable scaffolds. *Transplantation Proceedings,* 38 (1), pp. 320 – 322.

Lucey, B. P., Nelson-Rees, W. A., and Hutchins, G. M., 2009. Henrietta lacks, HeLa cells, and cell culture contamination. *Archives of Pathology and Laboratory Medicine,* 133 (9), pp. 1463-1467.

Lundmark, K., Westermark, G. T., Olsen, A., and Westermark, P., 2005. Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism. *Proceedings of The National Academy of Sciences of the United States of America,* 102 (17), pp. 6098-6102.

Lyons, F., Partap, S., and O'Brien, F. J., 2008. Part 1: scaffolds and surfaces. *Technology Health Care*, 16, pp. 305-317.

Ma, P. X., 2008. Biomimetic materials for tissue engineering. *Advanced Drug Delivery Review*, 60, pp. 184–198.

Masters, J. R, and Stacey, G. N., 2007. Changing medium and passaging cell lines. *Nature Protocols,* 2 (9), pp. 2276 – 2284.

Matthew, I. R., Browne, R. M., Frame, J. W., and Millar, B. G., 1995. Subperiosal behaviours of alginate and cellulose wound dressing materials. *Biomaterials,* 16 (4), pp. 275-278.

Mi, F. L., Shyu, S.S., Lin, Y. M., Wu, Y, B., Peng, C. K., and Tsai, Y. H., 2003. Chitin / PLGA blend microspheres as a biodegradable drug delivery system: a new delivery system for protein. *Biomaterials,* 24 (27), pp. 5023 – 5036.

Minary-Jolandan, M., and Yu, M.F., 2009. Nanomechanical heterogenecity in the gap and overlap regions of Type I Collagen fibrils with implications for bone heterogenecity. *Biomacromolecules,* 10 (1), pp. 2565-2570.

Minghou, J., Yujun, W., Zuhong, X., and Yucai, G., 1984. Studies on the M:G ratios in alginate. *Hydrobiologia*, 116, pp. 554–556.

Miyata, T., Taira, T., and Noishiki, Y., 1992. Collagen engineering for biomaterial use. *Clinical Materials,* 9 (3-4), pp. 139-148.

Mokarram, R., and Alonso, M. J., 2006. Preparation and evaluation of chitosan nanoparticles containing Diphtheria toxoid as new carriers for nasal vaccine delivery in mice. *Archives of Razi Institute,* 61 (1), pp. 13-25.

Moran, C. L., Ramesh, A., Brama, P. A. J., O'Byrne, J. M., O'Brien, F. J., and Levingstone, T. J., 2016. Benefits and limitations of animal models for translational research in cartilage repair. *Journal of Experimental Orthopedics,* 3 (1), pp. 1-12.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. *Journal Of Immunology,* 65 (1)*,* pp. 55-63.

Müller, W. E., 2003. The origin of metazoan complexity: poriferan as integrated animals. *Integrative and Comparative Biology,* 43 (1), pp. 3 – 10.

Murillo-Àlvarez, J. I., and Hérnandez-Carmona, G., 2007. Monomer composition and sequence of sodium alginate extracted at pilot plant scale from three commercially important seaweeds from Mexico. *Journal of Applied Phycology,* 19, pp. 545-548.

Mushollaeni, W., 2011. The physicochemical characteristics of sodium alginate from Indonesian brown seaweeds. *African Journal of Food Science,*5 (1), pp. 349– 352.

Muyonga, J.H., Cole, C.G., and Duodu, K.G., 2004. Fourier transform infrared (FTIR) spectroscopic study of skin and bones of young adult nile perch. *Food Chemistry,* 86 (1), pp. 325-332.

Nagai, T., and Suzuki, N., 2000. Partial characterization of collagen from purple sea urchin *anthocidaris crassispina. International Journal of Food Science and Technology*, 35 (1), pp. 497–501.

Nagai, T., Suzuki, N., Tanoue, Y., Kai, N., and Nagashima, T., 2010. Characterisation of acid soluble collagen from skins of surf smelt *(hypomesuspretiosus japonicis brevoort). Food And Nutrition Sciences,* 1 (1)*,* pp. 59-66.

Nagarajan, M., and Shakila, R. J., 2013. Skin, bone and muscle collagen extraction from the trash fish, leather jacket (*Odonus niger*), and their characterisation. *Journal of Food Science and Technology,* 50 (6), pp. 1106 – 1113.

Narra, K., Dhanalekshmi, U., Tangaraj, G., Raja, D., Kumar, C. S., Reddy, P. N., and Mandal, A. B., 2012. Effect of formulation variables on rifampicin loaded alginate beads. *Iranian Journal of Pharmaceutical Research*, 11 (3), pp. 715–721.

Nazarpak, M. H., Pourasgari, F., and Sarbolouki, M., 2011. Preparation of a novel porous scaffold from poly (lactic-co-glycolic acid) / hydroxyapatite. *Asian Biomedicine*, 5 (4), pp. 513–518.

Nema, R., and Khare, S., 2012. An animal cell culture: Advance technology for modern research. *Advances in Bioscience and Biotechnology,* 3 (3), pp. 219 – 226.

O'Brien, F. J., 2011. Biomaterials and scaffolds for tissue engineering. *Materials Today,* 14 (3), pp. 88 – 95.

Oerther, S., Payan, E., Lapicque, F., Presle, N., Hubert, P., Muller, S., and Netter, P., 1999. Hyaluronate-alginate combination for the preparation of new biomaterials: investigation of the behaviour in aqueous solutions. *Biochimica et Biophysica*, 1426 (1), pp. 185-194.

Oostgaard, K., Knutsen, S. H., Dyrset, N., and Aasen, I. M., 1993. Production and characterisation of guluronate lyase from *Klebsiella pneumonia* for applications in seaweed biotechnology. *Enzyme and Microbial Technology,* 15 (9), pp. 756-763.

Özeroglu, C., and Birdal, A., 2009. Swelling properties of acrylamide-N,N'-methylene bis(acrylamide) hydrogels synthesised by using meseo-2,3-dimercaptosuccinic acid-cerium(IV) redox couple. *eXPRESS Polymer Letters*, 3 (3), pp. 168-176.

Panilaitis, B., Altman, G. H., Chen, J. Jin, H. J., Karageorgiou, V., and Kaplan, D. L., 2003. Macrophage responses to silk. *Biomaterials*, 24 (18), pp. 3079-3085.

Parades-Juarez, G. A., Spasojevic, M., Fass, M. M., and de Vos, P., 2014. Immunological and technical considerations in application of alginate-based microencapsulation systems. *Frontiers in Bioengineering and Biotechnology*, 2 (26), pp.1-15.

Parenteau-Bareil, R., Gauvin, R., and Berthod, F., 2010.Collagen-based biomaterials for tissue engineering applications. *Materials*, 3 (3), pp. 1863–1887.

Pati, F., Adhikari, B., and Dhara, S., 2010. Isolation and characterisation of fish scale collagen of higher thermal stability. *Bioresource Technology,* 101 (10), pp. 3737 – 3742.

Peng, Y., Glattauer, V., Werkmeister, J. a., and Ramshaw, J. a. M., 2004. Evaluation for collagen products for cosmetic application. *Journal of Cosmetic Science*, 55 (4), pp. 327–341.

Potaros, T., Raksakulthai, N., and Runglerdkreangkrai, J., 2009. Characteristics of collagen from nile tilapia (*Oreochromis niloticus*) skin isolated by two different methods. *Nature Science*, 43 (1), pp. 584–593.

Priya, P. V., and Rao, S. A., 2015. Evaluation of anticancer activity of *Tridax procumbens* leaf extracts on A549 and Hep G2 cell lines. *Asian Journal of Pharmaceutical and Clinical Research*, 8 (3), pp. 129-132.

Ratner, B. D., Hoffman, A. S., Schoen, F. J., and Lemons, J. E., (1997) *Biomaterials Science: An Introduction to Materials in Medicine*. 1st edition. United States of America: Elsevier Science (USA).

Rehm, B. H., and Valla, S., 1997. Bacterial alginates: biosynthesis and applications. *Applied Microbiology and Biotechnology,* 48 (3), pp. 281-288.

Renier, D., Bellato, P., Bellini, D., and Borrione, A., 2005. Pharmacokinetic behaviour of ACP gel, an autocrosslinked hyaluronan derivative, after interperitoneal administration. *Biomaterials,* 26 (26), pp. 5368-5374.

Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. *Progress In Polymer Science*, 31 (7), pp. 603-632.

Robert, J., Cote, J., and Archambault, J., 1992. Surface immobilisation of anchorage-dependent mammalian cells. *Biotechnology and Bioengineering,* 39 (7), pp. 697-706.

Sandhu, S. V., Gupta, S., Bansal, H., and Singla, K., 2012.Collagen in health and disease. *Journal of Orofacial Research*, 22 (33), pp. 153–159.

Sang, L., Luo, D., Xu, S., Wang, X., and Li, X., 2011. Fabrication and evaluation of biomimetic scaffolds by using collagen-alginate fibrillar gels for potential tissue engineering applications. *Materials Science and Engineering C*, 31 (2), pp. 262–271.

Saraswathi, S. J., and Babu, B., Rengasamy, R., 2003. Seasonal studies on the alginate and it's biochemical composition I: *Sargassum polycystum* (Fucales), Phaephyceae. *Phycological Research,* 51 (4), pp. 240 -243.

Schoen, F. J., and Levy, R. J., 1984. Bioprosthetic heart valve failure: Pathology and pathogenesis. *Cardiology Clinics*, 2 (4), pp. 717-739.

Sharma, S. R., Poddar, R., Sen, P., and Andrews J.T., 2008. Effect of vitamin C on collagen biosynthesis and degree of birefringence in polarisation sensitive optical coherence tomography (PS-OCT). *African Journal of Biotechnology,* 7 (12), pp. 2049 – 2054.

Sieuwerts, A. M., Klijn, J. G., Peters, H. A., and Foekens, J. A., 1995. The MTT tetrazolium salt assay scrutinised: how to use this assay reliably to measure metabolic activity of cell cultures in vitro for the assessment of growth characteristics, IC50-values and cell-survival. *European Journal of Clinical Chemistry and Clinical Biochemistry: journal of the Forum of European Clinical Chemistry Studies*, 33 (11), pp. 813-823.

Sigma Aldrich, 2016. Mass molarity calculator. *Sigma Aldrich.* [ONLINE] Available at: http://www.sigmaaldrich.com/chemistry/stockroom-reagents/learning-center/technical-library/mass-molarity-calculator.html. [Accessed 01 November 2016].

Sigma Aldrich., 2012. In vitro toxicology assay kit MTT based. *Sigma Aldrich*, pp. 3–4.

Silver, F. H., and Doillon, C., 1989. *Biocompatibility,* Vol.1, *Polymers*. VCH Publishers Inc., New York, NY.

Singh, L., Kumara, V., and Ratner, B.D., 2004. Generation of porous microcellular 85/15 poly (DL-lactide-co-glycolide) foams for biomedical applications. *Biomaterials,* 25 (1), pp. 2611-2617.

Sinthusamran, S., Benjakul, S. and Kishimura, H., 2013.Comparative study on molecular characteristics of acid soluble collagen from skin and swim bladder of seabass (*Lates calcarifer*). *Food Chemistry,* 138 (4), pp. 2435-2441.

Skaugrud, O., Hagen, A., Borgersen, B., and Dornish, M., 1999. Biomedical and pharmaceutical applications of alginate and chitosan. *Biotechnology and Genetic Engineering Reviews*, 16 (1), pp. 23-40.

Skierka, E. and Sakowska, M., 2007. The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod *(Gadus morhua)*. *Food Chemistry*, 105, pp. 1302-1306.

Smidsrod, O., and Skjak-Braek, G., 1990. Alginate as immobilisation matrix for cells. *Trends in Biotechnology,* 8 (3), pp. 71-78.

Song, E., Yeon Kim, S., Chun, T., Byun, H. J., and Lee, Y. M., 2006. Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials,* 27 (15), pp. 2951-2961.

Stegman, S. J., Chu, S., and Armstrong, R., 1988. Adverse reactions to bovine collagen implant: clinica and histologic features. *The Journal of Dermatologic Surgery and Oncology,* 14 (1), pp. 39 - 48.

Stockert, J. C., Blazquez-Castro, A., Canete, M., Horobin, R. W., and Villanueva, A., 2012. MTT assay for cell viability: Intracellular localisation of the formazan product is in lipid droplets. *Acta Histochemica*, 114 (8), pp. 785-796.

Stoddart, M. J., 2011. *Mammalian cell viability, methods and protocols*. 1st ed. London, United Kingdom: Humana Press.

Sugiura, H., Yunoki, S., Kondo, E., Ikoma, T., Tanaka, J., and Yasuda, K., 2009. In vivo biological responses and bioresorption of tilapia scale collagen as a potential biomaterial. *Journal of Biomaterials Science-Polymer Edition*, 20 (10), pp. 1353-1368.

Sujithra, S.,Kiruthiga, N., Prabhu, M. J., and Kumeresan, R., 2013. Isolation and determination of type I collagen from tilapia (*Oreochromis niloticus*) Waste. *International Journal of Engineering and Technology*, 5 (3), pp. 2181–2185.

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

Sun, L., Hou, H., Li, B., and Zhang, Y., 2017. Characterisation of acid- and pepsin-soluble collagen extracted from the skin of Nile Tilapia (*Oreochromis niloticus*). *International Journal of Biological Macromolecules,* 99 (1), pp. 8 – 14.

Suzuki, Y., Nishimura, Y., Tanihara, M., Suzuki, K., Shimizu, Y., Yamawaki, Y., and Kakimaru, Y., 1998. Evaluation of a novel alginate gel dressing: cytotoxicity to fibroblasts in vitro and foreign-body reaction in pig skin in vivo. *Journal of Biomedical Materials Research,* 39 (2), pp. 317-322.

Szekalska, M., Pucilowska, A., Szymanska, E., Ciosek, P., Winnicka, K., 2016. Alginate: Current use and future perspectives in pharmaceutical and biomedical applications. *International Journal of Polymer Science,* 1 (1), pp. 1- 18.

Takka, S., and Acarturk, F., 1999. Calcium alginate microparticle for oral administration: I: effect of sodium alginate type on drug release and drug entrapment efficiency. *Journal of Microencapsulation,* 16 (3), 275-290.

Tan, C. C., Karim, a. a., Latiff, a. a., Gan, C. Y., and Ghazali, F. C., 2013. Extraction and characterization of pepsin-solubilized collagen from the body wall of crown-of-thorns starfish (*acanthaster planci*). *International Food Research Journal,* 20 (6), pp. 3013–3020.

Tang, M., Chen, M., Weir, M. D., Thein-Han, and W., Xu, H.H., 2012. Human embryonic stem cell encapsulation in alginate microbeads in microporous calcium phosphate cement for bone tissue engineering. *Acta Biomaterialia*, 8 (9), pp. 3436 – 3445.

Tathe, A., Ghodke, M., and Nikalje, A, P., 2010. A brief review: biomaterials and their application. *International Journal of Pharmacy and Pharmaceutical Sciences,* 2 (4), pp. 19-23.

Thomas, S., 2013. Advances in Natural Polymers: Composites and Nanocomposites. 1st ed. London, United Kingdom: Springer-Verlag Berlin Heidelberg.

Thuy, L. T. M., Okazaki, E., and Osako, K., 2014. Isolation and characterisation of acid-soluble collagen from the scales of marine fishes from Japan and Vietnam. *Food Chemistry*, 149 (1), pp.264-270.

Tonder, v. A., Joubert, A. M., and Cromarty, A. D., 2015. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Research Notes*, 8 (47), pp. 1-10.

Tran, S-L., Puhar, A., Ngo-Camus, M., and Ramarao, N., 2011. Trypan blue dye enters viable cell incubated with the pore-forming toxin HlyII of *Bacillus cereus. PlosOne,* 6 (9), pp. 1-5.

Tronci, G., Grant, C. A., Thomson, N. H., Russell, S. J., and David, J. Multi-scale Mechanical Characterization of Highly Swollen Photo-activated Collagen Hydrogels. *Journal of The Royal Society Publishing,* 1 (1), 1-43.

Tylingo, R., Mania, S., Panek, A., Piatek, R., and Pawlowicz, R., 2016. Isolation and characterisation of acid soluble collagen from the skin of African catfish (*Clarias gariepinus*), Salmon (*Salmo salar*) and Baltic cod (*Gadus morhua*). *Journal of Biotechnology and Biomaterials,* 6 (2), pp. 1-6.

Uzunalan, G., Ozturk, M. T., Dincer, S., and Tuzlakoglu, K., 2013. A newly designed collagen-based bilayered scaffold for skin tissue regeneration. *Journal of Composites and Biodegradable Polymers,* 1 (1), pp. 8–15.

Vaissiere, G., Chevallay, B., Herbage, D., and Damour, O., 2000. Comparative analysis of different collagen-based biomaterials as scaffolds for long-term culture of human fibroblasts. *Medical and Biological Engineering and Computing,* 38 (2), pp. 205 – 210.

Van Staveren, W. C., Solis, D. Y., Hebrant, A., Detours, V., Dumont, J. E., and Maenhaut, C., 2009. Human cancer cell lines: Experimental models for cancer cells in situ? For cancer cells? *Biochimica et Biophysica Acta*, 1795 (2), pp. 659-672.

Vargo-Gogola, T., and Rosen, J. M., 2007. Modelling breast cancer: one size does not fit all. *Nature Review. Cancer,* 7 (9), pp. 659-672.

Viswanathan, S., and Nailamuthu, T., 2014. Extraction of sodium alginate from selected seaweeds and their physiochemical and biochemical properties. *International Journal of Innovative Research in Science, Engineering and Technology,* 3 (4), pp. 10998-11003.

Visweswara Rao, A., and Mody, I.C., 1964. Extraction of alginic acid and alginates from brown seaweeds. *Indian Journal of Technology,* 3 (8), pp. 261-262.

Wahl, D. a., and Czernuszka, J. T., 2006. Collagen-hydroxyapatite composites for hard tissue repair. *European Cells and Materials,* 11 (1), pp. 43–56.

Wallis, T. E. (1985). *Unorganised Drugs, Textbook of Pharmacognosy.* 5th edition. Delhi: CBS Publishers and Distributors, pp. 467-469.

Walum, E., 1998. Acute oral toxicity. *Environmental Health Perspectives,* 106 (2), pp. 497–503.

Wahl, D. a., and Czernuszka, J. T., 2006. Collagen-hydroxyapatite composites for hard tissue repair. *European Cells and Materials,* 11 (1), pp. 43–56.

Wallis, T. E. (1985). *Unorganised Drugs, Textbook of Pharmacognosy.* 5th edition. Delhi: CBS Publishers and Distributors, pp. 467-469.

Walum, E., 1998. Acute oral toxicity. *Environmental Health Perspectives,* 106 (2), pp. 497–503.

Wang, H. L., Modarressi, M., and Fu, J. H., 2012. Utilizing collagen membranes for guided tissue regeneration-based root coverage. *Periodontology,* 59 (1), pp. 140 – 157.

Wang, L., An, L., Yang, F., Xin, Z., Zhao, L., and Hu, Q., 2008. Isolation and characterisation of collagens from the skin, scale and bone of Deep-sea redfish *(Sebastes mantella). Food Chemistry*, 108 (2), pp. 616-623.

Werkmeister, J. A., Ramshaw, J. A. A., and Ellender, G., 1990. Characterisation of a monoclonal antibody against native human type I collagen. *European Journal of Biochemistry,* 187 (2), pp. 439 – 443.

Wohlrab, J., Wojlrab, D and Neubert, R. H., 2013. Comparison of non cross-linked and cross-linked hyaluronic acid with regard to efficacy of the proliferative activity of cutaneous fibroblasts and keratinocytes *in vitro*. *Journal of Cosmetic Dermatology,* 12 (1), pp. 36 – 40.

Yamada, S., Nagaoka, H., Terjima, M., Tsuda, N., Hayashi, Y., and Yamauchi, M., 2013. Effects of fish collagen peptides on collagen post-translational modifications and mineralisation in an osteoblastic cell culture system, *Dental Materials Journal,* 32 (1), pp. 88 – 95.

Yamamoto, S., Deguchi, K., Onuma, M., Numata, N., and Sakai, Y., 2016. Absorbtion and urinary excretion of peptides after collagen tripeptide ingestion in humans. *Biological and Pharmaceutical Bulletin,* 39 (3), pp. 428-434.

Zhang, F., Wang, A., Li, Z., He, S., and Shao, L., 2011. Preparation and characterisation of collagen from freshwater fish scales. *Food and Nutrition Sciences,* 2 (8), pp. 818–823.
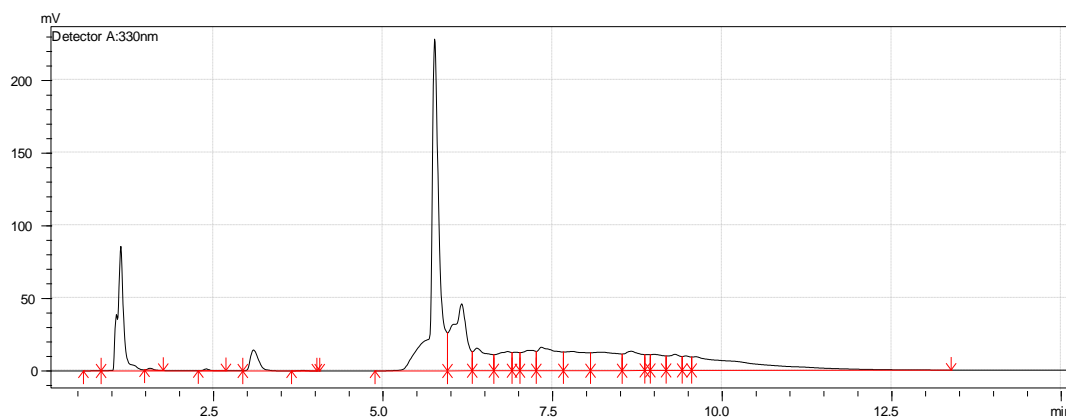
Zhao, Y. H., and Chi, Y. J., 2009. Characterisation of collagen from eggshell membrane. *Biotechnology,* 8 (2), pp. 254-258.

Zuber, M., Zia, F., Tabasum, S., Salman, M., and Sultan, N., 2015.Collagen based polyurethanes — a review of recent advances and perspective. *International Journal of Biological Macromolecules,* 80 (1), pp.366-374.

APPENDICES

Appendix A

HPLC Analysis of Collagen at 330 nm

Peak#	ID#	Name	Ret. Time	Conc.	Area	Height	Mark	Peak Start	Peak End	Area%
1			0.758	0.00000	2420	241		0.583	0.842	0.0389
2			1.128	0.00000	562153	85811	SV	0.842	2.933	9.0397
3			1.558	0.00000	6887	1234	T	1.483	1.758	0.1107
4			2.389	0.00000	7036	1261	T	2.275	2.683	0.1131
5			3.085	0.00000	125008	14485	SV	2.933	4.067	2.0102
6			3.818	0.00000	2407	301	TV	3.650	4.025	0.0387
7			5.757	0.00000	1855850	228472		4.883	5.950	29.8430
8			6.154	0.00000	666649	46090	V	5.950	6.317	10.7200
9			6.382	0.00000	244621	15582	V	6.317	6.633	3.9336
10			6.835	0.00000	197646	13219	V	6.633	6.900	3.1782
11			6.947	0.00000	88597	12793	V	6.900	7.017	1.4247
12			7.194	0.00000	195700	14024	V	7.017	7.258	3.1470
13			7.330	0.00000	341666	16168	V	7.258	7.658	5.4942
14			7.790	0.00000	305493	13292	V	7.658	8.058	4.9125
15			8.221	0.00000	341775	12714	V	8.058	8.525	5.4959
16			8.653	0.00000	242207	13276	V	8.525	8.858	3.8948
17			8.908	0.00000	55119	11033	V	8.858	8.942	0.8863
18			8.995	0.00000	149366	11088	V	8.942	9.175	2.4019
19			9.299	0.00000	145082	11204	V	9.175	9.408	2.3330
20			9.460	0.00000	82271	10001	V	9.408	9.550	1.3230
21			9.607	0.00000	600758	9441	V	9.550	13.375	9.6605

Appendix B

HPLC Analysis of Collagen at 280 nm
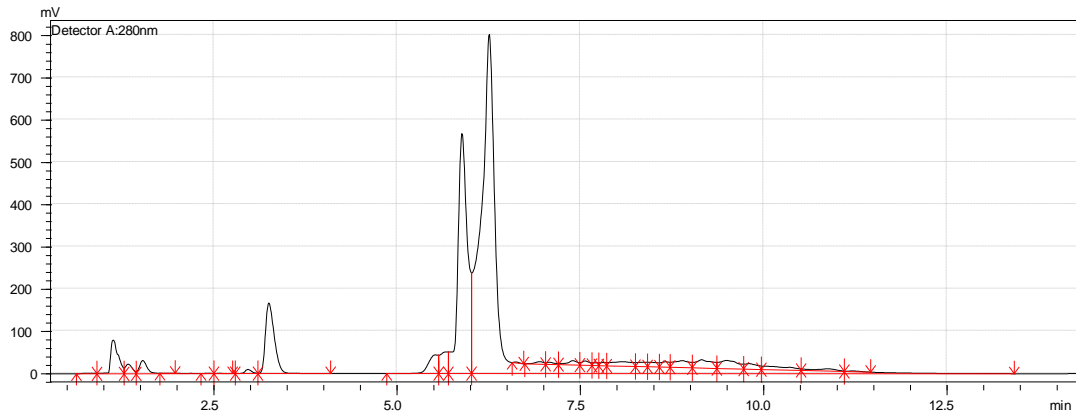
Peak#	ID#	Name	Ret. Time	Conc.	Area	Height	Mark	Peak Start	Peak End	Area%
1			0.793	0.00000	14949	1290		0.625	0.908	0.0596
2			1.124	0.00000	576803	79603	V	0.908	1.275	2.2988
3			1.329	0.00000	149518	22751	V	1.275	1.442	0.5959
4			1.528	0.00000	254789	31289	SV	1.442	2.792	1.0155
5			1.822	0.00000	1325	263	T	1.767	1.975	0.0053
6			2.411	0.00000	2217	412	T	2.325	2.500	0.0088
7			2.569	0.00000	1748	268	TV	2.500	2.758	0.0070
8			2.963	0.00000	64593	9817	V	2.792	3.100	0.2574
9			3.247	0.00000	1405142	167162	V	3.100	4.092	5.6001
10			5.515	0.00000	417503	44052		4.858	5.567	1.6639
11			5.660	0.00000	390444	51269	V	5.567	5.700	1.5561
12			5.881	0.00000	5181115	567968	V	5.700	6.017	20.6492
13			6.252	0.00000	14251652	802837	SV	6.017	13.417	56.7995
14			6.614	0.00000	10299	2163	T	6.567	6.725	0.0410
15			6.941	0.00000	53999	6116	T	6.742	7.025	0.2152
16			7.072	0.00000	40998	5307	TV	7.025	7.200	0.1634
17			7.390	0.00000	105899	10522	TV	7.200	7.492	0.4221
18			7.562	0.00000	80220	10248	TV	7.492	7.658	0.3197
19			7.700	0.00000	36321	6668	TV	7.658	7.750	0.1448
20			7.825	0.00000	48420	7817	TV	7.750	7.858	0.1930
21			8.073	0.00000	221543	10656	TV	7.858	8.250	0.8830
22			8.348	0.00000	103126	10834	TV	8.250	8.417	0.4110
23			8.450	0.00000	101411	11050	TV	8.417	8.575	0.4042
24			8.656	0.00000	108582	12981	TV	8.575	8.725	0.4327
25			8.882	0.00000	258297	16781	TV	8.725	9.025	1.0294
26			9.148	0.00000	323850	19883	TV	9.025	9.358	1.2907
27			9.488	0.00000	352913	19610	TV	9.358	9.725	1.4065
28			9.793	0.00000	163734	13235	TV	9.725	9.967	0.6526
29			10.351	0.00000	218752	6940	TV	9.967	10.508	0.8718
30			10.866	0.00000	127132	5681	TV	10.508	11.100	0.5067
31			11.216	0.00000	23857	1791	TV	11.100	11.458	0.0951

HPLC analysis of Amino Acid Standard (Henderson and Brooks, 2016)
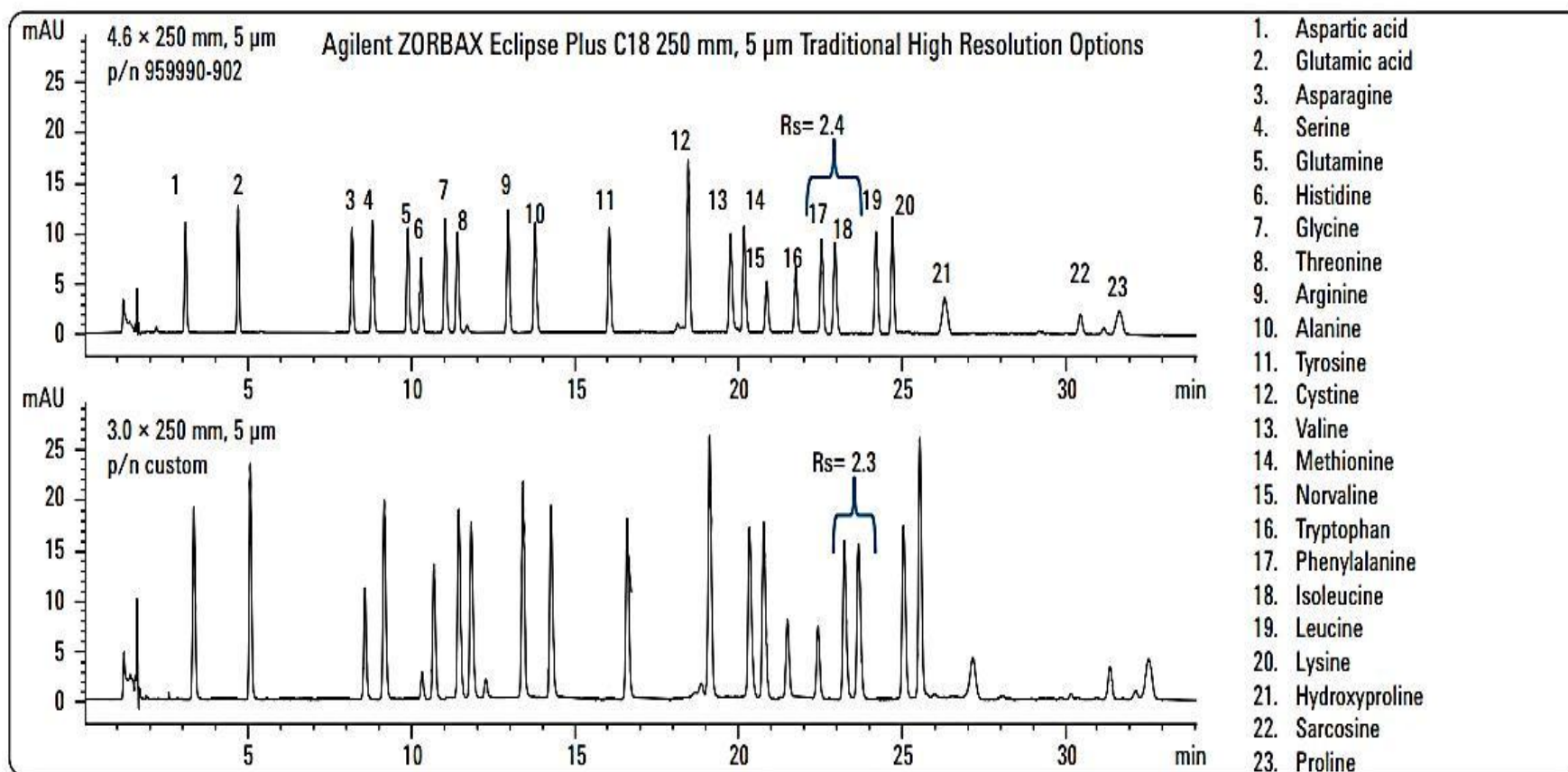
Appendix D

HeLa cancer cells as viewed from haemocytometer
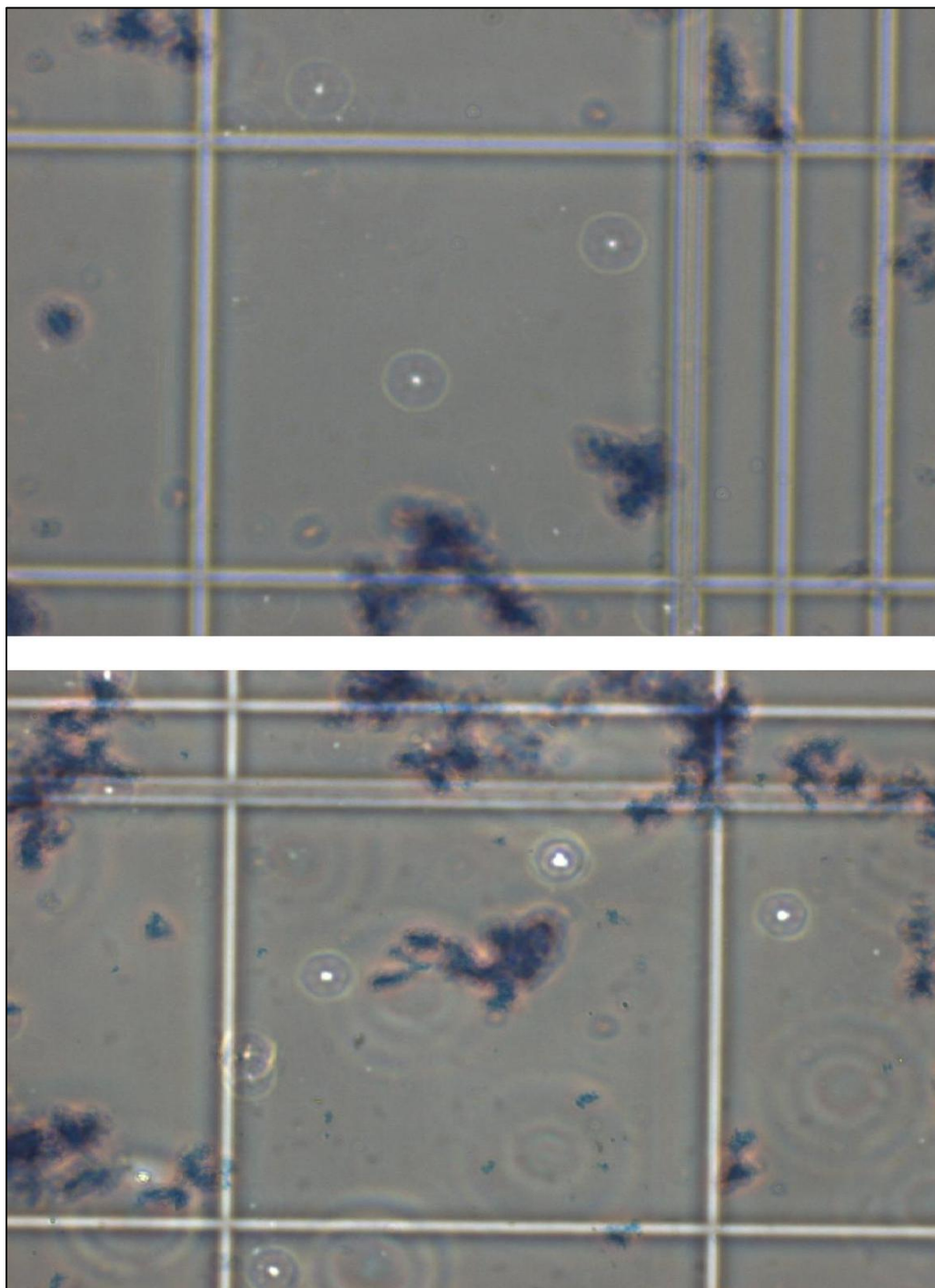
Appendix E

HeLa cancer cells at 100 % confluency as viewed from light microscope
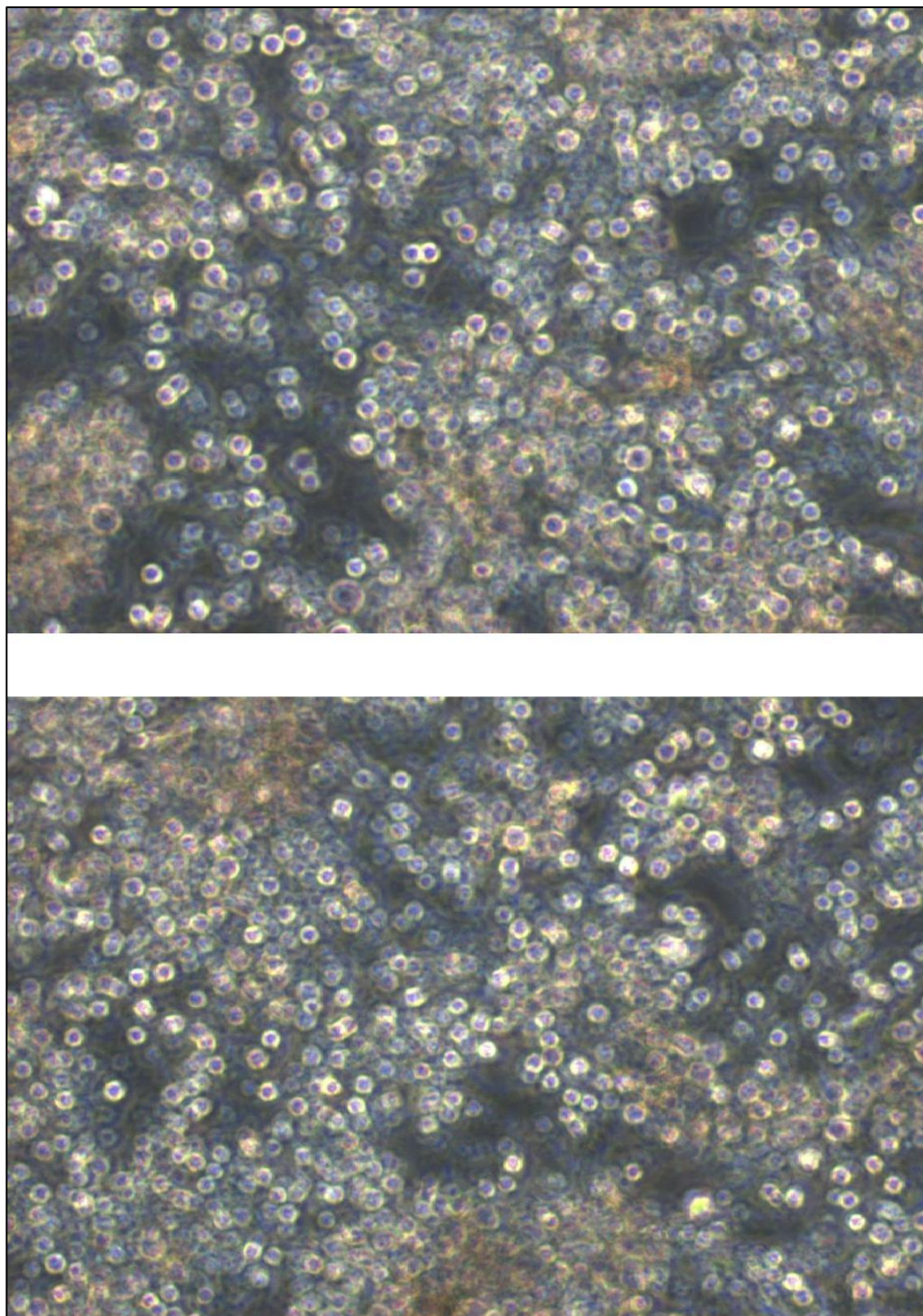
Flow chart of collagen extraction method (Nagai and Suzuki, 2000)

Fish skin cut and mixed with 0.1 M NaoH		Yield recorded as dry weight (g). Stored at -20 ℃ until use
↓		↑
Alkali solution changed every 3 hours		Lyophilised for 48 hours
↓		↑
Rinsed with distilled water until neutral pH		Precipitate dialysed with 0.5 M acetic acid against 0.21 M acetic acid
↓		↑
Mixed with 10 % butanol		Centrifuged at 3'000 × g for 15 minutes at 4 ℃ to collect precipitate
↓		↑
Washed with distilled water		Salted out with 2.5 M NaCl in 0.05 M Tris
↓		↑
Extraction with 0.325 M acetic acid	→	Filtered with tea bag

Appendix G

**Flow chart of sodium alginate extraction method
(Visweswara and Mody, 1964)**

W seaweeds to remove epiphytes	Ground into powder. Stored in dessicator at room temperature until use
↓	↑
Dry seaweeds at room temperature	Sodium alginate yield is recorded (g)
↓	↑
Soak dried seaweeds in 1 % CaCl	Dry precipitate at 50 ℃ overnight
↓	↑
Rinse with distilled water	Supernatant mixed with pure ethanol until precipitate forms
↓	↑
Acidification of seaweed with 5 % Hcl	Centrifuge at 3'000 × g for 5 minutes to collect supernatant
↓	↑
Rinse with distilled water	→ Alkaline extraction of sodium alginate with 3 % NaCO₃

Flow chart of collagen-sodium alginate biomaterial fabrication method (Sang et al, 2011)

Lyophilised collagen dissolved in 0.5 M acetic acid to form 0.7 % w/v solution		
↓		Lyophilised sodium alginate dissolved in phosphate buffer saline to form 10 % w/v sodium alginate solution
Mixed with 2 M NaOH until neutral pH obtained	→ Collagen and sodium alginate mixed until homogenised ←	

Collagen and sodium alginate were mixed. Allowed to gelate overnight

Biomaterials were frozen at −20 ℃ prior to lyophilisation

Fabricated biomaterials labelled accordingly and stored in dessicator until further use