PHYSICOCHEMICAL PROPERTIES OF BIOMATERIAL FABRICATED FROM FISH SKIN COLLAGEN AND BROWN SEAWEED ALGINATE

SOON KEAN SENG

MASTER OF SCIENCE

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN
MAY 2018

ABSTRACT

PHYSICOCHEMICAL PROPERTIES OF BIOMATERIAL FABRICATED FROM FISH SKIN COLLAGEN AND BROWN SEAWEED ALGINATE

Soon Kean Seng

Collagen based biomaterials are used widely in tissue engineering industry. *Smooth wolf herring* skin that was thrown by the local fish processing industry was used as source for collagen extraction in present study. Collagen from the fish skins was obtained with acid solubilisation method. Marine collagen was selected in this study due to the fact that most of the collagen was extracted from land based animal such as bovine and porcine. However, these animals are forbidden for consumption in some religious. Furthermore, there is risk of animal transmitted diseases in using land based animals as source of collagen. Marine collagen is thus far safe from these disturbances. Although, collagen has been intensively applied in biomaterials, these structural proteins found in the connective tissues of animal are not sufficient to achieve the biodegradation and mechanical properties for such applications. Therefore, natural polysaccharide alginate was incorporated. Polysaccharide used to enhance the mechanical properties of fish skin collagen was obtained from *sargassum polycystum* (brown seaweed) at 50°C by using calcium carbonate. The properties of collagen were examined, including amino acid composition, denaturation temperature and the molecular structure with FTIR. The yield obtained from the fish skin was confirmed as collagen with a few signature band of Amide A, B, I, II, and III bands in FTIR spectra. In addition, IR ratio between Amide III band and peak at 1450cm^{-1} region of approximately 1 indicated the presence of triple helical structure in collagen. Imino acid content and hydroxylation ratio from the HPLC analysis of collagen in this study were 2.6% and 46.22%, respectively. Beside that, denaturation temperature of collagen revealed from DCS analysis was 35.23°C. Collagen Alginate Composite films of different alginate and collagen ratio (CA 0/100, CA 20/80, CA 30/70, CA 50/50, CA 70/30, CA 80/20 and CA 100/0) were fabricated by lyophilisation with pure alginate

and pure collagen film as controls. The effects of alginate on porosity, swelling behavior, tensile strength, collagenase degradation, surface structure, denaturation temperature, and FTIR spectra of the biomaterial films were studied. Swelling behavior improved with alginate content, 50% alginate film achieved 1254.75 % swelling after incubation of 24 h. All films possessed porosity of more than 80 % apart from the film with 80% collagen (65.41%). 100 % alginate possessed the highest porosity (94.30 %). Highest young modulus (27.05 MPa) and tensile strength (1585.87 kPa) was discovered in 50% alginate film. Moreover, collagenase degradation was reduced with alginate content, 80% alginate film demonstrated lowest degradation rate. In terms of FTIR analysis, an additional band at around 3280 cm^{-1} indicated the hydroxyl group (O-H) of alginate was bonded to amino group (N-H) of collagen was observed in all CA composite films. Apart from that, result shows that addition of alginate in certain amount can improved the thermal denaturation temperature of CA films. Highest denaturation temperature (83.11°C) was found in 30% alginate composite film. Surface structure of CA films were different in different alginate concentration. Results showed that alginate is effective in enhancing some physicochemical and mechanical properties of the composite film.

ACKNOWLEDGEMENTS

Firstly, I would like to thank everyone who had contributed to the successful completion of this project. Secondly, I would like to express my gratitude to my research supervisor and co-supervisors, Dr. Woo Kwan Kit and DR. Leong Loong Kong for their advice, guidance and enormous patience throughout the development of the research.

In addition, I would also like to express my gratitude to my loving parent and friends who had helped and given me encouragement during the development of this research. Finally I would like to dedicate my thanks to Restaurant *Sin Hao Jing* for the contribution of fish waste which make this project conducted smoothly.

APPROVAL SHEET

This dissertation/thesis entitled "**PHYSICOCHEMICAL PROPERTIES OF BIOMATERIAL FABRICATED FROM FISH SKIN COLLAGEN AND BROWN SEAWEED ALGINATE"** was prepared by SOON KEAN SENG and submitted as partial fulfillment of the requirements for the degree of Master of Science in Faculty of Engineering Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. WOO KWAN KIT) Date:……..
Professor/Supervisor
Department of Chemical Engineering
Lee Kong Chian Faculty of Engineering and Science
Universiti Tunku Abdul Rahman

_____

(Dr. Leong Loong Kong) Date:……..
Professor/Co-supervisor
Department of Chemical Engineering
Lee Kong Chian Faculty of Engineering and Science
Universiti Tunku Abdul Rahman

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 05/05/2018

SUBMISSION OF THESIS

It is hereby certified that ***Soon Kean Seng*** (ID No: ***15UEM01376***) has completed this thesis entitled "PHYSICOCHEMICAL PROPERTIES OF BIOMATERIAL FABRICATED FROM FISH SKIN COLLAGEN AND BROWN SEAWEED ALGINATE" under the supervision of Dr. Woo Kwan Kit (Supervisor) and DR. Leong Loong Kong (Co-Supervisor) from the Department of Chemical Engineering, Lee Kong Chian Faculty of Engineering and Science.

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

Yours truly,

_____

(*Soon Kean Seng*)

DECLARATION

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

Name _____

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS / ABBREVIATIONS

CA	collagen-alginate
FACIT	fibril-associated collagen
BSE	bovine spongiform encephalopathy
FTIR	fourier transform infrared spectroscopy
ASC	acid soluble collagen
PSC	pepsin soluble collagen
T_d	denaturation temperature
M	β- D- mannuronate
G	α- L- guluronate
F_{GG}	guluronic blocks
F_{MM}	mannuronic block
DSC	differential scanning calorimetry
W_{dry}	dry weight
PBS	phosphate buffer saline
W_{wet}	wet weight
S	swelling ratio
V_s	geometrical volume
V_p	pore volume
SEM	scanning electron microscope
SE	secondary electron
BSE	back-scattered electron

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background and Problem Statement

Biomaterial is a natural or synthetic material that possessed specific properties that allowed them to be in contact with living tissues, without lead to adverse immune rejection (Gilbert & Oksana Budinskaya, 2016). Biomaterials have been used for decades in fabricating joint replacement materials, artificial heart, skin repair device, drugs delivery system and contact lens (Tathe, Ghotdke, & Nikalja, 2010).

Industrial applied biomaterial can be derived naturally from the natural resources or man-made. Examples of synthetic biomaterials are ceramics, polyethylene, and calcium phosphate. On the other hand, examples of natural polymers are collagen, chitosan (from insect), polysaccharides (from plant), and keratin (from hair). The advantages of using natural biomaterials may overcome the occurrence of toxicity which encountered by synthetic biomaterials (Davis, 2003). Thus, collagen and alginate are chosen as the raw materials in fabricating the composite biomaterial film in this study.

Collagen is a fibrous protein that found in animal's skin and bone connective tissue (Foegeding, Lanier, & Hultin, 1996). Approximately, 20-30% of the total body protein is made up of collagen (Wang, Yang, & Du, 2008). Throughout the years, collagen has been applied widely in tissue engineering, biomedical, and cosmeticeutical industry due to its great biocompatibility and weak antigenicity (Lee, Singla, & Lee, 2001).

For example, collagen in the form of mini pellet is used as a drug delivery system. In addition, collagen biomaterial is used to regenerate and reconstruct tissue, these applications have showed that collagen plays an important role in mediating cell migration and adhesion because of its potential interaction with cells (Mccardy, Vachhani, & Lida, 1996). Thus, collagen has been extracted from various sources for such applications.

For the past few decades, collagen was mainly extracted from porcine and bovine (Woo, Yua, Cho, Lee, & Kim, 2008). However, collagen isolated from porcine is not accepted by Islam and Judaism (Duan, Zhang, Du, Yao, & Konno, 2009). Besides, collagen derived from the bovine sources are at the risk of contamination with bovine spongiform encephalopathy (BSE) (Choi & Regenstein, 2000). Thus, marine collagen is used as the main sources of collagen in the current industry.

Marine collagen is widely extracted from the byproduct produced during fish filleting process. Total of byproduct (fish skin, fins and bones) produced during filleting process can be as much as 75% of the total weight of the fish (Woo et al., 2008). In order to ease the problem of waste management in the fish industries, fish

byproducts such as skin can be used to produce value added product. Therefore, in present study, collagen is extracted from the skin of *smooth wolf herring* which is normally discarded from the fish meat processing industry. Extraction of collagen using disposed fish skin will be a useful platform in diversifying the revenue of fish processing industries.

Even though marine collagen is widely used today, but its application in tissue engineering industry is still unable to fulfil certain requirements due to its weak mechanical and ease degradation property (Sang, Luo, Wang, & Li, 2011). As a result, collagen is combined with alginate to strengthen the mechanical properties of collagen. In recent years, alginate has been used widely for encapsulating the cell, and fabricating scaffolds due to its great biodegradation property, biocompatible property and elasticity (August, Kong, & Mooney, 2006).

Alginate is linear anionic polysaccharides with 1-4 linked α-L-guluronate and β-D-mannuronate residues that arranged in an irregular block wise pattern along the chain (Lee & Mooney, 2011). Commercial alginate are usually extracted from brown seaweed *laminaria species* and *ascophyllum nodosum* where they reached up to 40% of the dry weight in yield (Rinaudo, 2007). Current study utilized alginate obtained from the brown seaweed (*sargassum polycystum*) of the local variety which is under utilized and ready to be harvested.

As alginate and collagen are used widely in the industry of tissue engineering, the development of collagen-alginate composite film with improved properties is thus essential. At present, collagen based biomaterials are mostly produced directly by

lyophilized the pure collagen or collagen composite blend (Gao, Wang, & Shen, 2003). In this study, pure collagen, alginate and collagen-alginate composite films are prepared by lyophilized the respective blends.

1.2 Aims and Objectives

Current study is aimed to utilize the waste from the fish industry (*smooth wolf herring* skin) in fabricating the collagen based biomaterials film. In addition, another objective of this study is to hybridize the collagen based biomaterial with alginate obtained from the brown seaweed (*sargassum polycystum*) of the local variety to enhance the physicochemical and mechanical properties of the biomaterial film.

Moreover, current study is aimed to characterize the physicochemical and mechanical properties of collagen, alginate and collagen-alginate films. Lastly, the objective of this study is to investigate the effects of alginate amounts on the tensile property, enzymatic degradation, denaturation temperature, porosity, swelling behavior, surface morphology and FTIR spectra of CA composite films.

CHAPTER 2

LITERATURE REVIEW

2.1 Biomaterials

In medical terminology, biomaterial is defined as any synthetic or natural material that introduced into living tissues as a part of medical implant (Gilbert & Oksana, 2016). Over the past few decades, biomaterials are used in total hips replacement, knee implant, fabricating artificial skin and etc. An ideal biomaterial should be biocompatible which able to avoid adverse tissue infections. Other than that, both synthetic and natural biomaterials should have excellent degradation resistance and good mechanical strength (Ekta, Keerti, Saurabh, Suravi & Nidhi, 2016).

Synthetic biomaterials such as polyethylene, polyvinylchloride and polyamide usually are toxic to our body. Thus, biomaterial derived from natural sources such as collagen, gelatin, keratin and alginate are usually used in tissue engineering and purposes (Davis, 2003). In this study, collagen and alginate which extracted from *smooth wolf herring* skin and brown seaweed *sargassum polycystum* are used as raw materials in fabricating collagen-alginate film.

2.2 Collagen

Collagen is a type of fibrous protein which accounting for approximately 20-30% of total body protein (Wang et al., 2008). Collagen is considered as a structural element in all connective tissues due to its ability to maintain the stability and structural integrity of tissues and organs (Gelse, Pöschi, & Aigner, 2003). Besides, the shape of collagen is unique compared to other types of proteins.

Collagen is a type of protein which present in the shape of triple helical structure because it is formed from a rod-shape protein (tropocollagen) that consist of three polypeptide unit (α-chain) (Wong, 1989). Three α-chain are held together mainly by hydrogen bonding between carbonyl and amino group (Lee et al., 2001).

Other than that, the characteristic linkages of all collagens are represented by repeating structure of $(Gly\text{-}X\text{-}Y)_n$. Glycine residue is the structural prerequisite for triple helix while the X and Y positions are usually occupied by hydroxyproline and proline (Gelse et al., 2003). In short, collagen is a type of fibrous protein that appears in triple helical structure which formed from 3 α-chains that stabilized together by hydrogen bonding. Figure 2.1 shows the arrangement of collagen fibril in collagen fibre.

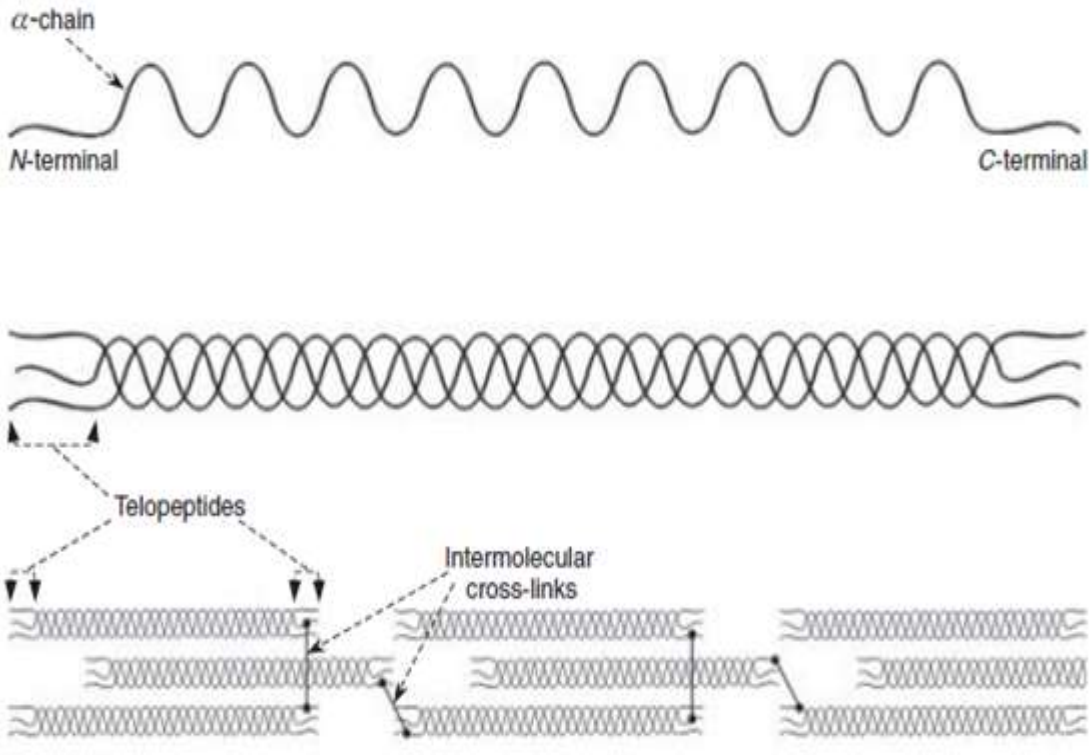


Figure 2.1: Arrangement of Collagen Fibril in Collagen Fibre: (a) Collagen Polypeptide, (b) Tropocollagen and (c) Collagen Fibril (Benjakul et al., 2012).

2.3 Members of Collagen Family

Collagen is a protein that constructed by triple helix of three polypeptides chains. These supramolecular structures in the extracellular matrix can be found in all members of the collagen family even if the size, function and tissue distribution of collagen are different. At present, there are 26 types of collagens have been discovered, and these collagens have been classified into different groups (Gelse et al., 2003).

In general, collagens were classified into fibril-forming collagens, fibril-associated collagen (FACIT), network-forming collagen, anchoring fibrils, transmembrane collagen, basement membrane collagen, and others with unique function according to their structure and supramolecular organization (Gelse et al., 2003).

Among the groups of collagen, fibril-forming collagen is the most abundant collagen found in animal's body (Birk, Fitch, Babiarz, & Linsenmayer, 1988). According to Gelse et al. (2003), it accounts about 90% of the total collagen in our body. The allocation of different type of collagen in our body is showed in Table 2.1.

It is noticed that different type of collagens is distributed in distinct kind of tissues (Table 2.1). For example, type I and V collagen fibril can be found in the bones while type II and XI collagen are primarily present in articular cartilage (Birk et al., 1988; Mayne & Brewton, 1993).

Apart from that, type XVI collagen is found in hyaline cartilage and skin. Moreover, type IX collagen is mostly contributed in cartilage and the vitreous body (Lai & Chu, 1996). In summary, distinct type of collagens are distributed in different part of our body.

Table 2.1: Distribution of Different Type of Collagen in our Body (Gelse et al., 2003)

Type	Tissue distribution
Fibril-forming collagens	
I	bone, dermis, tendon, ligaments, cornea
II	cartilage, vitreous body, nucleus pulposus
III	skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)
V	lung, cornea, bone, fetal membranes; together with type I collagen
XI	cartilage, vitreous body
Basement membrane collagens	
IV	basement membranes
Microfibrillar collagen	
VI	widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils	
VII	skin, dermal C epidermal junctions
Hexagonal network-forming collagens	
VIII	endothelial cells, Descemet's membrane
X	hypertrophic cartilage
FACIT collagens	
IX	cartilage, vitreous humor, cornea
XII	perichondrium, ligaments, tendon
XIV	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	human rhabdomyosarcoma
XX	corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	blood vessel wall
Transmembrane collagens	
XIII	Skin epidermis, follicle of hair, lungs, intestine, and liver
XVII	Dermal-epidermal junctions

Table 2.1: (Continue)

Type	Tissue distribution
Multiplexins	
XV	fibroblasts, smooth muscle cells, kidney, pancreas,
XVI	fibroblasts, amnion, keratinocytes
XVIII	lungs, liver

2.4 Sources of Collagen

In general, collagen are extracted extensively from various sources like bovine and porcine. For example, collagen has been extracted from bovine pericardium and used as a biomaterials in scaffold preparation by Simpson et al. (2012). Other than that, the collagen isolated from porcine skin was utilized in tissue engineering field by Parenteau-Bareil et al. (2011). However, the collagen derived from both porcine and bovine encountered with some limitations.

For instant, the porcine collagen are restricted for some religions such as Islam and Judaism (Duan et al., 2009). While the collagen derived from the bovine sources are at the risk of contamination with bovine spongiform encephalopathy (BSE) and prohibited for Sikhs and Hindus (Choi & Regenstein, 2000; Fernandez, Gomez, & Montero, 2001). As a result, the alternative source of collagen such as marine collagen have received increasing attention by many researchers.

In recent years, marine collagen has been isolated and characterized from various fish species such as *grass carp, striped catfish, tilapia, minkle whale* and etc. In this study, collagen was isolated from the skin of *smooth wolf herring species* by using acid extraction method.

2.5 Extraction of Collagen

Generally, fish collagen can be extracted from fish waste through acid solubilisation process by using some acid solution such as acetic acid, citric acid and hydrochloric acid (Simpson et al., 2012). The collagen obtained from the acid solubilisation process is known as acid soluble collagen, ASC (Bae et al., 2008).

In acid solubilisation process, extraction of collagen is performed in acidic condition in which the collagen polypeptides (positively charged) become dominant. As a result, ASC is obtained as the repulsion between the tropocollagen is enhanced and thus leading to the increased of collagen solubilization in acidic solution (Benjakul, Nalinanon, & Shahidi, 2012).

After the extraction of ASC, the remnant is known as non-acid soluble collagen which are strongly crosslinked collagen fibrils. Further extraction of collagen can be performed by using pepsin (Gefen, 2011). The collagen extracted through pepsin solubilization process is known as pepsin soluble collagen, PSC (Bae et al., 2008).

Predominantly, pepsin solubilization process generate a higher yield compare to acid solubilization process. Nalinanon, et al. (2007), which stated that the yield of collagen from *bigeye snapper* with acid and pepsin extraction were 5.31% and 18.74% respectively. The higher yield can be due to the ability of pepsin to cleave the telopeptide region of collagen (Gefen, 2011). Thus, the yield of collagen is higher when the pepsin is utilized in the extraction process.

Collagen can be extracted from fish waste by using both acid and pepsin. However, collagen extracted by using pepsin is able to provide a higher yield compare to acid because pepsin is able to digest the telopeptide region of the collagen. In this study, collagen is extracted from *smooth wolf herring* species with the aid of acetic acid.

2.6 Function of Collagen

Throughout the years, collagen has been used in both medical and biomedical industry because of its great biodegradability biocompatible property (Ma et al., 2003). For example, collagen in the form of collagen sponges is used for the treatment of wounds and burns such as pressure sores (Rao, 1995). Besides, collagen is applied as a drug delivery system in the form of collagen shields (Kaufman, 1988). In the form of collagen film and sheet, collagen is used in the treatment of tissue infection, such as infected corneal tissue (Bloomfield et al., 1978).

Other than the application in biomedical industry, collagen is widely used in tissue engineering and cosmetic field. In cosmetic field, collagen hydrolysate is utilized as an ingredient in cosmetic formulation to enhance the structure of the skin. For tissue engineering purposes, collagen based scaffold is used for the regeneration of bones and skin (Lee et al., 2001). In addition, collagen based scaffold provide a 3D structure for cell adhesion, proliferation and secretion of extracelluar matrices to initiate the formation of new tissue (Sang et al., 2011).

Collagen is used extensively in biomedical, cosmetic and tissue engineering industry for decades because of its excellent biocompatibility. As a result, the physiochemical and mechanical properties of collagen from different sources is important to be studied before it is implemented into the industry.

2.7 Physicochemical Properties of Collagen

Collagen had been extracted from fish waste for decades. Many researchers have characterised the physiochemical and mechanical properties of collagen before it is used as a biomaterial in biomedical industry. Among the properties of collagen, the amino acid composition, fourier transform infrared spectroscopy (FTIR) and thermal stability are usually studied.

2.7.1 Amino Acid Composition of Fish Collagen

The amino acid of fish collagen from different species is shown in Table 2.2. It can be observed that fish collagen is rich in proline, glycine and alanine. However, cysteine and tryptophan are usually absent in fish collagen (Benjakul et al., 2012).

According to Singh, Benjakul, Maqsood, and Kishimura (2011), collagen is the only protein that rich in hydroxyproline. As a result, hydroxyproline is usually used as a measured of collagen content in foods (Foegeding et al., 1996). Other than hydroxyproline, imino acid (proline and hydroxyproline) is another important component in collagen.

It is well known that the imino acid played an important role in maintaining the stability of collagen (Nagai, Nagashima, & Suzuki, 2008). This is because the pyrrolidine rings of proline and hydroxyproline assisted to support the triple helix structure of collagen (Wong, 1989).

As illustrated in Figure 2.2, the hydroxyl group of hydroxyproline stabilized the helix by interchain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to a carbonyl group (Wong, 1989). Thus, the higher the imino acids content, the more stable the helical structure of collagen.

Apart from that, the collagen derived from different fish species possess distinct imino acid content. The variation in imino acid content amongst distinct fish species is mainly due to the living habitat. In general, cold water fish had a lower imino

acid content compare to warm water fish. For example, the imino acid content of cod (cold water fish species) is lower than the grass carp (warm water fish species) as shown in Table 2.2 (Duan et al., 2009; Zhang, Liu, Li, Shi, Miao, & Wu, 2007). Thus, collagen extracted from warm water fish is more stable than those extracted from cold water fish.

Table 2.2: Amino Acid Composition of Fish Collagen (Simpson et al., 2012).

	Cod Skin	*Bigeye Snapper Skin*	*Grass Carp Skin*	*Calf Skin*
Amino Acids	Cold Water Fish	Water Fish	Warm Water Fish	Water Fish
Asparagine	53	51	42	45
Glutamine	80	78	61	75
Glycine	342	286	334	330
Hydroxyproline	51	77	65	94
Proline	103	116	121	121
Serine	59	36	39	33
Isoleucine	12	5	10	11
Leucine	22	24	22	23
Lysine	29	31	23	26
Valine	19	22	31	21
Threonine	23	29	24	18
Tyrosine	4	4	2	3
Histidine	8	10	5	5
Hydroxylysine	7	10	8	7
Methionine	15	12	10	6
Phenylalanine	12	15	17	3
Cysteine	0	0	4	0
Imino Acid	154	193	186	215

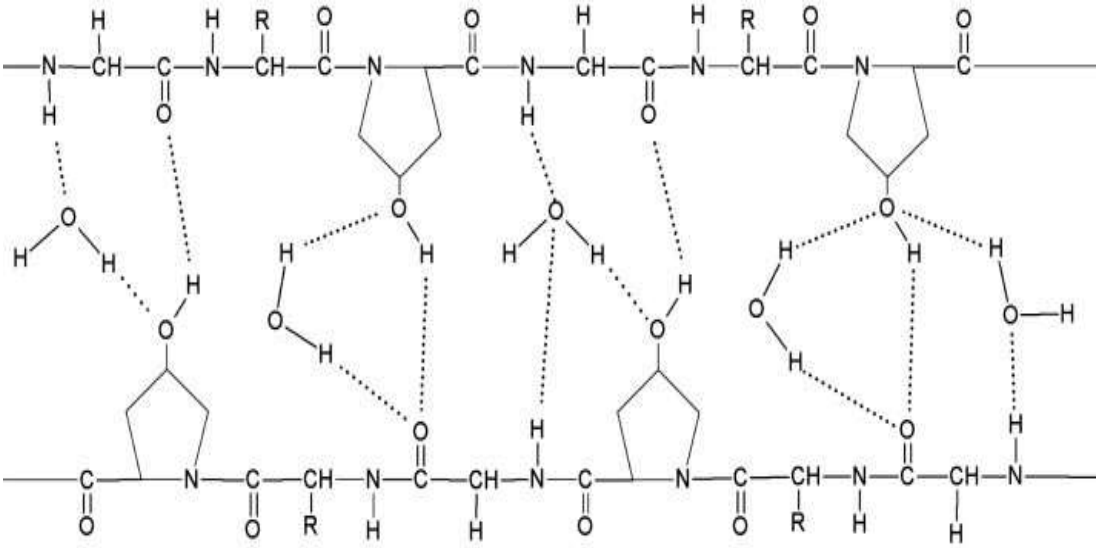


Figure 2.2: Contribution of Hydroxyproline in Hydrogen Bonding between Collagen Polypeptides (Simpson et al., 2012).

2.7.2 Thermal Stability of Fish Collagen

The thermal stability of fish collagen is mainly governed by the imino acid content (proline and hydroxyproline). In general, the collagen with higher imino acid content possessed higher denaturation temperature compared to the collagen with low imino acid content (Singh et al., 2011). The denaturation temperature (T_d) of collagen from different species are listed in Table 2.3.

From Table 2.3, it can be observed that the collagen from *carp* is lower than *calf*. This is due to the imino acid content of collagen from *calf* is higher than *carp* (Duan et al., 2009). As mention previously, the imino acid is responsible to maintain the helical structure of collagen (Ikoma, Tanaka, Kobayashi, Walsh & Mann, 2003; Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Thus, the higher the content of imino acid, the greater the thermal stability of collagen.

Apart from that, the thermal stability of fish collagen is correlated to the living environment of fish, particularly environmental temperature (Kittiphattanabawon et al., 2010; Nagai et al., 2008). Basically, the denaturation temperature of cold water fish collagen is lower than the warm water fish collagen.

For example, the denaturation temperature of cod (cold water fish species) is 15^{o}C which is lower than the denaturation temperature (28.4^{o}C) of *grass carp* (warm water fish species) (Duan et al., 2009; Zhang et al., 2007). This is because the imino acid concentration of collagen from cold water fish is lesser than warm water fish (Jongjareonrak et al., 2005; Zhang et al., 2007). In general, the collagen stability can be affected by the content of imino acid which is correlated to the habitat of fish.

Table 2.3: The Denaturation Temperature (T_d) of Acid Soluble Collagen from the Skin of Several Fish Species (Simpson et al., 2012).

Various Collagen Sources	Imino Acid Content (%)	Denaturation
	Acid Soluble Collagen (ASC)	Temperature (T_d, ^{o}C)
Calf	21.5	36.3
Yong Nile Perch	19.3	36.0
Adult Nile Perch	20.0	36.0
Black Drum	20.0	34.2
Bigeye Snapper	19.3	32.5
Carp	19.0	28.0
Cod	15.4	15.0
Arabesque Greenling	15.9	15.7

2.7.3 Solubility of Collagen

In general, all collagens exhibited high solubility in between pH 2.0 to 5.0, with a relative solubility over 80%. On the other hand, solubility of collagen will decreased gradually when the collagen is dissolve in a neutral pH solution (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). For example, acid soluble collagen (ASC) from *bigeye snapper* exhibited lowest solubility at pH 7.0 (Kittiphattanabawon, et al., 2005). Other than that, distinct type of collagen possessed different solubility in the pH range of 6.0 to 10.0 (Nalinanon et al., 2007). For instance, the solubility of pepsin soluble collagen (PSC) and ASC are differ in the pH range of 6.0 to 10.0.

Study have proved that, the solubility of PSC is higher than ASC at pH 7.0 and above 7.0 (Nalinanon, et al., 2007). This can be due to the poorer degree of cross-linking or weaker bonds in PSC than ASC (Jongjareonrak et al., 2005). Apart from that, the solubility of both PSC and ASC is observed to be impaired when both collagen dissolve in extreme acidic pH (pH 1.0) (Montero, Gómez-Guillén, & Borderías, 1999). This can be explained by the denaturation of both collagens in extremely low pH (Vojdani, 1996). Beside that, the solubility of the collagen is affected by the present of NaCl in the solution as well (Simpson et al., 2012).

According to Nalinanon, et al. (2007), the solubility of collagen will decline gradually when the concentration of NaCl is inclined. This can be due to the effect of "salting out" whereby the hydrophobic interaction and aggregation of collagen is increased when the salt concentration is higher (Vojdani, 1996). In addition, study has shown that the solubility of PSC and ASC is different when both of the collagen was dissolve in salt solution (Simpson et al., 2012). For example, the PSC from bigeye snapper exhibited a higher solubility compare to ASC when the NaCl concentration is above 3% (Nalinanon et al., 2007). This can be due to the lower chain strength of PSC compare to ASC (Jongjareonrak et al., 2005). In short, the solubility of ASC and PSC is affected by both pH and salt concentration of the solution. However, PSC may possess a higher solubility compare to ASC as the chain strength is poorer.

2.8 Alginate

Alginates are the most abundant naturally occurring polysaccharides found in brown seaweed (Torres et al., 2007). It has been used extensively in biomedical field due to its excellent biodegradability and biocompatibility (Sun & Tan, 2013). In recent years, alginate is defined as linear polymers containing blocks of (1,4)- linked β- D-mannuronate (M) and α- L- guluronate (G) residues (Lee & Mooney, 2011).

As shown in Figure 2.3, the blocks are made up of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM), and alternating G and M residues. The M and G contents as well as the length of each block in alginate are distinct from different sources (Lee & Mooney, 2011). For example, the alginate synthesized from *pseudomonas species* does not contained G-block as compared to brown seaweed alginate (Thomas et al., 2013).

According to George and Abraham (2006), only G-blocks of alginate are believed to participate in intermolecular cross-linking with divalent cations (Ca^{2+}) to form hydrogel. Thus, the composition (M/G ratio) and block structures are critical factors which affect the physical and mechanical properties of alginate (Hay, Rehman, Ghafoor, & Rehm, 2010).

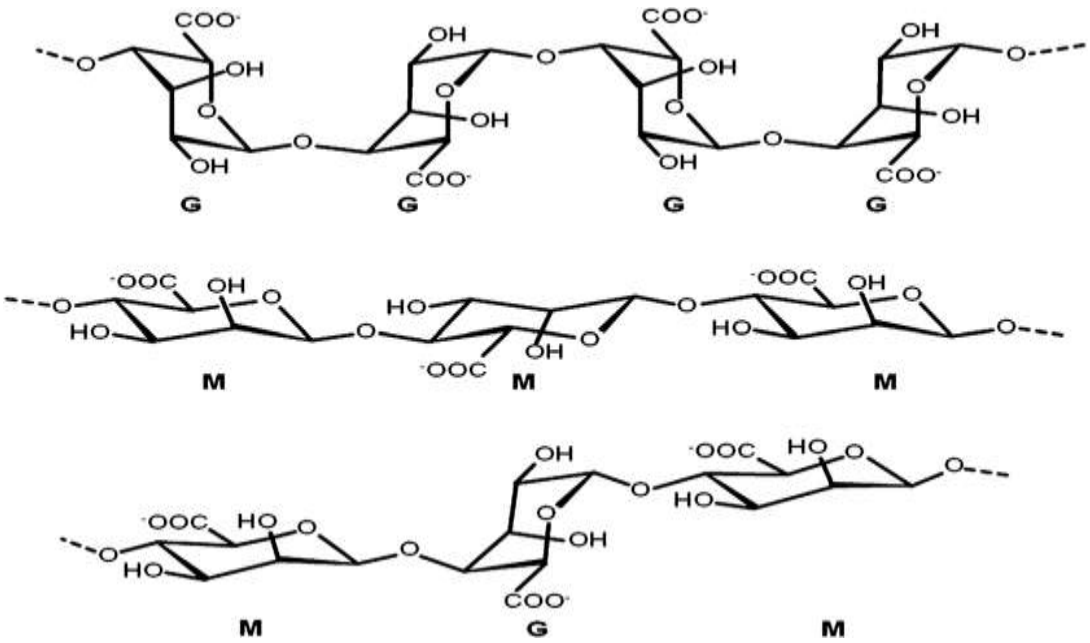


Figure 2.3: Chemical Structures of G-block, M-block, and Alternating Block in Alginate (Lee and Mooney, 2012).

2.9 Physical and Chemical Properties of Alginate

Throughout the years, alginate has been extracted from variety species of brown seaweed. Beside that, the physical and chemical properties of alginate have been characterized by researchers prior applying in both biomedical and food industry. Among the properties of alginates, the compositions, block structure, fourier transform infrared spectroscopy, molecular weight and intrinsic viscosity are usually analysed.

2.9.1 Compositions and Block Structure of Alginate

Basically, the gelling properties of alginate is greatly influenced by the uronic acid composition (guluronic and mannuronic acid ratio) (Penman & Sanderson, 1972). Elastic gel are resulted from the alginate with high M/G ratio, whereas low M/G ratios yielded brittle gel (Fenoradosoa et al., 2010). Apart from M/G ratio, the existence of homopolymeric block structures in alginate also has an influence on the gelling properties (Torres et al., 2007).

According to Fertah, Belfkira, Dahmane, Taourirte, and Brouillette (2014), the gel formation is mainly depends on the abundance of guluronic blocks (F_{GG}) in alginate. This is because the divalent cation (Ca^{2+}) tends to bind towards the G-block instead of M-block. This characteristic became more important in industrial application when alginate is used as a bio-absorbent for heavy metals, such as cadmium ions (Cd^{2+}) (Davis, Llanes, Volesky, & Mucci, 2003).

From Table 2.4, alginate from different brown seaweeds possessed distinct compositions and block structure. For instance, the M/G ratio of alginate from *sargassum fluitans* and *sargassum polycystum* are 0.52 and 0.21 respectively. Besides, the F_{GG} of alginate from *sargassum oligocystum* and *sargassum polycystum* are different as stated in Table 2.4 (Davis et al., 2003).

Other than that, the compositions of alginate is affected by the growing environment of brown seaweed (Fenoradosoa et al., 2010). Referring to Table 2.4, the compositions and block structure of *Sargassum Fluitans* alginate from Florida and Cuba are distinct.

Table 2.4: Compositions of Alginate Extracted from various Brown Seaweeds Species (Torres et al., 2007).

Species	Origin	M/G Ratio	F_{GG} (block structure)	F_{MM} (Block Structure)
Sargassum Fluitans	Cuba	0.52	0.57	0.25
Sargassum Fluitans	Florida	1.18	0.28	0.36
Sargassum Oligocystum	Australia	0.62	0.55	0.31
Sargassum Dentifolium	Egypt	0.52	0.55	0.23
Sargassum Thunbergii	Korea	0.53	0.48	0.17
Sargassum Muticum	England	0.31	0.59	0.07

2.9.2 Purity of Alginate

Alginate is a type of polysaccharide that used extensively in biomedical field in fabricating biomaterial. Unpurified alginate can caused the overgrowth of fibrotic cell around the alginate microcapsules (Torres et al., 2007). Therefore, purification of alginate is needed before it is applied in the biomedical field.

The main contaminants found in alginate are polyphenol, proteins, and endotoxins. The purification degree of alginate can be determined through florescence spectroscopy which identified the presence of polyphenolic impurities often found in alginate (Dusseault et al., 2006).

Dissolution and re-precipitation of alginate with ethanol is an effective method to remove the contaminants in alginate (Fertah et al., 2014; Torres et al., 2007). For example, the fluorescence intensity of contaminants in both *laminaria hyperborean* and *sargassum vulgare* alginate are reduced by 63 and 52.7% respectively by reprecipitation of alginate with ethanol (Torres et al., 2007). In short, the re-precipitation of alginate with ethanol is a useful method to reduce the polyphenolic impurities which presence in alginate.

2.10 Functions of Alginate

Alginate have been used extensively in biomedical, pharmaceutical, and food industries. In food industry, alginate is used to stabilize and changed the texture of the food (Torres et al., 2007). Apart from that, alginate is used as a scaffolding material in biomedical industry due to its excellent biocompatibility, mechanical strength and biodegradability (Sun & Tan, 2013).

Alginate in the form of gel is used to deliver a variety of low molecular weight drugs (Boontheekul, Kong, & Mooney, 2005). In addition, it is an excellent material for the delivery of proteins drugs. This is because denaturation and degradation of protein is prevented when the protein is incorporated into the alginate-based formulation (Lee & Mooney, 2011).

Beside that, alginate-based wound dressings is used for the treatment of acute and chronic wound (Baoteng, Matthews, Stevens, & Eccleston, 2008). Other than that, alginate in the form of gel is increasingly being used as a model system for mammalian cell culture in biomedical research (Lee & Mooney, 2011). Other reported study include the combination of collagen with alginate to enhance the mechanical properties of collagen based biomaterial and it delivered a positive outcome (Sang et al., 2011).

2.11 Types of Collagen Composite Scaffold/Films

In recent years, collagen have been used widely to fabricate functional biomaterials such as scaffold, film, sponges and in the form of shield in tissue engineering and biomedical industry. However, the role of collagen based biomaterial was limited by its weak mechanical properties and biodegradability (Lee, Alsberg, & Mooney, 2001).

As a consequences, many researchers have hybridized collagen with other potential material to improve the overall performances of collagen based biomaterial (Chen, Ushida, & Tateishi, 2002). For example, minerals such as silica was incorporated with the collagen by Perumal et al. (2015) to enhance the performances in terms of biodegradation and tensile strength.

Apart from that, natural polymers like chitosan, alginate and pectin (polysaccharides) were blended with collagen in different composition to overcome the limitations of pure collagen (Arpornmaeklong, Pripatnanont, & Suwatwirote, 2008; Sang et al., 2011). Other than natural polymers, mineral such as hydroxyapatite was introduced into the collagen based biomaterial as well (Feng, Liang, Feng, Qi, & Tang, 2015).

Thus, both natural polymers and minerals can be used to improve the properties of collagen based biomaterials. Natural polymer was used widely to cross linked with collagen because of its low toxicity to human cell (Sionkowska & Kozłowskaet, 2013). Many studies have reported that the overall performance of collagen based biomaterials can be strengthen by blending the collagen with other natural materials together.

2.12 Mechanical and Physicochemical Properties of Collagen Composite Biomaterials

In recent years, many potential materials such as natural polysaccharides and minerals have incorporated with collagen to yield composite materials with improved properties. When collagen is hybridized with other material, physicochemical and mechanical properties of collagen based biomaterials such as tensile strength, biodegradability, denaturation temperature and porosity are changed.

2.12.1 Mechanical Properties of Collagen Composite Biomaterials

Mechanical properties of biomaterials are mostly measured in terms of tensile strength. Collagen with a weak mechanical property usually yielded a scaffold or film with weak tensile strength due to its high degradation rate (Liu & Ma, 2004). Thus, many researchers have blended other materials with collagen to improve the tensile strength of collagen based scaffold.

According to the study of Sang et al. (2011), tensile strength and elongation at break of scaffolds were improved when the collagen was hybridized with alginate. For instance, tensile strength of collagen based scaffolds are increased from around 200 kPa to 350 kPa, when the concentration of alginate is 20% in total formulation of the composite blend. The improvement in both tensile strength and elongation at break of composite scaffolds are due to the addition of alginate with great elasticity into the collagen blend (Sang et al., 2011).

Other than that, Wang, Sang, and Li (2011) reported that tensile strength of collagen chitosan composite scaffolds were improved with the increased of chitosan amount. For example, tensile strength of collagen chitosan composite scaffold was enhanced from 248.2 to 350.5 kPa, when the chitosan/collagen ratio was increased from 0.2 to 0.5 (Wang et al., 2011). Apart from natural polysaccharide, biopolymer such as ELP (elastin like polypeptide) can be used to improve the mechanical properties of collagen based biomaterial as well.

Amruthwar and Janorkar (2013) reported that the tensile strength of collagen based scaffold was raised from 0.34 to 0.99 MPa with the hybridization of ELP with collagen. Moreover, young modulus of scaffold was enhanced as well when the collagen was combined with ELP (Amruthwar & Janorkar, 2013).

Based on these reports, it can be generalised that incorporation of natural polysaccharides or biopolymer into collagen blend was an effective strategy to enhance the tensile strength of collagen based scaffold. Other than tensile strength, biodegradability of collagen based scaffold can be improved with the addition of other potential materials into collagen blend.

2.12.2 Biodegradability of Collagen Composite Biomaterials

Biodegradation of collagen based biomaterials are usually determined by measuring the degradation rate of biomaterial in collagenase solution. Other enzymes are rarely used because their degradation efficacy against collagen are not obvious. Wang et al. (2013) reported that collagen based scaffold is easily degraded by collagenase. On the other hand, no obvious degradation was observed when the collagen scaffolds are soaked in the lysozyme and hyaluronidase solution. This can be explained by the active site of both lysozyme and hyaluronidase are inactive on collagen molecules (Wang et al., 2013). Thus, enzymatic degradation of collagen based biomaterial is usually measured by collagenase solution.

Biodegradation behaviour of scaffold played an important role in the tissue engineering process (Sionkowska & Kozłowska, 2013). This is because the degradation behaviour of scaffold will affected the cell vitality and tissue regeneration (Tan, Gong, Lao, Mao, & Gao, 2007). It is well known that the scaffold or biomaterial fabricated from pure collagen usually exhibited a weak biodegradation property. Thus, many researchers had attempts to mix the collagen with other materials to overcome the weak degradation property of collagen.

For instance, study of Yang, Guo, Fan, and Zhang (2013) have showed that crosslinking of collagen with alginate can lower the biodegradation rate of collagen based hydrogel. This is because the addition of alginate have made the collagen fibre structure to become denser; and strengthened the mechanical property of the hydrogel. Thus, composite hydrogel with denser and stronger mechanical strength will possessed higher resistance to biodegradation (Yang et al., 2013).

Apart from that, Feng et al. (2015), reported that incorporation of pectin into collagen blend is an effective strategy to overcome the ease degradation of pure collagen based scaffold. This is because the hydroxyl groups of pectin are able to bind to the amino groups of collagen, leading to the enhancement of intra- and intermolecular crosslinking in composite scaffold (Feng et al., 2015). As a result, the stability of the composite scaffold is stronger and thus reduced the degradation rate.

Similar to pectin, chitosan with plenty of hydroxyl groups have been incorporated with collagen to produce composite scaffolds with improved biodegradation property (Shrestha, Friedman, & Kishen, 2011; Arpornmaeklong et al.,

2008; Martínez, Blancoa, Davidenkob, & Cameron, 2015). Apart from that, study have showed that mineral such as silica can be cross-linked with collagen to improve the resistance of collagen based scaffold against biodegradation as well (Perumal et al., 2015).

Biodegradation property of collagen seems to be improved by incorporating useful natural polysaccharides and minerals into the collagen blend based on these reported studies. Higher degradation rate of scaffold will limit its application in tissue engineering industry. Therefore, fabricating collagen composite biomaterial is an effective strategy to solve the ease degradation problem encountered by pure collageneneous biomaterials.

2.12.3 Porosity of Collagen Composite Biomaterials

Porosity is the percentage of void spaces of solid materials (Leon, 1998). Porosity is an important property to scaffold material as pores are needed to allow the proliferation of mesenchymal cells and osteoblast (Kuboki et al., 1998). Porous biomaterials can be prepared through various techniques such as freeze drying, gas foaming and sintering (Karageorgiou & Kaplan, 2005).

Among the techniques, freeze drying is commonly used to produce scaffolds with high porosity (Sang et al., 2011; Lin, Tan, Marra, Jan, & Liu, 2009). According to Wang et al., the porous structure of the scaffold or biomaterials are mostly depended on the fabrication technique. Apart from that, porosity of scaffold can be affected by the type of material used in the fabrication of scaffold as well (Karageorgiou & Kaplan, 2005).

For example, the porosity of collagen alginate (CA) scaffold are different to the collagen silica composite scaffold (Sang et al., 2011; Perumal et al., 2015). Other than that, Sionkowska and Kozłowska (2013) reported that the collagen hydroxyapatite composite scaffolds exhibited porosity of 84.9 to 96.5% which are different to the previous stated samples.

It is well known that porosity is an important property of scaffold to allow the cells proliferation and migration. Porosity of scaffold or biomaterial is depended primarily on the fabrication technique. In addition, compositions of the scaffold played an important role in determining the porosity of the scaffold as well. Thus, different materials used in the sample fabrication will resulted different porosity of the scaffold.

2.12.4 Swelling Behaviour of Collagen Composite Biomaterials

Swelling behaviour revealed the water holding capacity of a biomaterial (Wang & Rhim, 2015). Swelling behaviour is very important to scaffolds or biomaterial films as water retaining ability is essential for cell proliferation, migration and attachment (Hu et al., 2013). In other words, the ability of scaffold to retained biological fluid is a very important index to evaluate the suitability of scaffold as a cell delivery vehicle in various tissue engineering procedures (Engler et al., 2004).

In general, the swelling characteristic of scaffold is highly dependent on the scaffold composition. Due to the low hydrophilicity of collagen, pure collagen scaffold or film usually exhibited lower water uptake capacity (Martínez et al. 2015). Thus, natural polysaccharides with plenty of hydroxyl groups such as chitosan and alginate are usually blended with collagen to produce collagen composite biomaterial with improved water adsorption characteristic (Karri et al., 2016).

For instance, both studies of Sang et al. (2011) and Karri et al. (2016), have revealed that the swelling behaviour of collagen alginate composite scaffolds were far better than pure collagen scaffold. The improvement of water uptake capacity in collagen alginate composite scaffolds are primarily due to the presence of alginate with high hydrophilicity (Karri et al., 2016). Thus, water adsorption is higher in collagen alginate composite scaffold.

Apart from natural polysaccharides, silica can be mixed with collagen to overcome the poor water uptake ability of pure collagen based scaffold as well (Perumal et al., 2015). According to the study of Perumal et al. (2015), the swellability of collagen silica composite scaffold is increased with the silica amount. For example, swelling ratio of the composite scaffold increased from 3014.42 to 3237.29%, when the amount of silica is increase to 20% in total formulation. This is mainly because of the presence of increased concentration of silica which have higher water adsorption ability (Perumal et al., 2015).

In summary, blending collagen with high hydrophilicity material is an effective strategy to improve the water uptake capacity of collagen based scaffold. It is important to produce a biomaterial with suitable swellability as it evaluate the efficacy of scaffold in various tissue engineering procedures (Kumar et al., 2012). Lastly, an ideal scaffold should possessed suitable water uptake capacity to allow cell proliferation and removed excessive exudates.

2.12.5 Thermal Stability of Collagen Composite Biomaterials

Thermal stability of collagen based biomaterials can be obtained via differential scanning calorimetry (DSC). Thermal stability of collagen based biomaterial is an important parameter because it reveal the protein denaturation temperature. Besides, the durability of collagen based biomaterial is affected by the thermal stability as well (Perumal et al., 2015). Thus, many researchers have incorporated other functional materials into collagen blend in order to improve the thermal stability of collagen based biomaterial.

For instance, Perumal et al. (2015) reported that the denaturation temperature of collagen based scaffold is improved when silica is incorporated into the collagen blend. Besides, the denaturation temperature of collagen silica composite scaffold is increased with the increased of silica content. Denaturation temperature of composite film increase from 76.20 to 93.90°C with the increase of silica content (Perumal et al., 2015). Apart from silica, natural polysaccharides such as chitosan is reported to be an effective material to enhance the thermal stability of collagen based scaffold as well.

Ramasamy and Shanmugam (2015) reported that the denaturation temperature of collagen chitosan composite film (113.34°C) is higher than the denaturation temperature of pure collagen scaffold (70.57°C). Other than that, composite membrane fabricated from the mixture of collagen and alginate dialdehyde showed a higher thermal stability compare to the pure collagen membrane (Hu et al., 2014). The improvement of thermal stability in all types of collagen composite biomaterials can be due to the hydrogen bonding interaction between the collagen and cross-linker (Perumal et al., 2015, and Hu et al., 2014).

Hybridization of collagen with other functional materials such as natural polysaccharides and minerals could yielded a collagen composite biomaterial with enhanced thermal stability. The improvement of thermal behaviour is mainly due to the increase of intra and intermolecular bonding in collagen composite biomaterials. Therefore, it is important to produce a collagen based biomaterial with improved thermal stability, as it affect the durability of biomaterial during the application in tissue engineering and biomedical industry.

CHAPTER 3

METHODOLOGY

3.1 Preparation of Raw Materials for Collagen Extraction

The *smooth wolf herring* fish skin was collected from the restaurants at Gotong Jaya, Pahang, after the fileting process. Next, the collected skin was washed with cold distilled water and cut into smaller pieces (1 cm × 1 cm) by using a scissors. The skin was then kept in the polystyrene bag and kept at -18 °C until used.

3.2 Preparation of Raw Materials for Alginate Extraction

Brown seaweed (*sargassum polycystum*) species was obtained from Port Dickson, Negeri Sembilan. The collected seaweed sample was firstly washed with distilled water for 3 times and brushed gently to remove the impurities such as sand. Next, the seaweed sample was rinsed 3 times with salt water and allow to air-dry for 5 days. Then, the dry seaweed sample was grinded into fine pieces by using a blender. The seaweed sample were finally kept in a dessicator until used.

3.3 Chemical Reagent

All the chemicals were analytical grade. Sodium hydroxide, butyl alcohol, acetic acid, tris(hydroxymethyl)aminomethane, potassium phosphate, sodium chloride, calcium chloride, sodium carbonate, HCl, ethanol, amino acid standards (1 nmol/μL), OPA (o-phthalaldehyde) and FMOC (fluorenylmethyloxycarbonyl chloride), methanol (HPLC grade), acetonitrile (HPLC grade), collagenase (265 Umg^{-1}), and EDTA (ethylenediaminetetraacetic acid) were purchased from the local supplier.

3.4 Preparation of Collagen from Fish Skin

Acid soluble collagen (ASC) was isolated from *smooth wolf herring* skin by using the method of Nagai and Suzuki (2008) with minor modification. All the preparation procedures were carried out below 4 °C.

3.4.1 Pretreatment of Fish Skin

Fish skin was firstly soaked in 0.1 M NaOH at a sample/alkali ratio of 1:8 (w/v) to eliminate the non-collagenous protein. The mixture was allowed to stand for 6 h and the alkaline solution was replaced every 3 h. Secondly, the treated skin was cleaned with cold distilled water repeatedly until the washing water achieved neutral pH.

Next, the treated skin was sodden in 10 % butyl alcohol at a sample/solvent ratio of 1:10 (w/v) for 24 h to remove the fats. The butyl alcohol was replaced every 12 h. The defatted skin was then rinsed with distilled water repeatedly to remove the butyl alcohol that remained on the skin.

3.4.2 Extraction of Acid Soluble Collagen from Fish Skin

Firstly, the defatted skin was immersed in 0.5 M acetic acid at sample/solvent ratio of 1:2.5 (w/v) to extract the collagen. The mixture was allow to stand for 24 h. Thereafter, the mixture was filtered through a cotton cloth to remove the residue and the extract was collected.

The ASC was then salted out from the extract by adding NaCl to a final concentration of 2.5 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane at pH 7.0. The mixture was stirred until the white precipitate was observed prior to centrifugation.

The mixture was centrifuged at 14000 × g for 45 min subsequently. After that, the white pellet was collected and then dissolved in a minimum volume of 0.5 M acetic acid. Thereafter, the sample was dialysed against 50 volumes of 0.1 M acetic acid and distilled water respectively.

Finally, the ASC (acid soluble collagen) was collected after the dialysate was lyophylised for 24 h. The yield of the ASC was calculated based on dry weight basis and expressed in percentage (g/g) by using the equation below:

$$\text{Yield of ASC (\%)} = \frac{Dry\ weight\ of\ ASC\ (g)}{Dry\ weight\ of\ fish\ skin\ (g)} \times 100\% \quad (3.1)$$

3.5 Extraction of Alginate from Brown Seaweed

Alginate was isolated from brown seaweed (*sargassum polycystum*) according to the method of Chee, Wong, and Wong (2010). To extract the alginate, 20 g of air dried sample was firstly soaked in 300 mL of 1% $CaCl_2$ solution at room temperature for 18h. Secondly, the sample was wash with 300mL of distilled water three times and then stored in 300 mL of 5% HCl for 1 h.

After the acid treatment, the sample was rinsed again with 300 mL of distilled water three times to remove the HCl stained on the sample. Thereafter, the sample was sodden in 300 mL of 3% Na_2CO_3 solution for 1 h at room temperature. Next, 250 mL of distilled water was added into the mixture and then stored in an oven at 50 °C for 3 h.

After that, the residue was separated from the viscous mixture by centrifuged at 14000 × g for 10 min. The supernatant was first collected and the residues were disposed. The extracted sodium alginate was precipitated from the supernatant by adding 150 mL of 95 % absolute ethanol. The precipitate was filtered through a cotton cloth and washed with 50mL of 95 % absolute ethanol subsequently.

Finally, the alginate was obtained after the treated precipitated was dried in a vacuum oven at 50 °C for 24 h. The yield of alginate was calculated on dry weight basis and expressed in percentage (g/g) by using the equation below:

$$\text{Yield of alginate (\%)} = \frac{Weight\ of\ Alginate\ (g)}{Weight\ of\ Sargassum\ Polycystum} \times 100\% \quad (3.2)$$

3.6 Fabrication of Collagen-Alginate (CA) Films

CA films were fabricated in accordance to the method of Sang et al. (2011) with minor modification. To prepare the collagen-alginate composite film, acid soluble collagen was dissolved in 0.5M acetic acid to produce 0.7 % (w/w) monomeric solution. Simultaneously, 2 % (w/w) of alginate solution was produced by dissolving the extracted alginate powder in the distilled water. The alginate and collagen solution were stirred for 2 h respectively.

After that, the acidic collagen solution was neutralized to pH 7.2 by adding 2 M NaOH at 4 °C. Then, the alginate solution was dropwise added to the neutral collagen solution to obtain collagen-alginate blend. A homogeneous blend with high clarity was produced after the collagen-alginate blend was stirred continuously for 2 h.

Then, the air bubbles in resultant blend was removed by degassing for 10 min. Thereafter, the blend was pour onto a petri dish and incubated at 25 °C for 20 h to initiate the gel formation through collagen fibrillogenesis, to form fibrillar hydrogel.

After that, the hydrogel was washed several times with deionized water. Thereafter, the hydrogel was kept at -18 °C for at least 12 h. Finally, the frozen hydrogel was freeze dried for 24 h to produce a collagen-alginate composite film.

Collagen-alginate composite films with collagen/alginate ratio of 100/0, 80/20, 70/30, 50/50, 30/70, 20/80 and 0/100 were prepared by weight; and then expressed as CA 100/0, CA 80/20, CA 70/30, CA 50/50, CA 30/70, CA 20/80 and CA 0/100 respectively in present study. Pure collagen and pure alginate film was fabricated respectively as controls.

3.7 Physicochemical Test of ASC and CA Films

3.7.1 FTIR Scanning of Collagen, Alginate and CA Films

Collagen, alginate and all CA films were subjected to Fourier transform infrared spectroscopy at 25 °C according to the method of Rochdi, Foucat, and Renou (2000) with slight modification. FTIR spectrometer (Thermo Scientific, United States) was used. The spectra of samples in the range of 400 - 4000 cm^{-1} with signal gain were obtained in 32 scans with 4 cm^{-1} resolution.

3.7.2 Amino Acid Composition of Collagen

Composition of amino acid of collagen in this study was obtained according to Nagai et al. (2008) method with minor modification by reversed phase HPLC (Shimadzu, Japan). Collagen sample (3 mg) was firstly hydrolysed in 6 M HCl (2 mL) at 150 °C for 5 h under reduced pressure by using microwave digester (Berghof, Germany). Then, the hydrolysates were derivatized with OPA (o-phthalaldehyde) and FMOC (fluorenylmethyloxycarbonyl chloride) to determine the primary and secondary amino acids of collagen respectively (Motoya et al., 2010).

Gradient mode with mobile phase I (acetonitrile: methanol: deionized water, 45:40:15) and mobile phase II (20 mM/L of potassium phosphate buffer, pH 6.9) were used in the test. The programme was set to firstly introduced mobile phase I (100%) for 5 min followed by mobile phase II (40 %) from 6^{th} min to 30^{th} min at of 0.5 mL/min. C18 column (Thermo Fisher Scientific, United States) with particles size of 5 µm, and dimension of 100 mm (l) × 2.1 mm (d) was use as a stationary phase.

Approximately 20 µL of sample was injected into HPLC. The elution profile of FMOC derivatized sample and OPA derivatized sample was visualised at 280 nm (OPA derivatized sample) and 330 nm (FMOC derivatized sample) respectively with a built in UV detector. Quantification and identification of amino acids in collagen were done by comparing the retention times and areas with an external pool of amino acid standard (Motoya et al., 2010 and Nagarajan et al., 2013).

The concentration (nmol/mL) of each amino acid in collagen was calculated by using the equation below:

$$\text{Concentration of amino acid in Collagen (nmol/mL)} = \frac{\text{peak area of amino acid in collagen}}{\text{peak area of amino acid in standard}} \times \text{Concentation of amino acid in standard} \quad (3.3)$$

After that the concentration of each amino acid in collagen was expressed in percentage according to equation below:

Concentration of amino acids in Collagen (%)

$$= \frac{\text{Concentration of amino acid in collagen}}{Total\ Concentration\ of\ amino\ acid\ in\ collagen} \times 100\% \quad (3.4)$$

Lastly, hydroxylation ratio (%) of collagen was determined with the equation below:

Hydroxylation ratio

$$= \frac{Hydroxyproline}{Hydroxyproline + Proline} \times 100\% \quad (3.5)$$

3.7.3 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) of collagen and CA films was performed according to Rochdi, Foucat, and Renou (2000) method with minor modification. DSC was conducted by using a differential scanning colorimeter (Mettler Toledo, Switzerland). Indium standard was run to calibrate the temperature. The collagen and CA films (8 mg) were firstly weighted into aluminium pan and sealed.

After that, collagen sample was scanned over the range of 20-50 °C at scanning rate of 1 °C/min. On the other hand, CA films were scanned at the rate of 5 °C /min over the range of 25-180 °C (Montoya et al., 2010). A reference was set up using an empty aluminium pan. Lastly, denaturation temperature of collagen and CA films were determined from the first peak of thermogram.

3.7.4 Swelling Behaviour of CA Films

The swelling behavior of CA film discs was accessed by firstly weighing the CA films disc (diameter 8 mm, thickness 1mm) and expressed as W_{dry}. Next, CA film discs were sodden in a closed tube which contain 5 mL PBS (1×, pH 7.4) for define period (Sang et al., 2011).

The samples were removed and dried with a filter paper at 2, 4, 6, and 24 h soaking interval. Then, the samples were weight as W_{wet}. The swelling ratio (S) of each CA film disc at distinct soaking intervals calculated by equation below and express as percentage:

$$\text{Swelling ratio (S)} = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100\% \quad (3.6)$$

3.7.5 Porosity of CA Films

Porosity of all CA films was obtained through the method of Sang et al. (2011). Geometrical volume (V_s) of CA films discs was calculated by firstly measuring the diameter and height. Then, pore volume (V_p) of each CA films discs were determined by using ethanol displacement method.

Weighted CA films discs (W_o) were sodden in absolute ethanol and placed in a desiccator under a reduced pressure. Then, the samples were removed and blotted gently with a filter paper and weighed immediately (W_e). Lastly, porosity of CA films was calculated using the equation below:

$$D = \frac{V_p}{V_s} \times 100\ \% \qquad (3.7)$$

Where, V_p is defined as $(W_e - W_o)/\rho_e$ and ρ_e represents the density of ethanol (0.789 $mgmL^{-1}$).

3.7.6 Mechanical Measurement of CA Films

Universal testing machine (Instron, United States) were used to measure the tensile property of sample at room temperature. Samples were prepared in the size of 10 mm × 50 mm × T, where T referred to the thickness of CA films. Load cell of 2000 N was used. Three specimens of each group of CA films in dry state were tested (Sang et al., 2011).

3.7.7 Microstructure Observation of CA Films

The cross-sectional morphologies of all CA films were observed by using scanning electron microscope (SEM, Hitachi S-3400N, Japan). All CA films were coated with a layer of gold and the working voltage was 10 kV. Lastly, every samples

were scan through the signal of SE (secondary electron) and BSE (back-scattered electron) with magnification of 200× and 800× respectively.

3.7.8 Enzymatic Degradation of CA Films

The resistance of CA films disc to collagenase degradation was tested through the method of Wang et al. (2013) with minor modifications. The weight of CA films discs were obtained by weighing the initial weight ($W_{initial}$). Next, the CA film discs were soaked in 5 mL of 0.1 M tris-HCl with 0.05 M $CaCl_2$. (pH 7.4) containing 20 U/mL Collagenase at 37°C.

The digestion was ended by adding 1 mL of 0.25 M EDTA into the samples at time intervals of 1, 2, 3, 4, 5 and 24 h. After that, the samples were washed 3 times with distilled water and the sample were weight after lyophilized (W_{final}). The degradation percentage of CA films were calculated by equation below:

$$\text{Percentage of degradation} = \frac{W_{initial} - W_{final}}{W_{initial}} \times 100\% \quad (3.8)$$

3.8 Experiment Design and Data Analysis

All tests were conducted in triplicate, results (yield of collagen and alginate, amino acid composition, swelling behavior, collagenase degradation, porosity, tensile strength and young modulus) are presented as mean and standard deviation.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Yield of Collagen

ASC (acid soluble collagen) was isolated with acid solubilisation method. The average yield of ASC from *smooth wolf herring* skin was 0.25 ± 0.03% in this study. The average yield was lower than the yield of ASC isolated from other tropical fishes such as *bigeye snapper* (6.4%), *striped catfish* (5.1%) and *brownbanded bamboo shark skin* (9.38%). (Jongjareonrak et al., 2005; Singh et al., 2011 and Kittiphattanabawon et al., 2010).

Lower yield of ASC in this study can be due to the *smooth wolf herring* skin was not completely solubilised in 0.5M of acetic acid during the extraction process (Singh et al., 2011). Generally, ASC was isolated using acidic condition, in which the positively charge of collagen polypeptide became dominant. As a result, repulsion force between the positively charge tropocollagen will enhanced which lead to the increased of solubilisation (Benjakul et al., 2012).

Solubility of collagen was low in this study because some of the positively charge amine group of protein was bind with CH_2COO^- (anion from acetic acid), leading to the reduction of repulsion force among the tropocollagen. As a consequences, the solubility of collagen was low which in turn decreased the yield of ASC in this study (Benjakul et al., 2012).

Other than that, lower yield of ASC in this study might due to the collagen molecule in *smooth wolf herring* skin were mostly cross-linked by covalent bonds via the condensation of aldehyde groups at telopeptide region, as well as the intermolecular crosslinking which reduced the solubility of collagen (Foegeding et al., 1996). Thus, the yield of ASC from *smooth wolf herring* skin was lower compared to other species of tropical fishes. However, this fish skin is believed to be a good source for collagen. The yield can be improved by some modification in extraction method such as using pepsin in digesting the fish skin to recover collagen.

4.2 Yield of Alginate

Alginate was extracted using sodium carbonate at 50°C in this study. The yield of alginate from *sargassum polycystum* was 20.68 ± 1.24%. The yield was higher compare to the yield of alginate from *sargassum vulgare* (16.9%) and *sargassum dentifolium* (3.25%) which isolated at 60°C. The variations in yield can be explained by the used of different extraction method as the extraction temperature was different (Torres et al., 2007).

With similar extraction method, average alginate yield in this study was lower than the yield of alginate from *sargassum baccularia* (26.7%) and *sargassum binderi* (38.7%) (Chee et al., 2010). The difference in yield was due to different species of brown seaweed used in extraction. As a result, present findings supported the fact that distinct extraction method and brown seaweed species may generated different yield of alginate (Torres et al., 2007, and Chee et al., 2010). As the seaweed used in the present study is not utilized as food source, and easily obtained at the coast of Port Dickson, it can be a useful raw material for alginate extraction. In terms of yield, it is believed that the method applied for alginate extraction can be further optimized to enhance the yield.

4.3 Amino Acid Composition of Collagen from *Smooth Wolf Herring* Skin

The amino acid composition of collagen in this study was determined by HPLC analysis and expressed in percentage. Table 4.1 shows the amino acid composition of collagen in this study. The major amino acid in collagen from *smooth wolf herring skin* was glycine (42 ± 3.74%) followed by serine (30.4 ± 2.82%). The result was in agreement to the finding of Nagai et al. (2008), Nagarajan, Shakila, and Jeyasekaran (2013) and Singh et al. (2011), which reported that glycine was the most abundant amino acid in fish collagen as well as mammalian collagen. Furthermore, glycine was the major amino acid in the collagen triple helix structure, which was the repeating arrangement of Gly-X-Y.

Apart from that, tryptophan, cysteine and sarcosine were absent in the *smooth wolf herring skin* collagen. Tryptophan, cysteine and sarcosine were found to be absent in the collagen from *cod* skin, *bigeye snapper* skin and *minke whale* species as well (Nagai et al., 2008, Kittiphattanabawon et al., 2010 and Duan et al., 2009). Present result confirmed the results reported elsewhere that tryptophan was absent in fish collagen. Beside that, hydroxyproline (1.2 ± 0.04%) and proline (1.4 ± 0.01%) were present in small amount in *smooth wolf herring* skin collagen.

The content of proline and hydroxyproline (imino acids) of collagen in this study (2.6%) were lower than the amount found in *nile perch's* skin collagen (19.26%) (Muyonga et al., 2004). This can be due to the fish species and the living habitat of *smooth wolf herring* (cold water) and *nile perch* (warm water) were different (Foegeding et al., 1996, and Nagai et al., 2008).

Generally, lower content of imino acids were found in cold water fish compared with warm water fish. This is because, only a small amount of imino acids were needed to maintain the helix structure (thermal stability) of collagen in cold environment compared to hot climate (Foegeding et al., 1996, and Jonjareonrak et al., 2005). Thus, low amount of imino acids was found in *smooth wolf herring* collagen.

Other than imino acids content, the thermal stability of collagen was affected by the hydroxylation ratio of proline as well (Nagarajan et al., 2013, and Nagai et al., 2008). Hydroxyproline was derived from proline by post-translational hydroxylation mediated by prolyl hydroxylase (Li, Fukunaga, Takenouchi, & Nakamura, 2005). The higher the hydroxylation ratio, the higher the thermal stability of collagen (Kittiphattanabawon et al., 2005, and Nagai et al., 2008).

For the collagen in this study, the degree of hydroxylation of proline (46.22 ± 1.03%) was higher than those found in *minke whale* collagen (39.2%). As a result, the thermal stability of collagen in this study (T_d = 35.23°C) was better than *minke whale* collagen (31.5°C) (Nagai et al., 2008). The thermal stability was further supported with the result of DSC discussed in next section (Section 4.4.1). Present study further confirmed that hydroxylation ratio and imino acid content of collagen played an essential role in determining the thermal stability of collagen.

Table 4.1: Amino Acid Composition of ASC from *Smooth Wolf Herring* Species.

Amino Acid	**Percentage, %**
Hydroxyproline	1.20 ± 0.04
Aspartic Acid	0.01 ± 0.00
Glutamine	7.24 ± 0.37
Serine	30.4 ± 2.82
Glycine	42.0 ± 3.74
Arginine	0.01 ± 0.00
Alanine	0.11 ± 0.01
Tyrosine	0.18 ± 0.02
Cystine	0.30 ± 0.01
Valine	4.10 ± 0.18
Methionine	1.30 ± 0.02
Phenylalanine	1.73 ± 0.21
Isoleucine	3.10 ± 0.19
Leucine	3.50 ± 0.92
Lysine	3.44 ± 0.84
Proline	1.40 ± 0.01

4.4 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was a useful technique to study the thermodynamics of protein stability. Besides, it was able to reveal the thermal denaturation temperature of a protein (Ramasamy & Shanmugam, 2015). In this study, thermodynamics behaviour of raw collagen and CA films were studied by using DSC analysis.

4.4.1 Thermal Denaturation Temperature (T_d) of Collagen

DSC analysis revealed the thermal stability of collagen in this study. Thermal stability was an important measure that determined the structure stability of the biomaterial. Figure 4.1 shows the DSC thermogram of collagen from *smooth wolf herring* species. The exothermic peak of the thermogram (35.23°C) represented the thermal denaturation temperature (T_d) of collagen in this study slightly explained the effect of heat towards collagen molecules. According to Nagarajan et al. (2013), the structure of collagen became rupture and converted into low molecular weights element at T_d.

The T_d of collagen from *smooth wolf herring* skin was higher than the T_d of *minke whale* (cold water fish) collagen (31.5°C). The difference can be explained by the degree of hydroxylation of proline in *smooth wolf herring* skin collagen (46.22%) which was higher than *minke whale* collagen (39.2%) (Nagai et al., 2008). This is in agreeable with the report from Kittiphattanabawon et al. (2010), and Nagai et al. (2008) which stated the higher the degree of hydroxylation, the higher the thermal stability of collagen.

Similar observation was found in some example of warm water fish such as T_d of *nile perch* (36°C) was slightly above the T_d of collagen in present study (35.23°C). As components in terms of proline and and hydroxyproline (imono acid) of *nile perch* contain about 9 times higher than collagen from this study which were 19.26% and 2.6%, respectively (Muyonga et al., 2004). These report generally supported the fact that imino acids played an essential role in stabilising collagen structure towards changes of temperature or climate in the living environment.

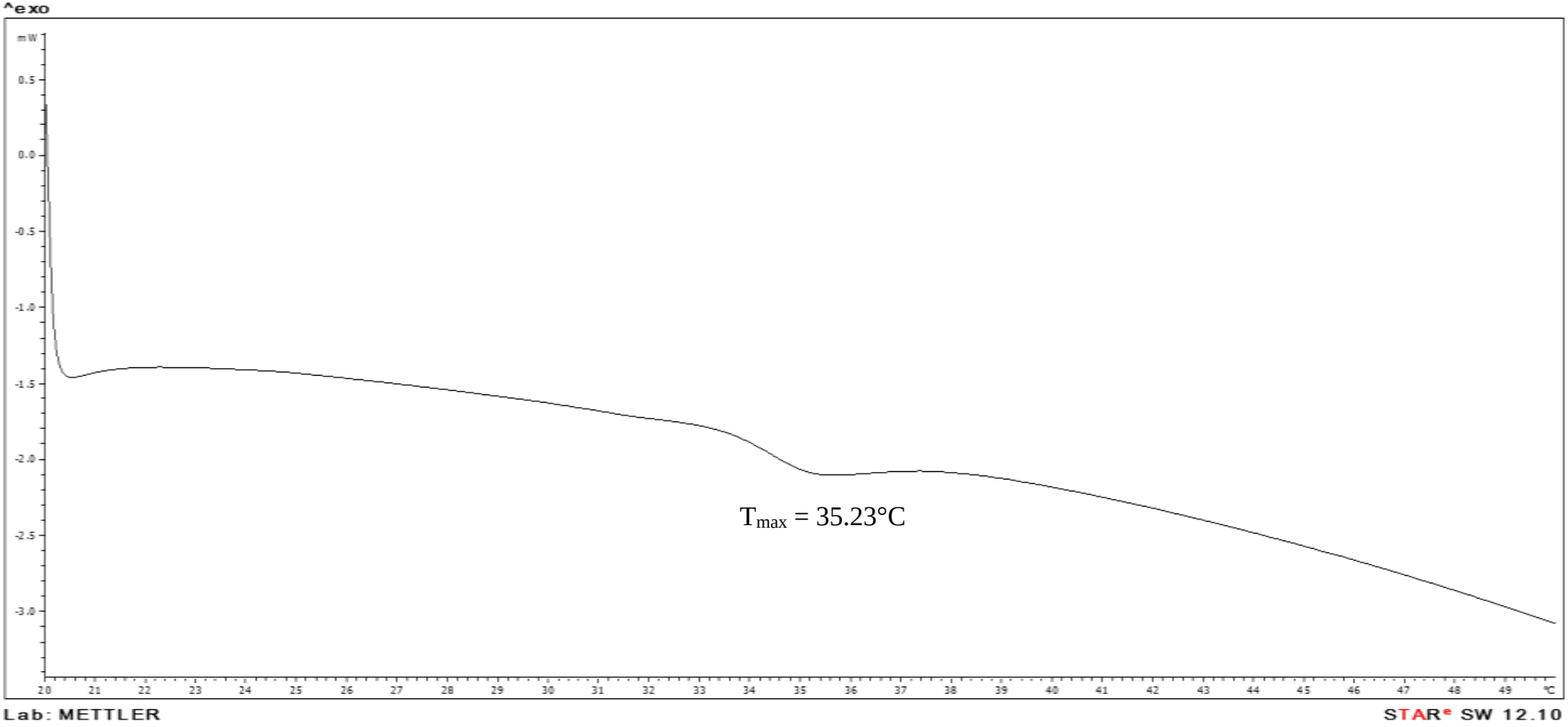


Figure 4.1: DSC Thermogram of ASC from *Smooth Wolf Herring* Species.

4.4.2 Thermal Denaturation Temperature of CA Films

Thermal stability was one of the important characteristics of biomaterials because it revealed the durability of biomaterial (Perumal et al., 2015). Figure 4.2 shows the denaturation temperature (T_d) of CA films. From the result obtained, it was noticed that the denaturation temperature of CA 80/20 (59.07 ± 0.73°C), CA 30/70 (59.86 ± 0.55°C) and CA 20/80 (59.76 ± 0.68°C) were slightly lower than CA 100/0 (61.26 ± 0.82°C).

However, the denaturation temperature of CA films were found to be increased in approximately 1.3 times when the concentration of alginate were added to 50% (CA 50/50) and 70% (CA 70/30). The improvement of denaturation temperature can be due to the increase of hydrogen bonding resulted from the crosslinking in between hydroxyl group of alginate and amino group of collagen (Hu et al., 2014).

The increment of hydrogen bonding in CA films was revealed in FTIR band at around 3280 cm^{-1} (Figure 4.4). The results obtained was in agreement to the result reported by Hu et al. (2014) which concluded that incorporation of alginate into collagen blend was a good strategy to enhance the denaturation temperature of collagen based films. Other than alginate, other types of natural polysaccharide such as chitosan was mixed with collagen to improve the denaturation temperature of collagen based scaffold by other researchers.

For instance, Ramasamy and Shanmugamaa (2015) reported that the thermal stability of the collagen based scaffolds were enhanced when collagen was hybridized with chitosan. The denaturation temperature of collagen film was increased from 70.57°C (pure collagen film) to 113.34°C (collagen chitosan composite film) when the chitosan content increased to 50%. Apart from that, Perumal et al. (2015) investigated that the denaturation temperature of collagen silica composite scaffolds were increased with the increased of silica content. The denaturation temperature of collagen silica composite film was increased from 76.20 to 93.90°C when the silica content was increased from 20 to 67%. Thus, increment of thermal denaturation temperature of collagen composite scaffolds could be due to hydrogen bonding interaction between the collagen and cross linker (Hu et al., 2014 and Perumal et al., 2015).

Thermal denaturation temperature of CA 100/0 in present study (61.26 ± 0.82°C) was increase to 78.2 ± 1.11°C and 83.11 ± 1.23°C, when the amount of alginate was increased to 30% and 50% respectively. Generally, present result in this study demonstrated that cross-linking of collagen with other functional material such as alginate in an amount of 30% and 50% in total weight was able to improve the thermal stability of collagen based films. This observation believed to be the incorporation of polysaccharide which is rich in hydrogen species was important in enhancing the intermolecular bonding. This strengthen the stability of the collagen molecule towards heat damage. The CA composite films showed improved thermal stability, thus application in human body in the future are optimist.

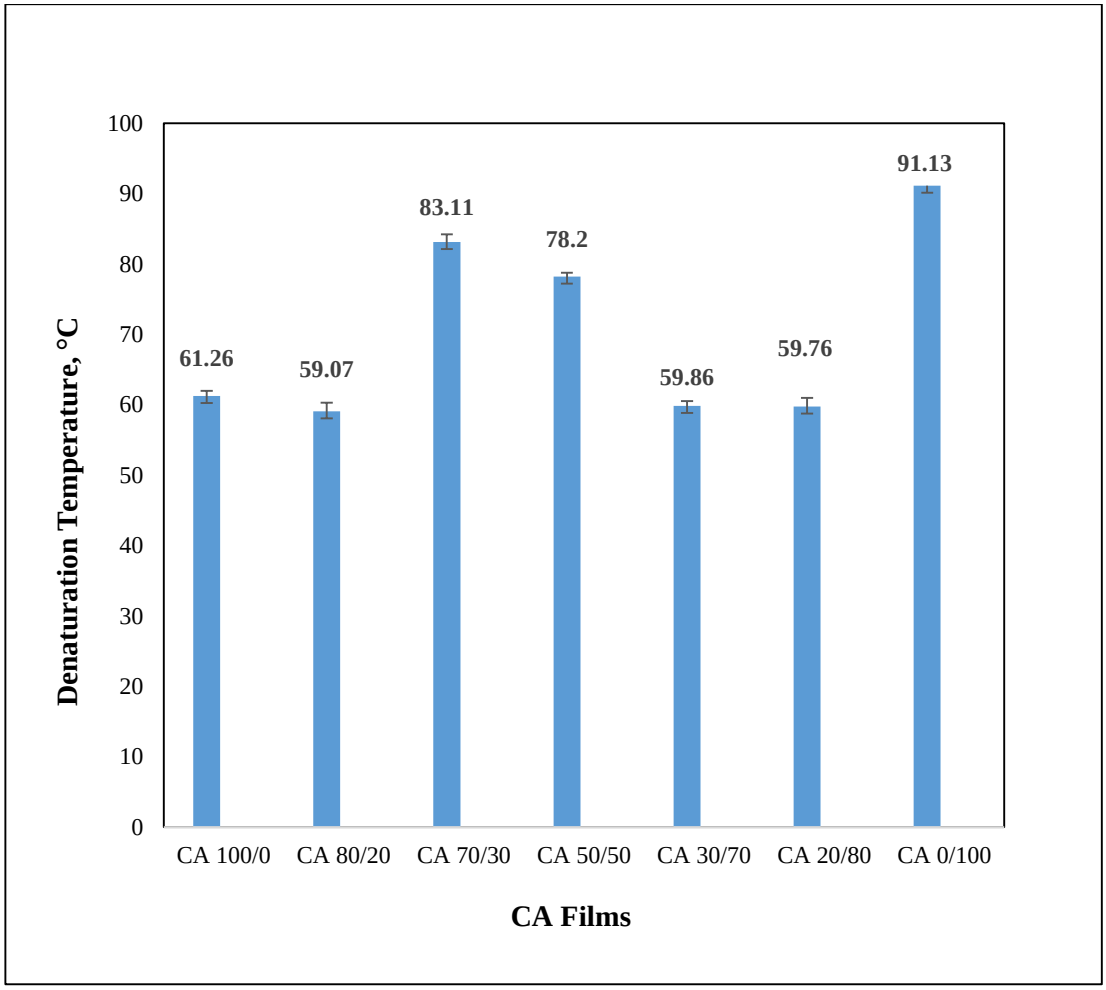


Figure 4.2: Denaturation or Degradation Temperature of CA films.

4.5 FTIR Spectra of Collagen, Alginate and CA Films

Figure 4.3a and 4.3b show the FTIR spectra of alginate from *sargassum polycystum* and CA 0/100 (pure alginate film). The characteristic bands of alginate from different species and CA 0/100 were summarized in Table 4.2. The band at 3295.36 cm^{-1} was assigned to hydrogen bonded O-H stretching vibration of alginate. Besides, bands at 2932.25 and 1596.64 cm^{-1} found in alginate in this study were attributed to the C-H stretching and asymmetric stretching vibration of carboxylate (O-C-O) respectively (Leal, Matsuhiro, Rossib, & Carusoc, 2008 and Fertah et al., 2014).

In addition, the C-O-H deformation with contribution of O-C-O symmetric stretching vibration of carboxylate group of alginate was situated at 1400.38 cm^{-1} (Fenoradosoa et al., 2010). The band at 1123.59 cm^{-1} indicated the C-O stretching, while the band at 1082.11 cm^{-1} was assigned to C-O and C-C stretching vibration of pyranose rings (Mathlouti & Koening, 1986; and Eva & Pillar, 2011).

Apart from that, the band at 1024.50 cm^{-1} represented C-O stretching vibration as well (Fertah et al., 2014). Signal obtained at 947.07 cm^{-1} indicated the C-O stretching vibration of uronic acid and 814.66 cm^{-1} were related to the vibration of mannuronic acid residues in alginate (Chandia et al., 2001; Fertah et al., 2014; and Fenoradosoa et al., 2010).

The characteristic bands of alginate in this studied was nearly similar to those obtained from *moroccan laminaria digitata* and *sargassum turbinarioides* (Table 4.2) (Fertah et al., 2014, and Fenoradosoa et al., 2010). In addition, the bands found in CA 0/100 (Figure 4.3b) was nearly similar to the raw alginate sample (Figure 4.3a) in this study as well. Thus, it demonstrated that the fabrication of CA film do not disturbed the functional groups present in alginate, and the result of FTIR spectra in this study was in remarkably good agreement with the findings reported from other researchers.

The characteristic bands of CA 0/100 (Figure 4.3b) was changed sharply when collagen solution was incorporated, especially in the region between 3000 to 3400 cm^{-1}. In the range of 3000 to 3400 cm^{-1}, only one O-H stretching vibration band (3252.72 cm^{-1}) was found in CA 0/100 (Figure 4.3b) originally. When alginate was blended with collagen solution (CA composite films), 2 additional characteristic bands were detected in the wavelength between 3000 to 3400 cm^{-1} (Figure 4.4).

Present result revealed an increase of hydrogen bonding in biomaterial films when alginate was hybridized with collagen (CA 50/50, Figure 4.4). Besides, the band near 3280 cm^{-1} shows a strong evidence of good intermolecular interaction between alginate with collagen. The band detected at around 3280 cm^{-1} was believed to be related the N-H stretching vibration of collagen bonded to O-H group of alginate (Nagai et al. 2008; and Dong, Wang, & Du, 2006).

Beside that, the spectra of ASC (Figure 4.5a), CA 100/0 (Figure 4.5b) and all composite films are nearly similar and summarized in Table 4.3. Spectra of CA 50/50 (Figure 4.4) was used as a representative spectra of composite films and the spectra of other composite films are listed in Appendix B. Amide A, B, I, II, and III bands were detected in ASC (Figure 4.5a), CA 100/0 (Figure 4.5b) and CA 50/50 (Figure 4.4). Amide A and B bands of CA 50/50 and CA 100/0 were seen at around 3400 and 3160 cm^{-1} respectively. However, the Amide A band of ASC in this studied was shifted to a lower frequency (3295.36 cm^{-1}) , because the N-H group of a peptide was involved in hydrogen bonding (Nagai et al., 2008, Singh et al., 2011; and Benjakul et al., 2012).

Amide A band was assigned to N-H stretching vibration, while amide B band was attributed to CH_2 asymmetrical stretching vibration (Sionkowska, Kozłowska, Skorupska, & Michalska, 2015). Furthermore, amide I band (around 1640 cm^{-1}) was associated to the stretching vibration of carbonyl group (C=O) along the polypeptide backbone (Barth, 2007 and Benjakul et al., 2012). Amide II and III band were observed at around 1550 and 1410 cm^{-1} respectively. According to Nagai et al. (2008), amide II was represented to N-H bending vibration; while amide III band was related to C-H stretching vibration.

Apart from that, the characteristic bands of ASC from *smooth wolf herring* skin, *striped catfish* skin, and *tilapia* skin were nearly similar (Table 4.3) (Sigh et al., 2011, and Chen et al., 2016). From the results obtained, current studied further confirmed that the ASC from different fish skins exhibited similar FTIR spectra (Benjakul et al., 2012). In addition, present findings also confirmed that there is an interaction (3280 cm^{-1}) between collagen and alginate when both materials are hybridized to form composite films.

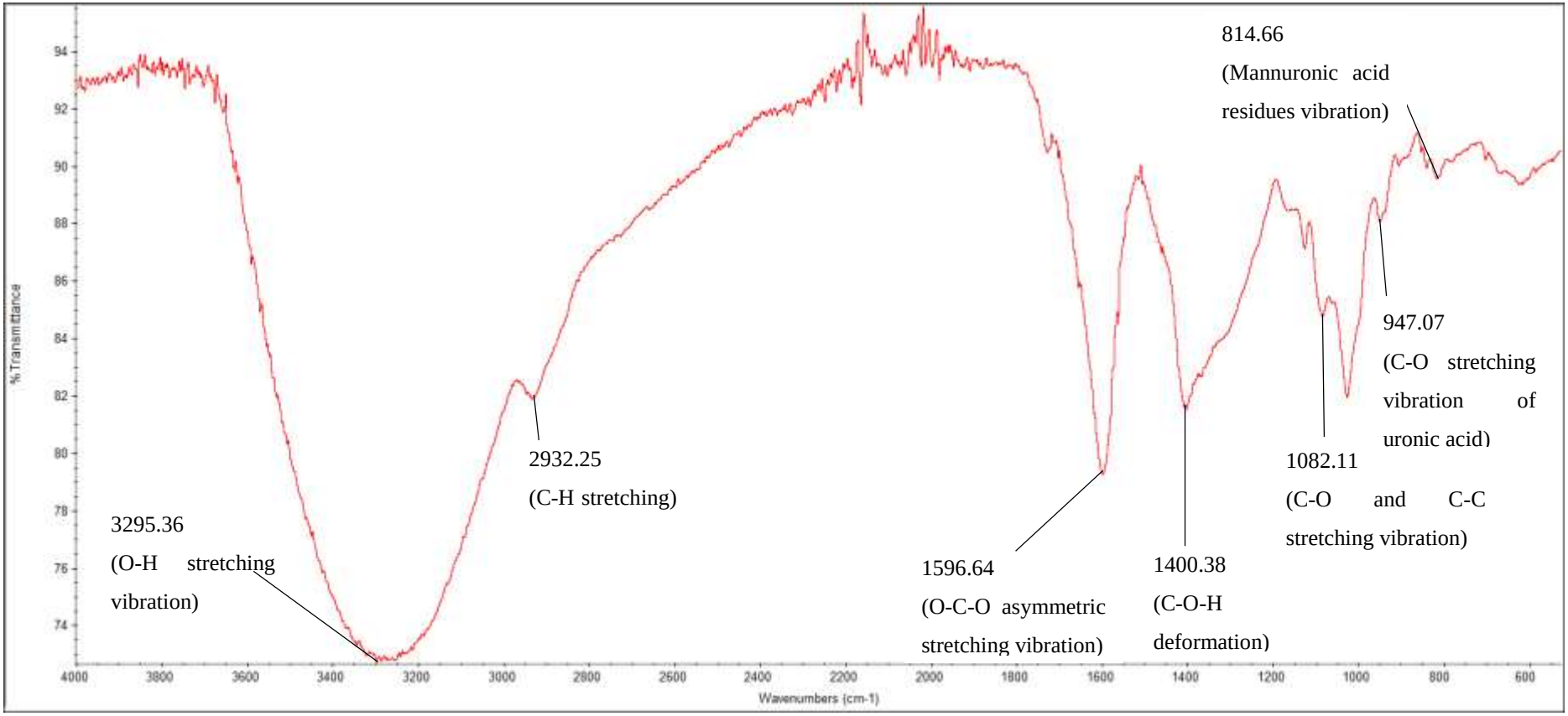


Figure 4.3a: FTIR Spectra of Alginate from *Sargassum Polycystum*.

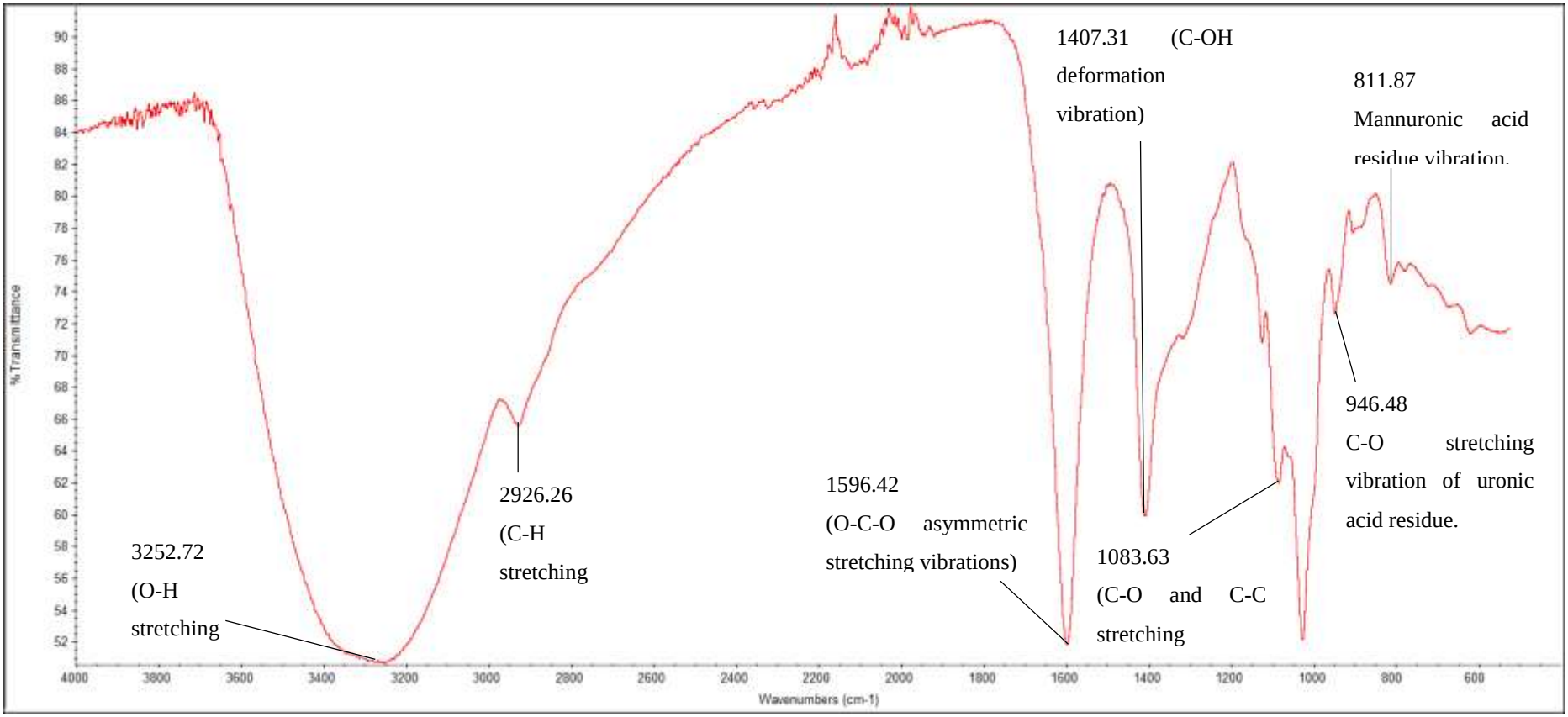


Figure 4.3b: FTIR Spectra of CA 0/100 (Pure Alginate Film).

Table 4.2: Characteristic Bands of Alginate (Fertah et al., 2014, and Fenoradosoa et al., 2010).

Bands, cm^{-1} **Source of Alginate**				
Sargassum Polycystum	***Moroccan Laminaria Digitata***	***Sargassum Turbinarioides***	**CA 0/100**	**Assignments**
3295.36	3421.05	3428	3252.72	O-H stretching vibration of alginate.
2932.25	2942.16	2929	2926.26	C-H stretching of alginate.
1596.64	1611.57	1608	1596.42	Asymmetric stretching vibration of carboxylate (O-C-O).
1400.38	1416.00	1415	1407.31	C-O-H deformation with contribution of O-C-O symmetric stretching vibration of carboxylate group.
1082.11	1094.66	1090	1083.63	C-O and C-C stretching vibration of pyranose rings.
1024.50	1035.60	1033	1025.30	C-O stretching vibration of alginate.
947.07	948.20	946	946.48	C-O stretching vibration of uronic acid.
814.66	818.76	Between 900 and 815	811.87	Vibration of mannuronic acid residues in alginate.

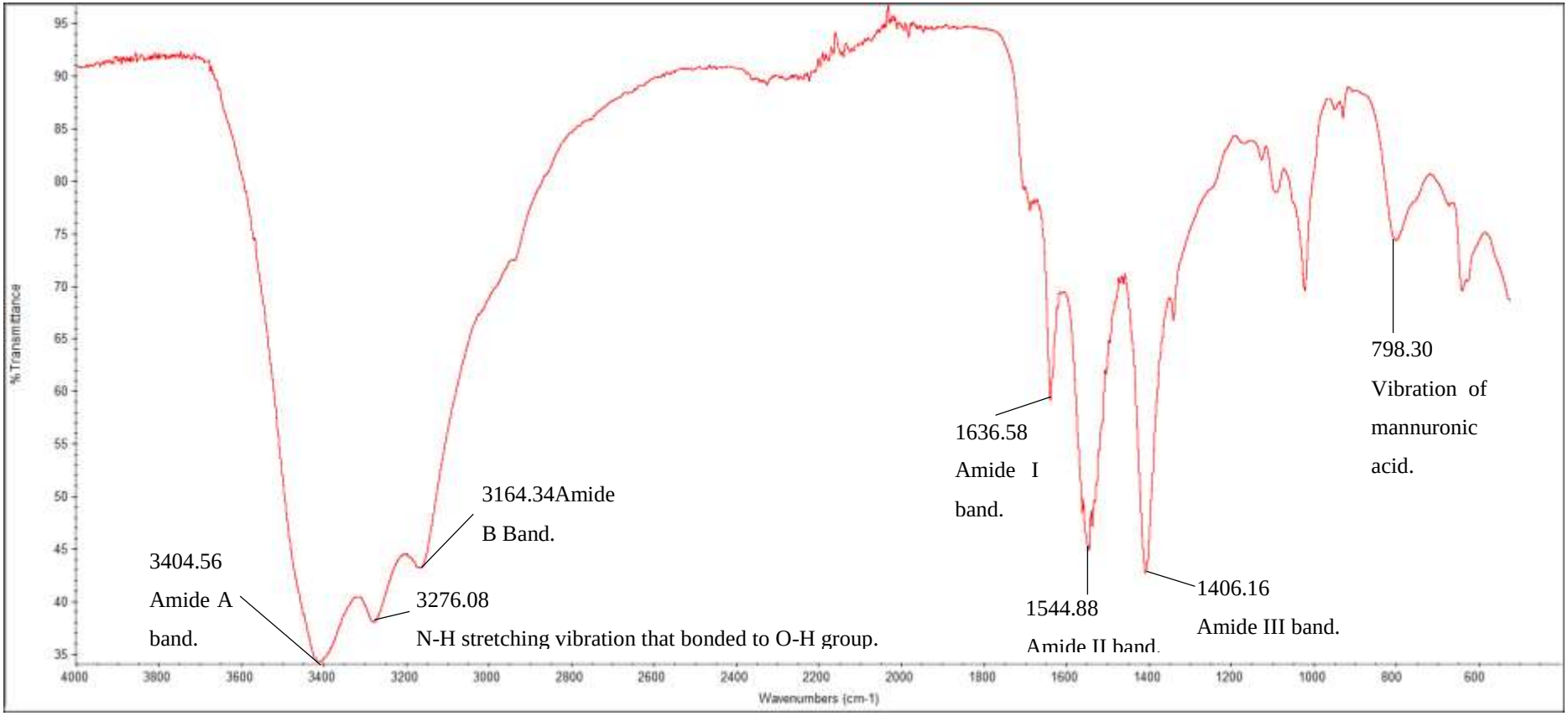


Figure 4.4: FTIR Spectra of CA 50/50.

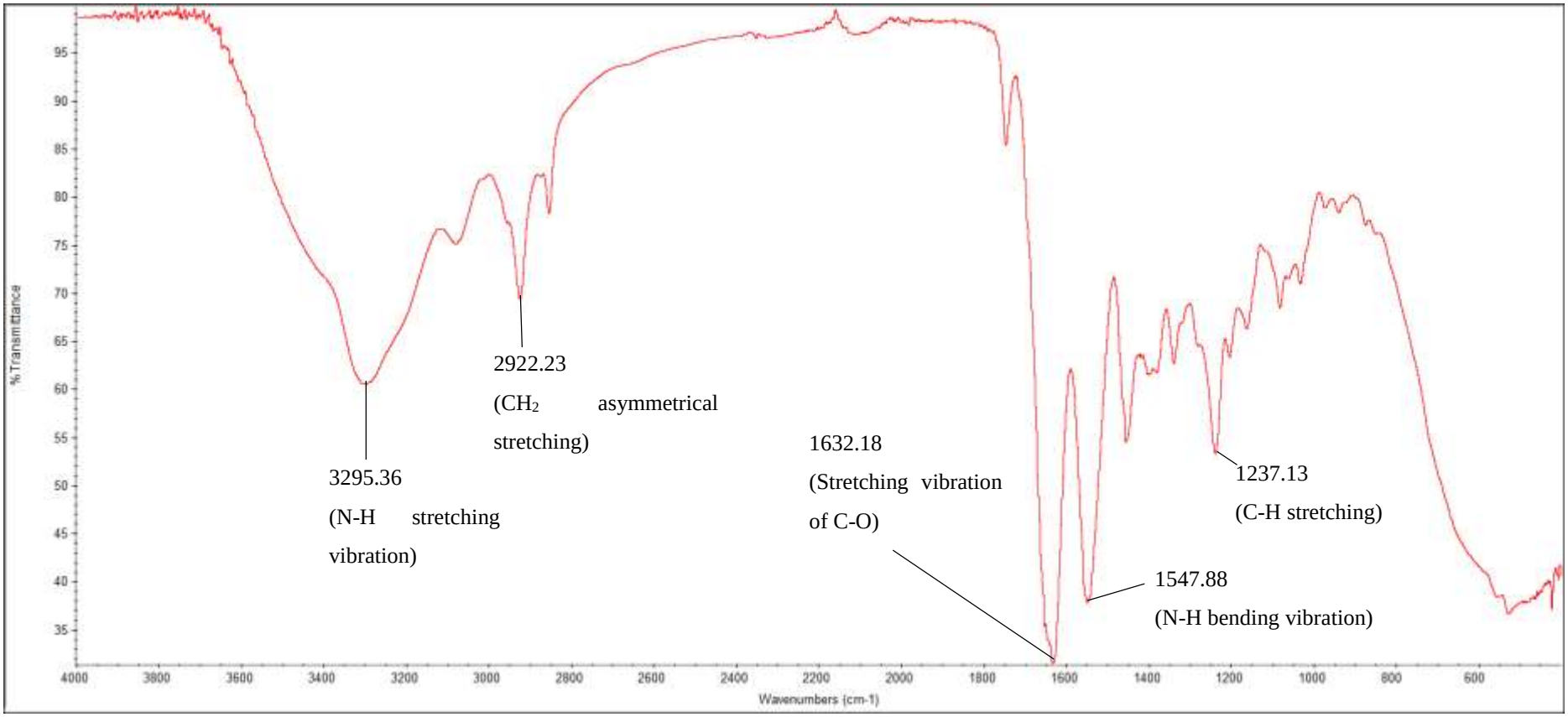


Figure 4.5a: FTIR Spectra of ASC (Acid Soluble Collagen) from *Smooth Wolf Herring Skin*.

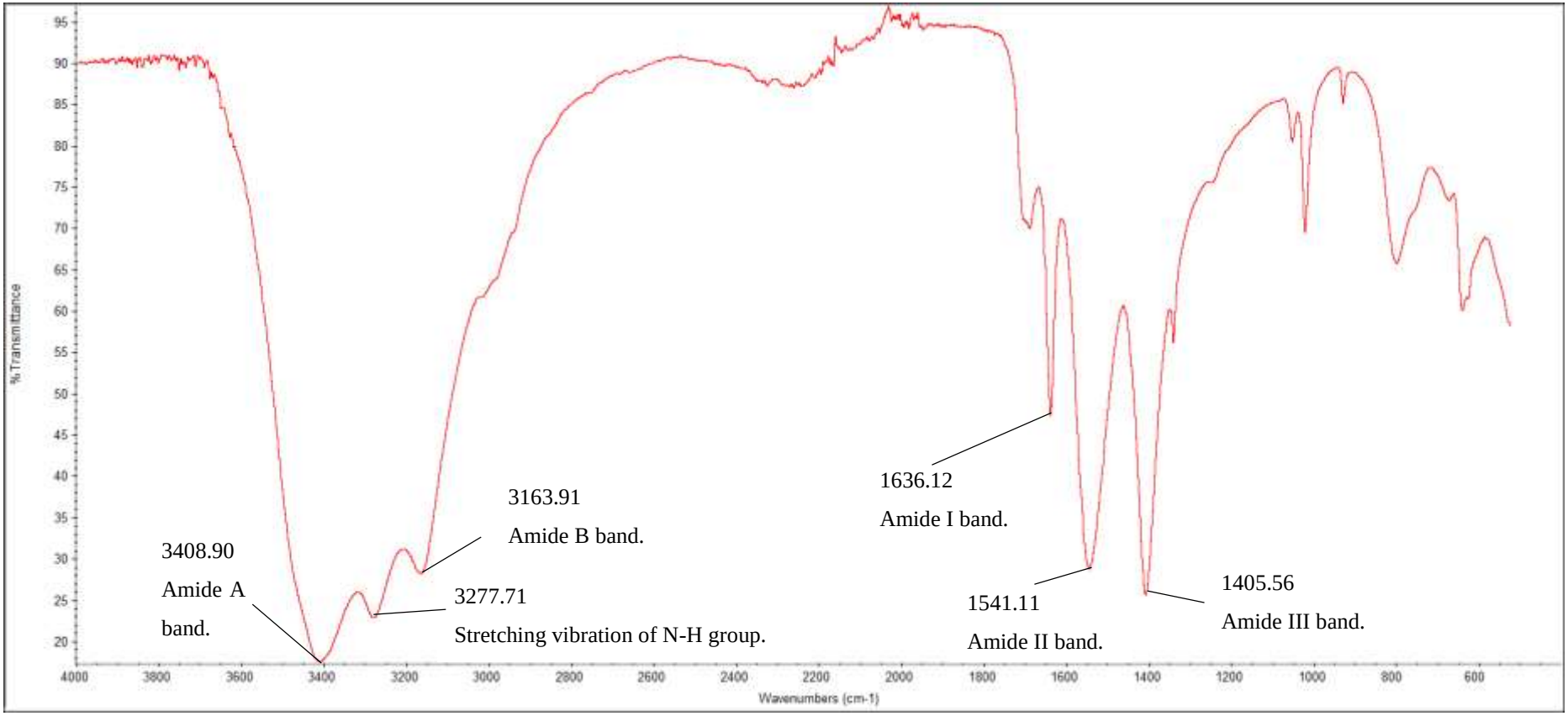


Figure 4.5b: FTIR Spectra of CA 100/0 (Pure Collagen Film).

Table 4.3: Characteristic Bands of ASC (Acid Soluble Collagen), CA 100/0 and CA 50/50 (Sigh et al., 2011, and Chen et al., 2016).

Bands, cm^{-1}					
Source of ASC (Acid Soluble Collagen)			**CA Films**		**Assignments**
***Smooth Wolf Herring* Skin**	***Striped Catfish* Skin**	***Tilapia* Skin**	**CA 100/0**	**CA 50/50**	
3295.36	3306	3321.55	3277.71	-	N-H stretching vibration which involved in hydrogen bonding (Amide A band).
-	-	-	3408.90	3404.56	N-H stretching vibration.
-	-	-	-	3276.08	N-H stretching vibration of collagen bonded to O-H group of alginate.
2922.23	2928	2924.26	3163.91	3164.34	Asymmetrical stretching of CH_2 (Amide B band).
1632.18	1640	1652.22	1636.12	1636.58	Stretching vibration of carbonyl group (C = O bond) along the polypeptide backbone (Amide I band).
1547.88	1541	1554.92	1541.11	1544.88	N-H bending vibration of ASC (Amide II band).
1237.13	1235	1244.23	1405.56	1406.16	C-H stretching of ASC (Amide III band).

4.6 Swelling Behaviour of CA Biomaterial Films

The swelling ratio represented the water uptake capacity of a substance (Wang & Rhim, 2015). It is important to note that, the ability of biomaterial films to retain water is an important index in determining its efficacy in tissue engineering and biomedical industry (Perumal et al., 2015). This is because an ideal biomaterial film should maintained a moisture environment for cell proliferation and transfer of cell nutrients (Perumal et al., 2015 and Karri et al., 2016).

According to Figure 4.6, the swelling behaviour of CA films were improved with the increased of alginate concentration. CA 20/80 showed the greatest swelling ratio among all the CA films after 2h of soaking in PBS. The swelling ratio of CA 20/80 was 1203.2 ± 27.89 %, which means that CA 20/80 able to hold 12.032 times of water than its dry weight.

On the other hand, swelling ratio of CA 100/0 (16.43 ± 5.25 %) was lowest compared to other CA films after incubated 2h in PBS. Other than that, swelling ratio of CA 80/20, CA 70/30, CA 50/50 and CA 30/70 were 115.3 ± 37.38 %, 256.1 ± 27.88 %, 436.31 ± 15.72 % and 1122.08 ± 22.11 % respectively after 2h of incubation time. However, when the amount of alginate increase to 70 % and above, swelling behaviour of samples were unable to measure due to the samples were partially dissolved in PBS solution.

The results obtained in this study was in same trend to the results of Sang et al. (2011), whereby the swelling ratio of CA films were improved with the raised of alginate amount. Incorporation of alginate in CA film will improved the water uptake capacity, because alginate was a polysaccharide with plentiful carboxyl and hydroxyl group which absorbed solution readily (August et al., 2006).

After incubation for 24h, swelling ratio of CA 100/0, CA 80/20 and CA 50/50 were increased to 83.32 ± 10.02 %, 711.60 ± 19.07 %, and 1254.75 ± 22.98 % respectively. Compared to CA 80/20 (nearly 950 %) and CA 50/50 (nearly 1450 %) from Sang et al. (2011), the samples with similar composition of collagen and alginate in this study exhibited a slight lower swelling ratio after incubated for 24 h. In addition, water uptake capacity of CA 100/0 (83.32 ± 10.02 %) in this study were much lower in contrast to CA 100/0 (nearly 900 %) from Sang et al. (2011). The difference might be due to the extraction methods applied. The latter was fabricated using PSC collagen.

In general, ASC possessed a greater stability than PSC. This is because the inter and intra-molecular crosslink of collagen was richer in ASC than PSC (Kang & Gross, 1970). It was well known that, improvement in stability of biomaterial will reduced the swelling ratio (Pieper, Oosterhof, Dijkstra, Veerkamp, & Kuppevelt 1999 and Sang et al., 2011). Thus, the swellability of CA 100/0 in this study was poorer as ASC with greater stability was used in sample fabrication.

Generally, water uptake capacity (swelling ratio) of CA films in present study were far higher compare to collagen/chitosan biomaterials (swelling ratio < 400%) in various ratio from Mahmoud and Salama (2016). However, water uptake capacity of

all CA films were lower than all chitosan/collagen sponges (average of 2500-3000 %) from Arpornmaeklong et al. (2008). Differences in swelling behaviour between the collagen composite biomaterials were mainly due to the composition and type of polysaccharides used in sample preparation (Martinez et al., 2015). As polysaccharides are made out of various monosaccharides, therefore, the hydrophilicity of these polysaccharides differ between each other.

The results obtained in this study revealed that the present strategy was able to improve the swelling ratio of collagen based biomaterial films effectively. In addition, the outcomes of this study further confirmed that presence of alginate in biomaterial films will enhanced the water holding capacity of biomaterial film (August et al., 2006 and Sang et al., 2011).

Thus, incorporation of alginate into collagen based biomaterial films was an ideal method to enhance the swelling ratio. In addition, CA composite films with good swelling properties demonstrated optimist features for further development such as wound dressing material which expected to absorb the exudate readily.

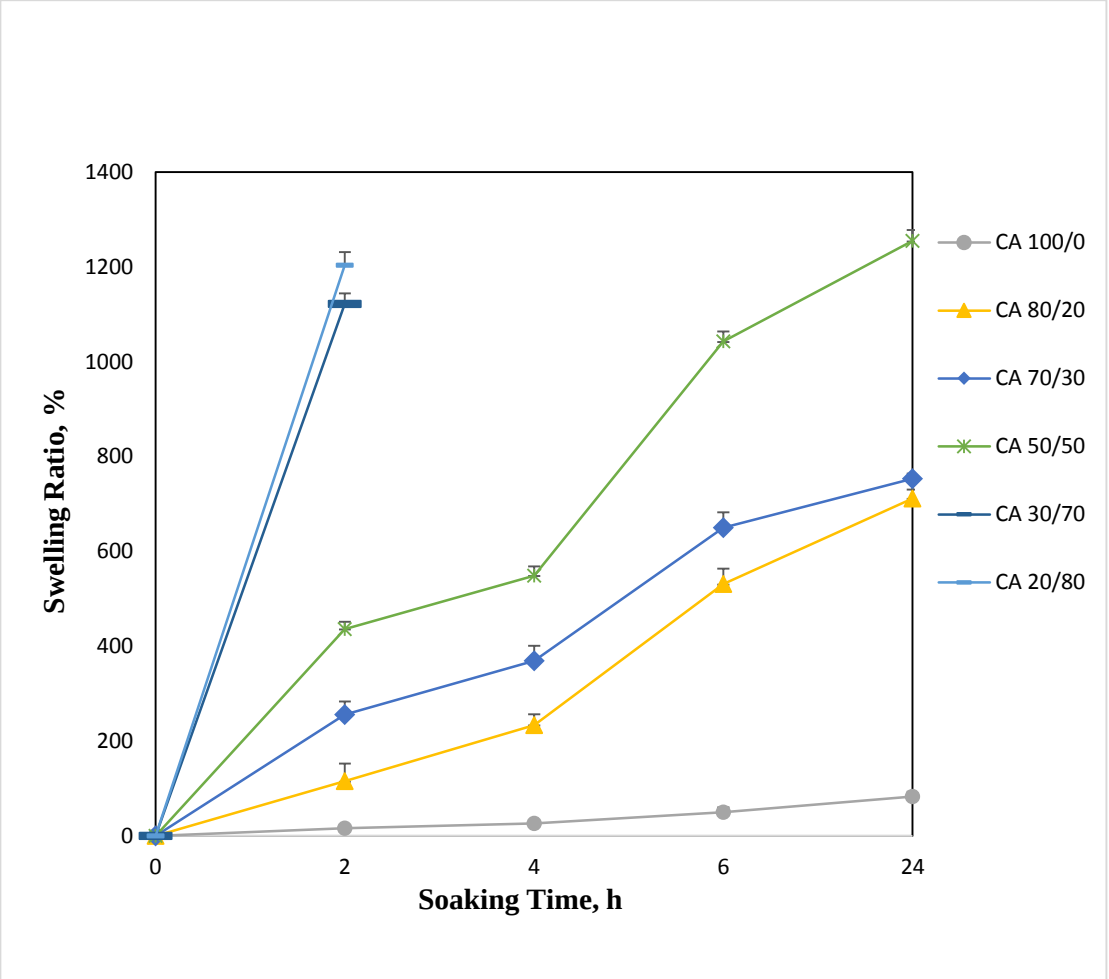


Figure 4.6: Swelling Ratio of CA 100/0, CA 80/20, CA70/30, CA 50/50, CA 30/70 and CA 20/80.

4.7 Collagenase Degradation of CA Biomaterial Films

Biodegradability was an important characteristic of biomaterial films and scaffolds. An ideal biomaterial film should degraded slowly enough to allow the released of bioactive substances (Lee et al., 2001). In this study, the biodegradability of CA films were determined by *in vitro* collagenase (20 U/mL) biodegradation test at 37°C.

Figure 4.7 shows the collagenase degradation of CA films. Among the CA films, CA 100/0 (pure collagen film) exhibited highest degradation rate. CA 100/0 (92.79 ±1.2 %) degraded rapidly after incubated in collagenase solution for 1h and fully degraded at the second hour of incubation in collagenase solution.

As shown in Figure 4.7, the enzymatic degradation of CA composite films were reduced with the raised of alginate amount in CA films. Among the composite films, CA 20/80 showed the lowest degradation at every time intervals of incubation. The degradation rate of CA films were found reduced with amount of alginate present in composite films were 30 % (CA 70/30) and above. Furthermore, it was noticed that all CA composite films were not fully degraded 24h of incubation.

Present results had revealed that incorporation of alginate into collagen blend was an effective strategy to reduce the collagenase degradation of CA film. This is because the hydroxyl group of alginate was able to bind with the amino group of collagen fibril which strengthened the resultant CA films (Feng et al., 2015). This was proved in the FTIR band (around 3280 cm^{-1}) of CA 50/50 (Figure 4.4) which showed that incorporation of alginate enable networking between collagen and alginate that inhibit the enzymatic degradation activity. Although the percentage of degradation is related to the amount of collagen used in the film, nonetheless, cross-linkage between collagen-alginate is believed to have established. This can be explained by the results from the FTIR spectrum.

The interaction of collagen with alginate had yield CA composite films with richer inter and intra molecular cross-linking compare to the CA 100/0 (pure collagen) film. Thus, CA composite films with stronger structure were more resistance to collagenase degradation as compared to the pure collagen film.

The results obtained in this study was in similar trend to the results from Yang et al. (2013), and Sang et al. (2011) which reported that increasing the alginate amount in CA composite biomaterials will reduced the collagenase degradation of samples. Besides, natural mineral such as hydroxyapatite and tricalcium phosphate were effective materials to enhance the biodegradability of collagen based biomaterial (Sionkowska & Kozłowska, 2015, and Antoniac, 2014). Biodegradation rate was reduced by half when the content of hydroxyapatite and tricalcium phosphate was increased to 50 % (wt.) in total formulation.

Incorporation of natural polysaccharides or minerals into collagen was an effective strategy to overcome the poor biodegradation property of collagen based biomaterial. In addition, present study had further confirmed that the alginate was a suitable material to cross-linked with collagen to produce collagen composite films with improved resistance against collagenase degradation. Furthermore, the biodegradation property of CA composite films can be altered by changing the amount of alginate in the composite blend. CA composite films in present study demonstrated potential to be applied in tissue engineering industry, as the biodegradability of samples were optimized.

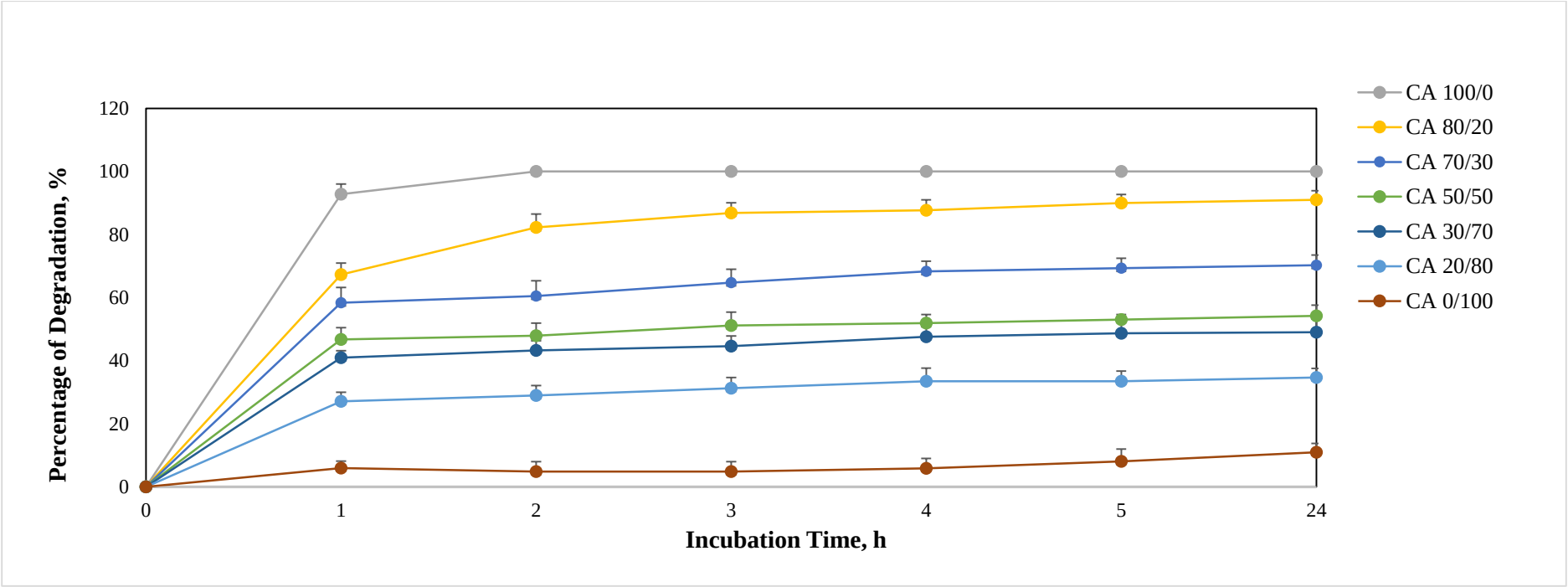


Figure 4.7: Collagenase Degradation of CA Films.

4.8 Porosity of CA Biomaterial Films

Porosity is the percentage of void space in a solid material (Leon, 1998). Porosity is an important characteristic for biomaterial as ideal biomaterial should possessed with high porosity that allowed the diffusion of nutrients and proliferation of cell. The porosity of CA biomaterial films in this study was presented in Figure 4.8. From the results obtained, all CA films possessed a porosity of more than 80 % except CA 80/20 (65.41 ± 5.98 %). In detailed, CA 0/100 showed the highest porosity (94.30 ± 4.21 %), followed by CA 100/0 (89.19 ± 7.21 %), CA 20/80 (88.99 ±2.98 %), CA 30/70 (86.85 ± 3.21 %), CA 50/50 (86.21 ± 6.02 %) and CA 70/30 (83.11 ± 6.31 %).

In general, the porosity of collagen based biomaterial films were reduced slightly when alginate was incorporated. However, the porosity of CA film were dramatically reduced to 65.41 ± 6.37 %, when the amount of alginate present was 20 % in total weight ratio (CA 80/20). Lower porosity in CA 80/20 was further supported by the SEM image (Figure 4.10m) which revealed the compact surface of CA 80/20. The low porosity in CA 80/20 can be explained by the dense deposition of both collagen and alginate.

Porosity of films (Figure 4.9) are governed by the type of materials applied in film fabrication (Karageorgiou & kaplan, 2005). Present study utilized marine based collagen showed different porosity against calf based collagen although alginate are incorporated in both films (Sang et al 2011). Besides, freeze drying conditions used during film fabrication as well played a major role in the film structure (Karageorgiou & kaplan, 2005, Sang et al, 2011).

In addition, distinct fabrication condition of CA biomaterials can lead to different porosity as well (Karageorgiou & Kaplan, 2005). According to Sang et al. (2011), it was found that the freeze drying condition was differ from the present study. It is believed to be one of the reasons that lead to the different structure formation of the biomaterials (CA films and CA scaffold).

It can be observed that porosity of the biomaterial was governed by the type of hybridization and cross-linkage material used as well. The biomaterial film fabricated from collagen, hydroxyapatite and β-tricalcium phosphate from Antoniac (2014) resulted lower porosity as compared to current study. For instance, CA 50/50 with a porosity of 86.21 ± 6.02 % was higher than porosity of collagen-hydroxyapatite- β-tricalcium phosphate film (57.5 %), even though collagen was present in 50 % in total.

On the other hand, the collagen-silica composite (96.56 ± 1.23) reported by Perumal et al. (2015) demonstrated higher porosity than CA 50/50 (86.21 ± 6.02 %) although both film contained the same amount of collagen (50 %). Thus, present result further confirmed that the porosity of biomaterial film can be affected by the type of cross-linker used. CA films with high porosity in present study possessed the potential to be used in tissue engineering and biomedical industry as high porosity biomaterial allowed the rapid proliferation of cells and diffusion of nutrients.

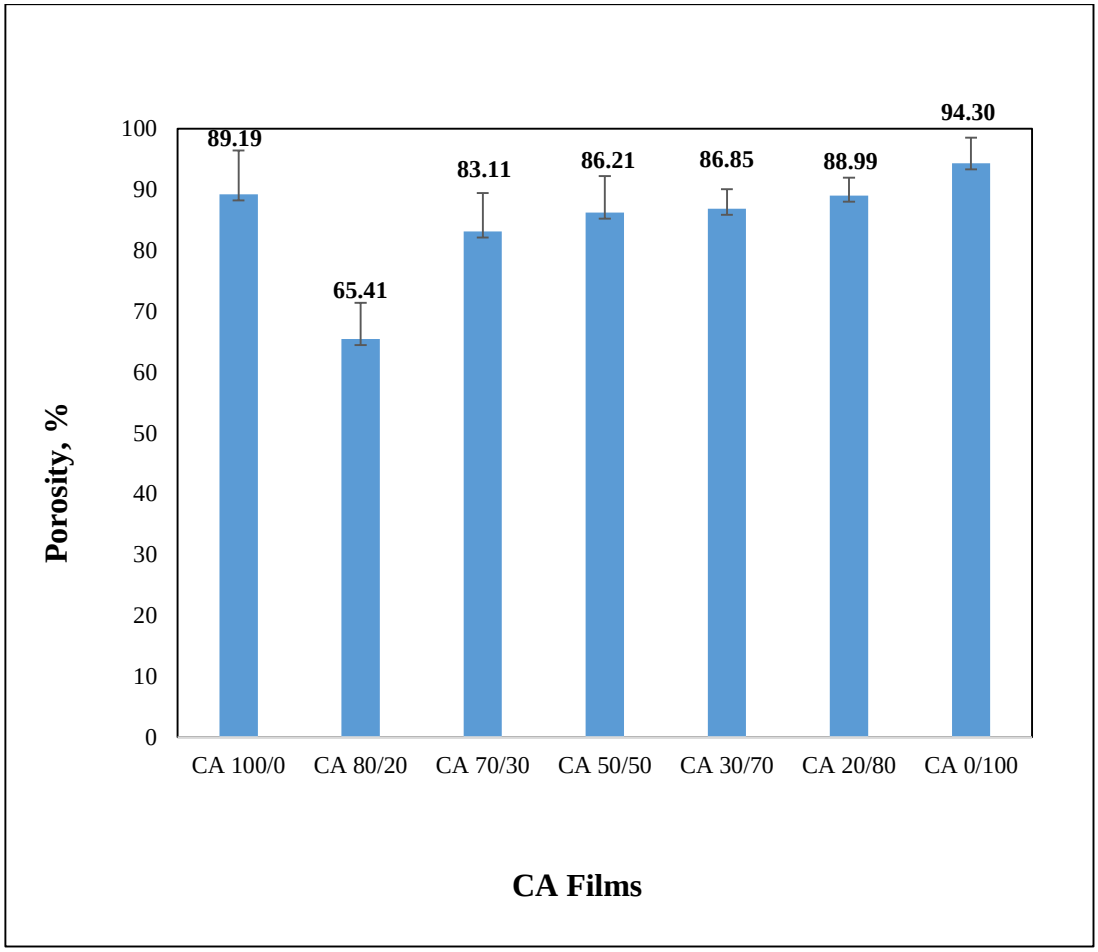


Figure 4.8: Porosity of CA Films.

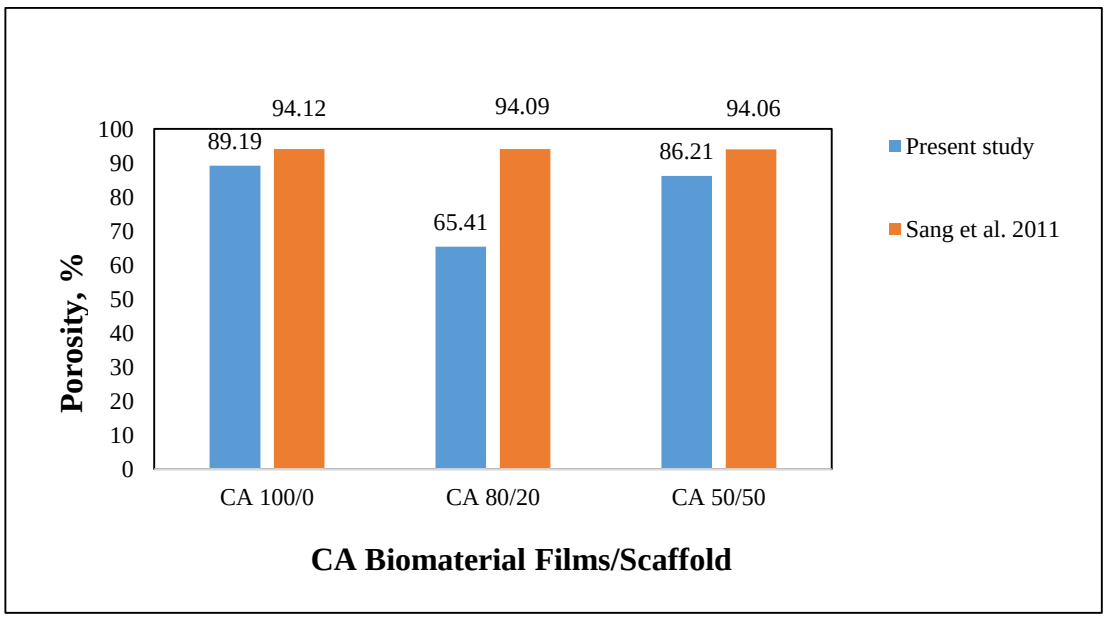


Figure 4.9: Porosity of CA Biomaterial Films/Scaffold (Sang et al., 2011).

4.9 Surface Morphology of Biomaterial Films

The surface morphology of all biomaterial films were examined by SEM. As shown in Figure 4.10a, the fibrous structure of pure collagen film (CA 100/0) was made up of small granules with various sizes. It can be observed as well the surface structure of CA 100/0 was highly fibrous and compact. The bulk porous layer of CA 100/0 was highly opaque and rough as compared to all the other biomaterial films (Figure 4.10b to g).

In contrary, the surface appearance of pure alginate film (Figure 4.10b) was smooth and possessed with high clarity. In comparison to the other samples, the clarity of pure alginate film (CA 0/100) was higher. In addition, plenty of tiny open pores with various sizes were observed on the porous surface of CA 0/100 (Figure 4.10h). The distribution of open pores on CA 0/100 was higher as compared to other CA films. When collagen was incorporated with alginate, the structure and surface morphology of biomaterial films were altered (Figure 4.10c to g).

From Figure 4.10i to e, it can be observed that the clarity of composite films decreased with the reduction of alginate concentration. Among the composite films, the film with highest clarity was CA 20/80 (Figure 4.10i). In contrary, the clarity of CA 80/20 (Figure 4.10m) was lowest among composite films.

In terms of surface appearance, CA films with higher collagen content tend to produce rough surface as compared to those with higher alginate amount (Figure 4.10c to 4.10g). This might be due to the rod like structure of collagen that cross-linked with alginate, which result in high granular surface structure of the CA films. These can be clearly observed in CA 20/80, 30/70, 50/50, 70/30 and 80/20 (Figure 4.10c to 4.10g).

Overall, the collagen granules in all CA films grow in size with the increased of collagen concentration. This can be observed in Figure 4.10c to 4.10g whereby the collagen granules were increasing in size. The smallest granules formation was found on CA 20/80 (4.10c), while the largest was observed in CA 80/20 (4.10g).

The SEM observations clearly showed that the porosity was affected by CA ratio. It can be observed that the overall pore sizes were reduce when the concentration of collagen in CA film getting higher (Figure 4.10h to n). Pore sizes were generally smaller with higher collagen content (Figure 4.10k to m). This can be due to the open pores in CA 50/50, CA 70/30 and CA 80/20 were filled up with vast amount of collagen granules.

Apart from that, the distribution of pores on the CA films were found reducing with increasing content especially when the collagen amount exceed 50%. (4.10k to m). A compact film was observed when the collagen was added up to 80% (Figure 4.10m). This can be due to the pores were mostly cross-linked with huge amount of collagen granules.

Based on the SEM results, it can be generalized that, adjusting the content of alginate and collagen may be used to generate biomaterial films with desired structure. This is due to the fact that alginate was able to establish cross linkage to collagen granules, and collagen granules were able to fill up the pores generated by alginate network structure.

Compared to the pure collagen films from Sang et al. (2011), the bulk fibrous structure of pure collagen film in this study demonstrated irregular folds instead of forming regular pores (Figure 4.10o). The distinct of surface topography of both pure collagen film can be due to the different freeze-drying conditions were used in fabricating the biomaterial (Sang et al., 2011 and Wang et al., 2011).

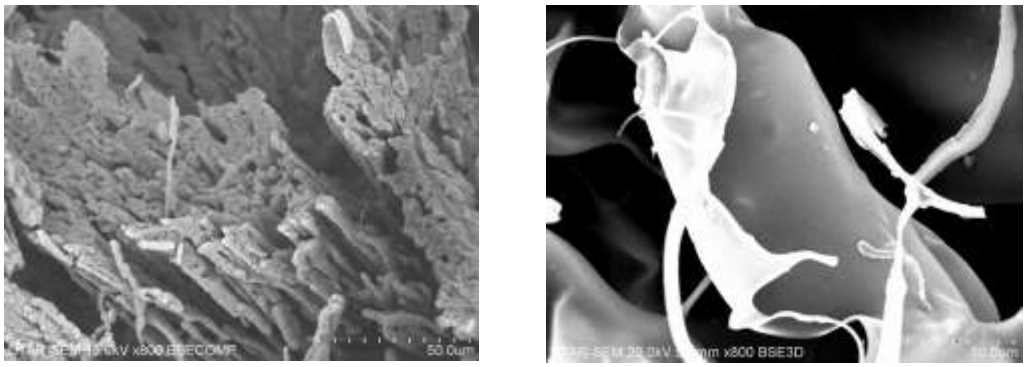
In terms of surface appearance, both of the pure collagen films exhibited rough surface. As shown in Figure 4.10o, coarse collagen granules were observed on the surface of both pure collagen films. This further supported that the pure collagen film were made up of tiny coarse collagen granules.

In contrast to the pure collagen film from Tang et al. (2015), pure collagen film in this study was rough and less compact (Figure 4.10p). This can be mainly due to the concentration of collagen stock solution used in both studies were different. Collagen stock solution with 0.7 wt % was used in this study, while 1wt/v % was used in the study of Tang et al. (2015). Biomaterial films fabricated with higher concentration of collagen will yielded a film with smoother and compact surface, as the space of open pores was fully filled with collagen granules.

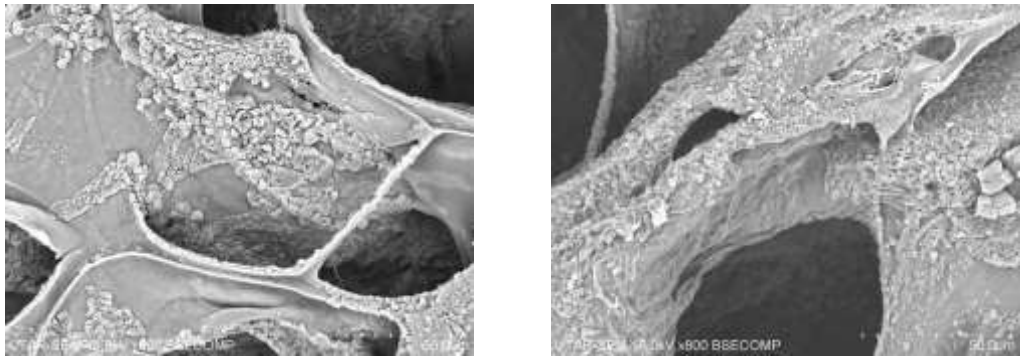
The surface morphology or topography of collagen composite film can be affected by the type of cross-linker used. For example, the collagen chitosan composite film in Figure 4.10q demonstrated a great distribution of pores compare to CA 50/50 in present study, even though both of the films were fabricated with 50 % collagen in weight. In addition, the number of pores on collagen-chitosan film were more than CA 50/50. Therefore, the type of cross-linker used in the study played an important role in determining the final structure of the composite films.

Other than that, stock concentration of both collagen and alginate solution can affected the structure of biomaterial film as well. From Figure 4.10r, pure collagen film was very compact when the concentration of collagen solution was increased to 1 and 1.4 %. In addition, porosity of both films were reduce dramatically as the pores were almost fully filled with collagen granules (Figure 4.10r).

Similar to pure collagen film fabricated with higher stock concentration, surface structure of pure alginate film fabricated from 3 wt % was very compact as well (Figure 4.10s). In addition, the porosity of pure alginate film was greatly reduced when the stock concentration was increased to 3 wt %. According to Sang et al. (2011), biomaterial films should possessed with high porosity to allow the cell proliferation and transfer of nutrients. Thus, the application both pure collagen film (1 and 1.4 wt %) and pure alginate film (3 wt %) were limited as the surface structure were too compact. Biomaterial films should fabricated with an appropriate collagen/alginate ratio and stock concentration (collagen and alginate solution) to produce structure which was useful in the industry.

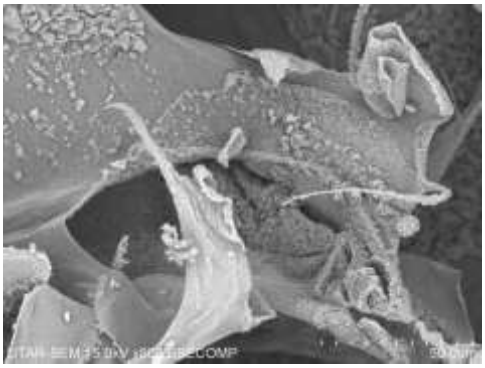


(a) **(b)**

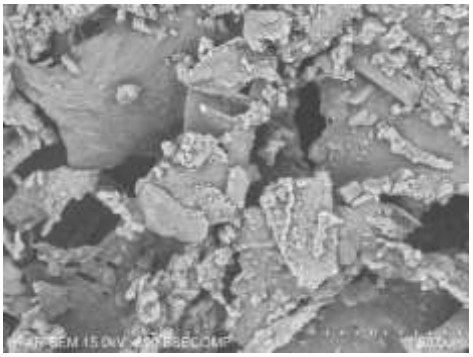


(c) **(d)**

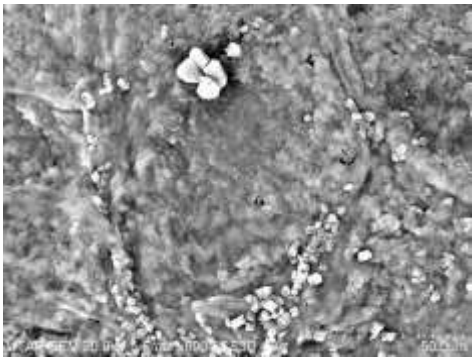
Figure 4.10: Cross-sectional SEM micrographs. (a), Pure Collagen Sponge (CA 100/0) at 50 µm; (b), Pure Alginate Film (CA 0/100) at 50 µm; (c), CA 20/80 at 50 µm; (d), CA 30/70 at 50 µm.



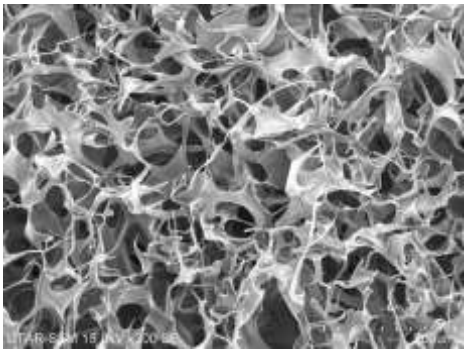
(e)



(f)

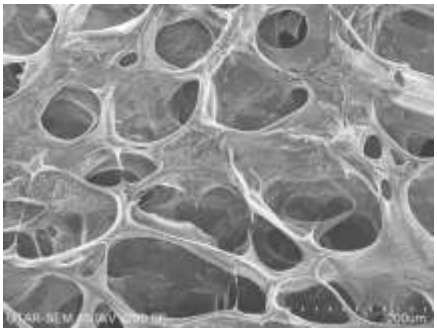


(g)

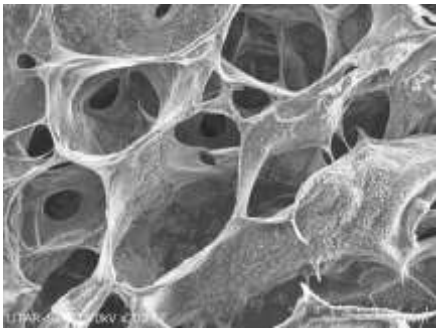


(h)

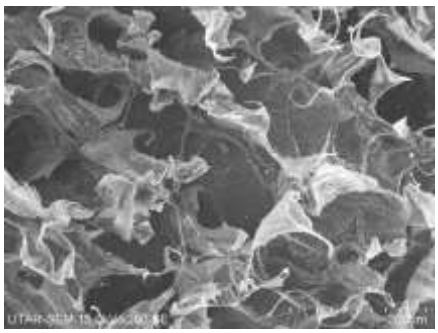
Figure 4.10: Cross-sectional SEM micrographs Cont. (e), CA 50/50 at 50 µm; (f), CA 70/30 at 50 µm; (g), CA 80/20 at 50 µm; (h), CA 0/100 at 200 µm.



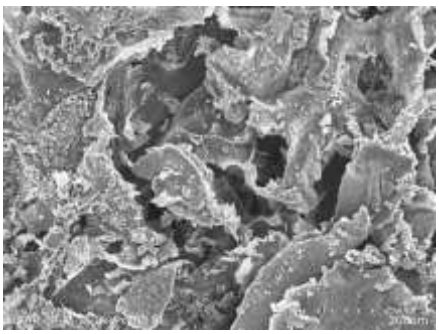
(i)



(j)

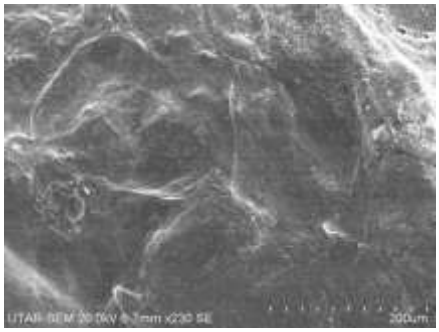


(k)

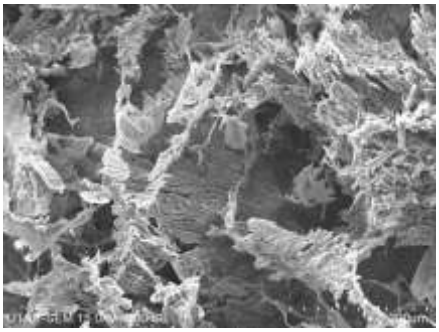


(l)

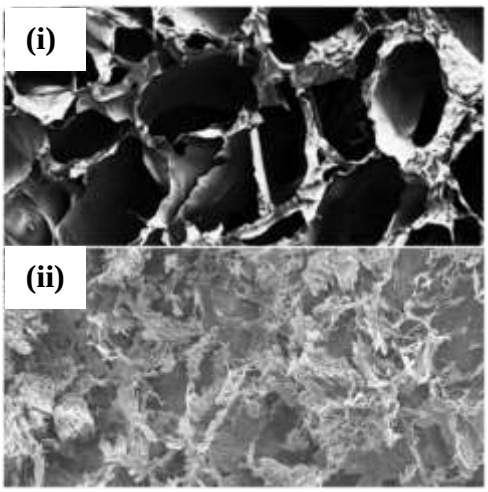
Figure 4.10: Cross-sectional SEM micrographs Cont. (i), CA 20/80 at 200 µm; (j), CA 30/70 at 200 µm; (k), CA 50/50 at 200 µm; (l), CA 70/30 at 200 µm.



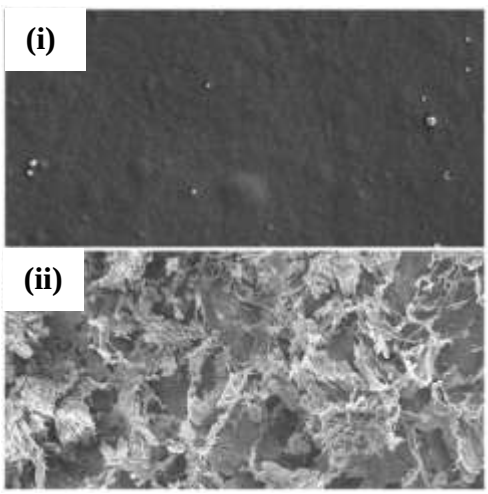
(m)



(n)

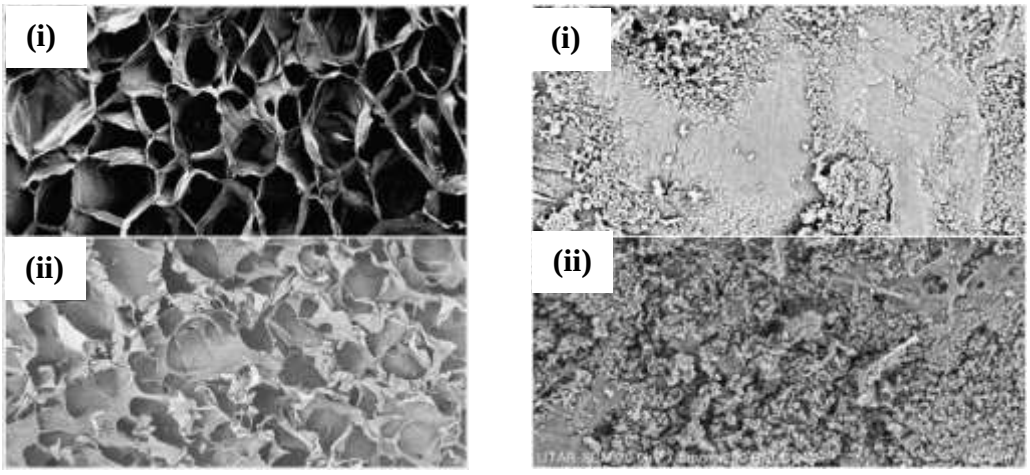


(o)

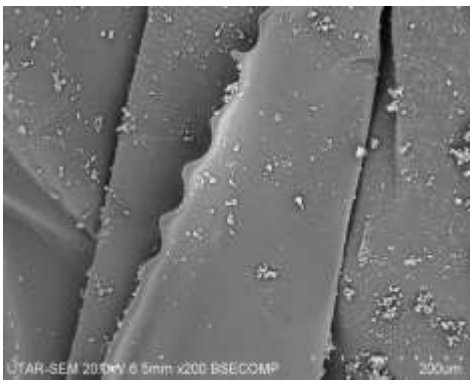


(p)

Figure 4.10: Cross-sectional SEM micrographs Cont. (m), CA 80/20 at 200 µm; (n), CA 100/0 at 200 µm; (o), (i) Pure Collagen Sponge (Sang et al., 2011) at 500, (ii), Pure Collagen Film in this study at 500 µm; (p), (i) Pure Collagen Sponge (Tang et al., 2015), (ii), Pure Collagen Film in this study at 500 µm.



(q) **(r)**



(s)

Figure 4.10: Cross-sectional SEM micrographs Cont. (q), (i) Collagen Chitosan 50/50 at 500 µm (Wang et al., 2011), (ii), CA 50/50 in this study at 500 µm ; (r), (i) Pure Collagen Film, 1.4% at 200 µm, (ii), Pure Collagen Film, 1% at 200 µm; (s), Pure Alginate Film, 3% in this study at 200 µm.

4.10 Mechanical Properties of CA Films

The mechanical properties of biomaterial films in present study were obtained with Instron testing machine. Figure 4.11a showed the tensile strength of biomaterial films in this study. In general, all CA (Collagen Alginate) composite films exhibited better tensile strength as compared to both pure collagen (CA 100/0) and alginate film (CA 0/100). From Figure 4.11a, it can be observed that the tensile strength of CA films were strengthened with the increased of alginate amount.

Among the CA composite films, CA 50/50 (1585.87 ±72.22 KPa) possessed the greatest tensile strength while the lowest was showed in CA 80/20 (413.22 ±18.89 KPa). Generally, collagen-alginate film with the ratio 50:50 (CA 50/50) showed highest tensile strength among all the film tested. Similar observation was found in collage-alginate film reported from Sang et al. 2011, however film from the present study was slightly higher in tensile strength (3 times higher).

The difference in tensile strength can be due to the type of collagen used in the fabrication of biomaterial films. According to Grover, Cameron, and Best (2012), different type of collagen used in fabrication of biomaterial can leaded to a large effect on the mechanical properties. This can be explained as the free amino group in collagen might be fully involved in the cross-linkage with alginate, thus, additional alginate will not result better tensile strength.

Apart from hybridizing the collagen and alginate, increasing the concentration of collagen stock solution can improved the tensile strength of biomaterial film as well.

According to Figure 4.11b, tensile strength of CA 100/0 was significantly elevated when the stock concentration of collagen solution was increased to 1.4 wt %. However, there was no improvement in the tensile strength of CA 0/100 even though the stock concentration of alginate solution was adjusted to 3 wt % (Figure 4.11c).

Other than elasticity (young modulus), the mechanical properties (tensile strength) of CA films were enhanced when the amount of alginate increases. Referred to Figure 4.11d, CA 50/50 (27.05 ± 0.79 MPa) showed the highest young modulus; while the lowest was found in CA 100/0 (2.25 ± 0.23 MPa). Thus, incorporation of alginate into collagen blend was a good strategy to improve the young modulus of collagen based biomaterial films, as the elasticity was enhanced. From the results obtained, it was noticed that CA 50/50 possessed the best tensile strength and elasticity (young modulus) among the CA films.

Present study confirmed that adding certain amount of polysaccharide such as alginate can improved the mechanical properties of collagen based biomaterials which is similar to some other reports (Wang et al., 2011; Arpornmaeklong et al., 2008; and Sang et al., 2011). Besides, present results also revealed that the sample preparation's methodology was able to yield biomaterials films with great mechanical properties which were comparable to the samples from other researchers. Lastly, CA composite films with improved mechanical properties were possible to be used as packaging material for drugs due to its stable structure.

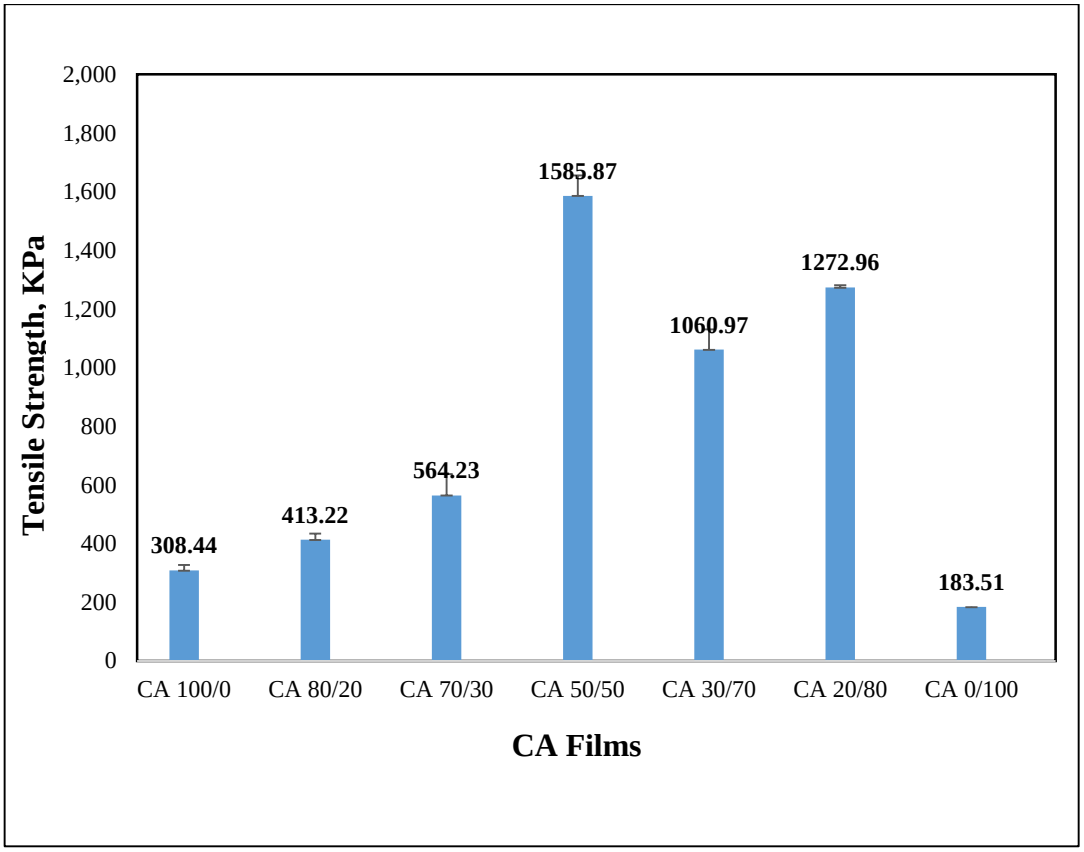


Figure 4.11a: Tensile Strength of CA Films.

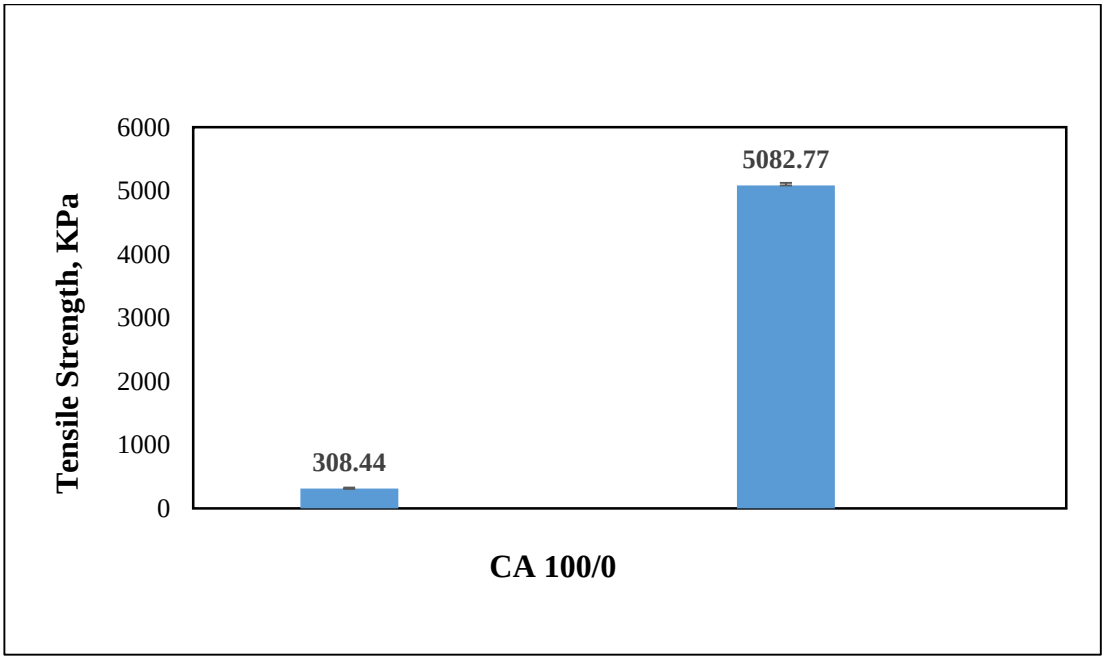


Figure 4.11b: Tensile Strength of CA 100/0 (Pure Collagen Film) with Different Collagen Stock Concentration.

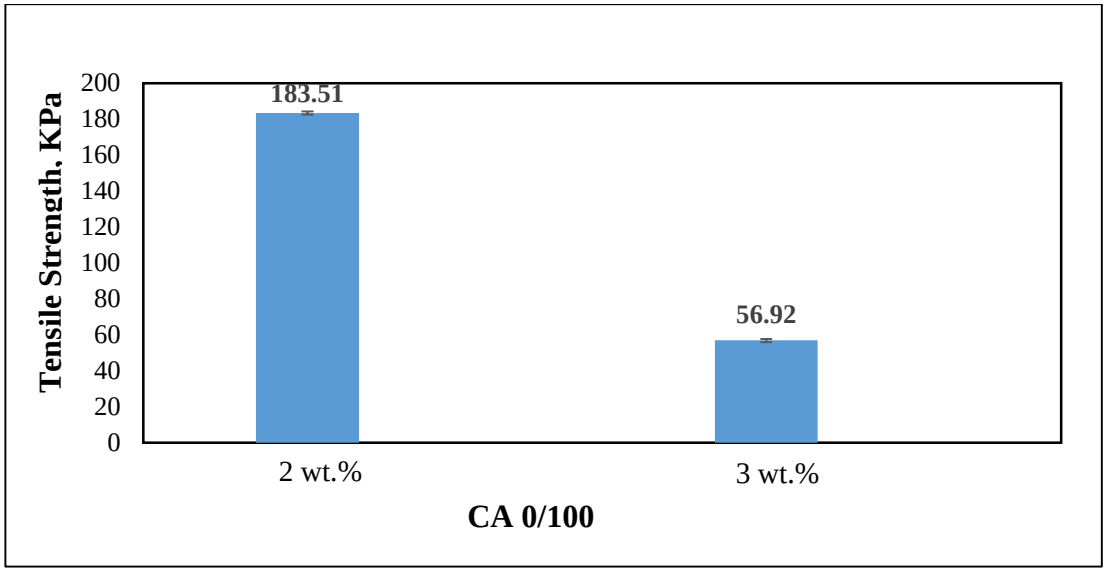


Figure 4.11c: Tensile Strength of CA 0/100 (Pure Alginate Film) with Different Alginate Stock Concentration.

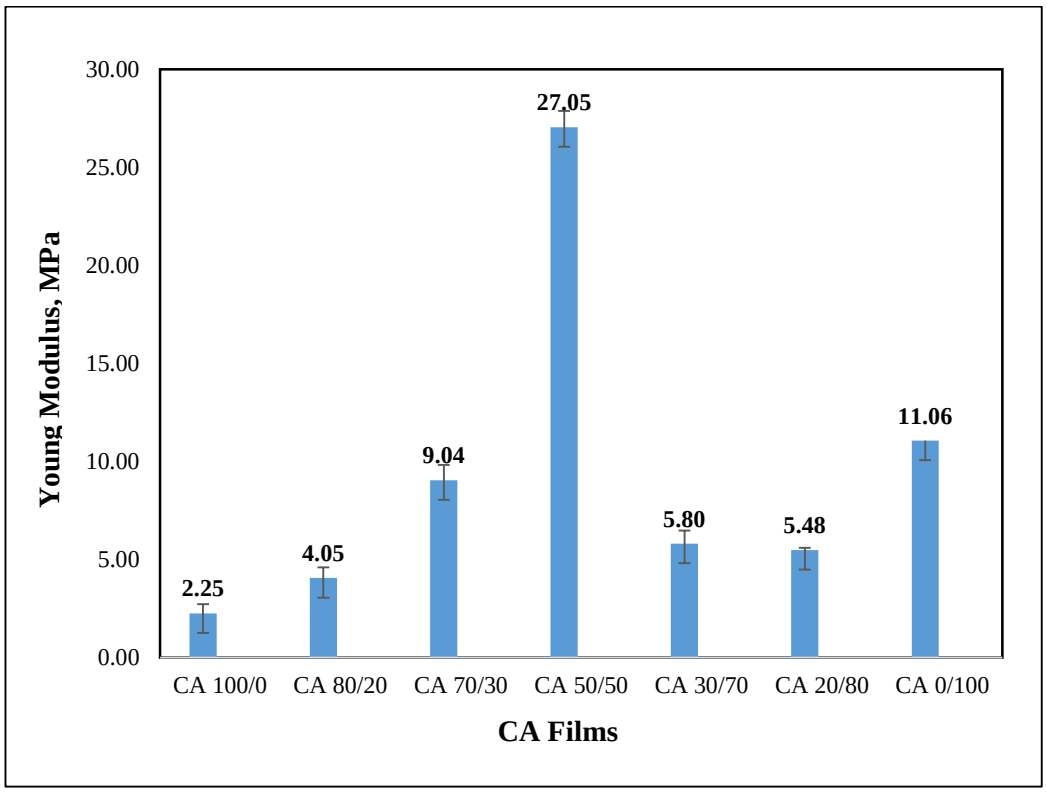


Figure 4.11d: Young Modulus of CA Films.

CHAPTER 5

CONCLUSION

5.1 Conclusion

Collagen was successfully extracted from *smooth wolf herring* skin by acid solubilisation method. The average yield of collagen extracted from *smooth wolf herring* skin was 0.25% in dry weight basis. Present study successfully utilized the waste from the fish industry in fabricating value added collagen based biomaterial. In addition, physicochemical properties of collagen and biomaterial film are characterized successfully in present study as well. The presence of amide A (3295.36 cm^{-1}), B (2922.23 cm^{-1}), I (1632.18 cm^{-1}), II (1547.88 cm^{-1}), and III (1237.13 cm-^{1}) bands in the FTIR spectra of collagen showed that the functional groups of collagen in this study were similar to those obtained from other species like *tilapia* and *striped catfish skin*.

Other than that, 16 types of amino acids were discovered in *smooth wolf herring* skin collagen. Like other collagen, glycine is the most abundant amino acids present in smooth wolf herring collagen, whereas tryptophan, cysteine and sarcosine were absent. Besides, present study further confirmed that the denaturation temperature of collagen was governed by hydroxylation ratio. The higher the hydroxylation ratio, the higher the denaturation temperature of collagen.

Alginate as a cross-linker in present study was isolated successfully from *sargassum polycystum* with an average yield of 20.68% by using calcium carbonate at 50°C. To overcome the weaknesses of collagen, a facile method was developed successfully in present study to develop CA composite films with enhanced physiochemical properties.

With the hybridization of collagen and alginate, resultant CA composite films possess a stronger resistance against the collagenase degradation. Moreover, swelling behavior of CA composite films were enhanced with the increase of alginate amount. Furthermore, tensile strength and denaturation temperature of CA films were improved with the addition of alginate.

Besides, all CA films exhibited porosity of more than 80% apart from CA 80/20. Apart from that, surface morphology of CA films were altered when alginate was incorporated. An additional band (around 3280 cm^{-1}) detected in CA composite films showed that there was an interaction between collagen and alginate.

Apart from that, CA films fabricated in present study exhibited high potentials to be applied in both tissue engineering and biomedical industries. For instance, CA 50/50 with stable structure (high tensile strength) and high swelling ratio have the potential to be used as a wound dressing material to absorb the exudates. Other than that, all CA films with high porosity (except CA 80/20) was possible to be applied in tissue engineering industry as the biomaterials are believed to allow rapid cell proliferation and nutrients diffusion. In conclusion, present study showed that hybridization of collagen with alginate was able to improve some physicochemical

properties of composite biomaterials; and developed variety of CA composite films to promote different applications.

5.2 Recommendations

Although collagen was successfully extracted with acid solubilisation method in this study, but the yield is low compared to the study of other researchers. Thus, pepsin solubilisation method was recommended to improve the yield of collagen. This is because, the cross-linking molecules at telopeptide region of collagen can be cleaved by pepsin which lead to the increase of collagen yield.

Apart from that, present study prove that hybridization of collagen and alginate was a good and simple strategy to improve certain physiochemical properties of the resultant composite films. To further improve the properties of composite films, chemical cross linking treatment was recommended. For example, composite films can be further cross-linked with chemical cross-linker such as EDC (1-ethyl-3-(3-dimethylaminopropyl) carboimide) and NHS (N-hydroxysuccinimide). In addition, biocompatibility study was recommended to ensure the biomaterials fabricated are compatible with the living tissues.

Last but not least, an additional natural cross-linkers such as chitosan can be added to further improve the strength of the composite films. Apart from chitosan, natural polysaccharides like pectin was suggested as well. Chitosan and pectin were recommended because these materials were reported to enhance the properties of composite films.

Both natural and chemical cross-linker were able to improve the physiochemical properties of composite films because they can stabilized the structure of the composite films. In addition, present of cross-linkers can increased the inter and intra-molecular crosslinking of composite films. Thus, the overall performances of composite films can be improved if chemical cross-linking treatment and natural cross-linker were introduced.

REFERENCES

Amruthwar, S., and Janorkar, A. (2013). In vitro evaluation of elastin-like polypeptide–collagen composite scaffold for bone tissue engineering. *Dental Materials*, 29, pp. 211 - 220.

August, A. D., Kong, H.J., & Mooney, D.J. (2006). Alginate hydrogels as biomaterials. *Macromolecular Bioscience*, 6, pp. 623 - 633.

Arpornmaeklong, P., Pripatnanont, P., and Suwatwirote, N. (2008). Properties of chitosan-collagen sponges and osteogenic differentiation of rat-bone-marrow stromal cells. *International Journal of Oral and Maxillofacial Surgery*, 37 (4), pp. 357 – 66.

Antoniac, (2014). Biodegradability of some collagen sponges reinforced with different bioceramics. *Key Engineering Materials*, 587, pp. 179 - 184.

Bae, I., Osatomic, K., Yoshidaa, A., Osakob, K., Yamaguchic, A., and Hara, K. (2008). *Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes.* Food Chemistry, 108 (1), pp. 49 - 54.

Boateng, J. S., Matthews, K. H., Stevens, H. N., and Eccleston, G. M. (2008). Wound healing dressings and drug delivery systems. *Journal of Pharmaceutical Science*, 97, pp. 2892 - 2923.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta*, 1767, pp. 1073 – 1101.

Benjakul, S., Nalinanon, S., and Shahidi, F. (2012). Fish collagen. *Food Biochemistry and Food Processing*, 2, pp. 365 - 387.

Birk, D. E., Fitch, J. M., Babiarz, J.P., and Linsenmayer, T.F. (1988). Collagen type I and type V are present in the same fibril in the avian corneal stroma. *The Journal of Cell Biology*, 106, pp. 999 – 1008.

Bloomfield, S.E., Miyata, T., Dunn, M. W., Bueser, N., Stenzel, K. H., and Rubin A. L. (1978). Soluble gentamycin ophthalmic inserts as a drug delivery system. *Arch. Ophtalmol*, 96, pp. 885 - 887.

Boontheekul, T., Kong, HJ., and Mooney, DJ. (2005). Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials*, 26, pp. 2455 - 2465.

Chandia, N. P., Matsuhiro, B., and Vasquez, A.E. (2001). Alginic acids in Lessonia trabeculata: characterization by formic acid hydrolysis and FT-IR spectroscopy. *Carbohydrate Polymers*, 46, pp. 81 - 87.

Chee, S., Wong, P., and Wong, C. (2010). Extraction and characterisation of alginate from brown seaweeds (*Fucales phaeophyceae*) collected from Port Dickson, Peninsular Malaysia. *Journal of Applied Physiology*, 23, pp. 191 - 196.

Chen, J., Long, L., Yi, R., Xu, N., Gao, R., and Hong, B. (2016). Extraction and characterization of acid-soluble collagen from scales and skin of tilapia (Oreochromis niloticus). *Food Science and Technology*, 66, pp. 453 - 459.

Chen, G., Ushida, T., and Tateishi, T. (2002). Scaffold design for tissue engineering. *Macromolecular Bioscience*, 2, pp. 67 - 77.

Choi, S., & Regenstein, J. M. (2000). Physicochemical and sensory characteristic of fish gelatin. *Journal of Food Science*, 65 (2), pp. 194 - 199.

Davis, T., Llanes, F., Volesky, B., and Mucci. (2003). Metal selectivity of Sargassum spp. and their alginates in relation to their α-l-guluronic acid content and conformation. *Environmental Science Technology*, 37, pp. 261 – 267.

Davis, J. D. (2003). Overview of Biomaterials and Their Use in Medical Devices. *Handbook of Materials For Medical Devices*, pp. 1 – 11.

Dong, Z., Wang, Q., and Du, Y. (2006). Alginate/gelatin blend films and their properties for drug controlled release. *Journal of Membrane Science*, 280, pp. 37 - 44.

Doyle, B., Bendit, E. G., and Blout, E. R. (1975). Infrared spectroscopy of collagen and collagen-like polypeptides. *Biopolymers*, 14, pp. 937 – 957.

Duan, R., Zhang, J., Du, X., Yao, X., & Konno, K. (2009). Properties of collagen from skin scale and bone of carp (*Cyprinus carpio*). *Food Chemistry*, 112, pp. 702 - 706.

Dusseault, J., Tam, S., Ménard, M., Polizu, S., Jourdan, G., Yahia, L., and Hallé, J. (2006). Evaluation of alginate purification methods: effect on polyphenol, endotoxin, and protein contamination. *Journal of Biomedical Materials Research*, 76, pp. 243 – 251.

Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M., and Discher, D. (2004). Substrate compliance versus ligand density in cell on gel responses. *Biophysical Journal*, 86, pp. 617 – 628.

Ekta, P., Keerti, S., Saurabh, G., Suravi, S., and Nidhi, M. (2016). Some Biocompatible Materials Used In Medical Practices- A Review. *International Journals of Pharmaceutical Science and Research*, 7, pp. 2748 – 2755.

Eva, G., and Pilar, R. (2011). FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. *Food Hydrocolloids*, 25, pp. 1514 - 1520.

Feng, W., Liang, G., Feng, S., Qi, Y., and Tang, K. (2015). Preparation and characterization of collagen–hydroxyapatite/pectin composite. *International Journal of Biological Macromolecules*, 74, pp. 218 – 223.

Fernandez, D., Montero, P., and Gomez-Guillen, M. C. (2001). Gel properties of collagens from skins of cod (Gadus morhua) and hake (Merluccius merluccius) and their modification by coenhancers magnesium sulphate, glycerol and transglutaminase. *Food Chemistry*, 74 (2), pp. 161 - 167.

Fenoradosoa, T. A., Ali, G., Delattre, C., Laroche, C., Petit, E., Wadouachi, A., and Michaud, P. (2010). Extraction and characterization of an alginate from the brown seaweed *Sargassum turbinarioides grunow*. *Journal of Applied Phycology*, 22, pp. 131 - 137.

Fertah, M., Belfkira, A., Dahmane E, Taourirte, M., and Brouillette, F. (2014). Extraction and characterization of sodium alginate from *Moroccan laminaria digitata*. *Arabian Journal of Chemistry*, pp. 3707 - 3714.

Foegeding, E., Lanier, T. C., & Hultin, H. O. (1996). Characteristics of edible muscle tissue. *Food Chemistry*, 3, pp. 849-972.

Gao, C. Y., Wang, D. Y., & Shen, J. C. (2003). Fabrication of porous collagen/chitosan scaffolds with controlling microstructure for dermal equivalent. *Polymer Advance Technology*, 14, pp. 373 - 379.

Gefen A., 2011. The Mechanical Environment of Cells in Collagen Gel Models *Cellular and Biomolecular Mechanics and Mechanobilogy*, pp. 201 - 247.

Gelse, P., Pöschi, E., and Aigner, T. (2003). Collagens- structure, function, and biosynthesis. *Advance Drug Delivery Reviews*, 55, pp. 1531 - 1546.

George, M., and Abraham, T. E. (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs. *Journal of Control Release*, 114, pp. 1 - 14.

Gilbert, R.T, and Oksana B. (2016). New Frontiers in Biomaterials. Oral and Maxillofacial Surgery Clinics of North America, 29, pp. 105 – 115.

Grover, C., Cameron, R., and Best, S. (2012). Investigating the morphological, mechanical and degradation properties of scaffolds comprising collagen, gelatin and elastin for use in soft tissue engineering. *Journal of the Mechanical Behavior of Biomedical Materials*, 10, pp. 62 – 74.

Hay, ID., Rehman, Z., Ghafoor, A., and Rehm., B. (2010). Bacterial synthesis of alginates. *Journal of Chemical Technology & Biotechnology*, 85, pp. 752 - 759.

Hu, Y., Liu, L., Dan, W., Dan, N., Gu, Z., and Yu, X. (2013). Synergistic effect of carbodi-imide and dehydrothermal crosslinking on a cellular dermal matrix. *International Journal of Biological Macromolecules*, 55C, pp. 221 – 230.

Hu, Y., Liu, L., Gu, Z., Dan, W., Dan, N., and Xixun Yu. (2014). Modification of collagen with a natural derived cross-linker, alginate dialdehyde. *Carbohydrate Polymers*, 102, pp. 324 – 332.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T., and Tanaka, M. (2005). Isolation and characterisation of collagen from bigeye snapper (*Priacanthus marcracanthus*) skin. *Journal of science of food and Agriculture*, 85, pp. 1203 - 1210.

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D., and Mann, S. (2003). Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas. *International Journal of Biological Macromolecules*, 32, pp. 199 – 204.

Karageorgiou, V., and Kaplan, D. (2005). Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials*, 26 (27), pp. 5471 - 5491.

Kang, A.H., and Gross, J. (1970). Relationship between the intra and intermolecular cross-links of collagen. *Proceedings of the National Academy of Sciences*, 67, pp. 1307 - 1314.

Karri, V. V. S. R., Kuppusamy, G., Talluri, S. V., Mannemala, S. S., Kollipara, R., Wadhwani, A. D., Mulukutl, S., Raju, K. R. S., Malayand, R. (2016). Curcumin loaded chitosan nanoparticles impregnated into collagen alginate scaffolds for diabetic wound healing. *International Journal of Biological Macromolecules*, 16, pp. 30449 - 30454.

Kaufman, H. E. (1988). Collagen shield symposium. *Journals of Cataract Refractive Surgery*, 14 (5), pp. 487 - 488.

Kuboki, Y., Takita, H., Kobayashi, D., Tsuruga, E., Inoue, M., and Murata, M. (1998). BMP-induced osteogenesis on the surface of hydroxyapatite with geometrically feasible and nonfeasible structures: Topology of osteogenesis. *Journal of Biomedical Materials Research*, 39, pp. 190 – 199.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H., and Shahidi, F. (2010). Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chemistry*, 119, pp. 1519 - 1526.

Kumar,P.T., Lakshmanan,V.K., Anilkumar,T.V., Ramya,C., Reshmi, P., Unnikrishnan, A.G., Nair, S.V., and Jayakumar,R. (2012). Flexible and microporous chitosan hydrogel/nano ZnO composite bandages for wound dressing: In vitro and in vivo evaluation. *Applied Materials and Interface,* pp. 2618 - 2629.

Lai, C., and Chu, M. (1996). Tissue distribution and development expression of type XVI collagen in the mouse. *Tissue and Cell*, 28 (2), pp. 154-164.

Leal, D., Matsuhiro, B., Rossib, M., and Carusoc, F. (2008). FT-IR spectra of alginic acid block fractions in three species of brown seaweeds. *Carbohydrate Research*, 343, pp. 308 – 316.

Lee, K., Alsberg, E., and Mooney, D. (2001). Degradable and injectable poly(aldehyde guluronate) hydrogels for bone tissue engineering. *Journal of Biomedical Materials Research*, 56, pp. 228 - 233.

Lee, C. H., Singla, A., & Lee, Y. (2001). Biomedical applications of collagen. *International Journal of Pharmaceutics* 221, pp. 1 – 22.

Leon, L. (1998). New perspectives in mercury porosimetry. *Advance Colloid Interface Science,* 76-77, pp. 341 - 372.

Lee, K.Y., & Mooney, D. (2011). Alginate: Properties and biomedical applications. *Progress in Polymer Science*, 37, pp. 106 - 126.

Li, G. Y., Fukunaga, S., Takenouchi, K., and Nakamura, F. (2005). Comparative study of the physiological properties of collagen, gelatin and collagen hydrolysate as cosmetic materials. *International Journal of Cosmetics Science*., 27(2), pp. 101 - 106.

Lin, Y., Tan, F., Marra, K., Jan, S., and Liu, D. (2009). Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications. *Acta Biomaterialia*, 5, pp. 2591-2600.

Liu, X., and Ma. (2004). Polymeric scaffolds for bone tissue engineering. *Annals of Biomedical Engineering*, 32, pp. 477 – 486.

Ma, L., Gao, C., Mao, Z., Zhou, J., Shen, J., Hu, X , and Han, C. (2003). Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. *Biomaterials*, 24 (26), pp. 4833 – 4841.

Mahmoud, A. A., and Salama, A. H. (2016). Norfloxacin-loaded collagen/chitosan scaffolds for skin reconstruction: Preparation, evaluation and in-vivo wound healing assessment. *European Journal of Pharmaceutical Sciences*, 83, pp. 155 - 165.

Mayne, R., and Brewton, R. G. (1993). New members of the collagen superfamily. *Current Opinion in Cell Biology*, 5 (5), pp. 883-890.

Martínez, A., Blancoa, M. D., Davidenkob, N., and Cameron, R. E. (2015). Tailoring chitosan/collagen scaffolds for tissue engineering: Effect of composition and different crosslinking agents on scaffold properties. *Carbohydrate Polymer*, 132, pp. 606 - 619.

Mathlouti, M., and Koening, J. (1986). Vibrational spectra of carbohydrates. *Advance Carbohydrate Chemistry Biochemistry*, 44, pp. 7 - 89.

Montero, P., Gómez-Guillén, M. C., and Borderías, A. J. (1999). Functional characterisation of muscle and skin collagenous material from hake *(Merluccius merluccius L.).* Food Chemistry, 65 (1), pp. 55-59.

Montoya, M. H., Moscoso, J. L., Jatomea, M., Ortega, H., Sandez, O., Lopez, J., Rios, E., and Brauer, J. (2010). Jumbo Squid (Dosidicius gigas) Collagen: extracion, charaztirization, and potential application in the preparation of chitosan-collagen biofilms. *Bioresource Technology*, 101, pp. 4212 - 4219.

Mohan, J. (2002). Infrared Spectroscopy. *Organic Spectroscopy Principle and Application*, 2, pp. 52 - 54.

Muyongaa, J.H., Coleb, C.G.B., and Duodub, K.G. (2004). Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chemistry*, 85, pp. 81-89.

Mccardy, J. B., Vachhani, B., & Lida, J. (1996). Cell Adhesion to Collegeneous Matrices. *Biopolymers*, 40, pp. 371 - 381.

Nagai, T., Suzuki, N., and Nagashima, T. (2008). Collagen from common minkle whale (*Balaenoptera acutorostrata*) unesu. *Food Chemistry*, 111, pp. 296 - 301.

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

Nalinanon, S., Benjakul, S., Visessanguan, W., and Kishimura, H. (2007). Use of pepsin for collagen extraction from the skin of bigeye snapper *(Priacanthus tayenus). Food Chemistry*, 104 (2), pp. 593-601.

Parenteau, B., Gauvin, R., Cliché, S., Gariepy, C., Germain, L., & Berthod, F. (2011). Comparative study of bovine, porcine, and avian collagens for the production of a tissue engineered dermis. *Acta Biomaterial*, 10, pp. 3757 – 3765.

Payne K.J., and Veis, A. (1988). Fourier transform IR spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for confirmation studies. *Biopolymers*, 27 (11), pp. 1749 - 1760.

Penman, A., and Sanderson G. (1972). A method for the determination of uronic acid sequence in alginates. *Carbohydrate Research*, 25, pp. 273 – 282.

Perumal, S., Ramadass, S. K., Gopinath, A., Madhan, B., Shanmugam, G., Rajadas, J., and Mandal, A. B. (2015). Altering the concentration of silica tunes the functional properties of collagen–silica composite scaffolds to suit various clinical requirements. *Journal of The Mechanical Behavior of Biomedical Materials*, 52, pp. 131-138.

Pieper, J.S., Oosterhof, A., Dijkstra, P.J., Veerkamp, J.H., and Kuppevelt, T.H. (1999). Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterial*, 20, pp. 847 - 858.

Plepis, A. M. D. G., Goissis, G., and Das-Gupta, D. K. (1996). Dielectric and pyroeectric characterisation of anionic and native collagen. *Polymer Engineering Science*, 36, pp. 2932 - 2938.

Rao, K.P. (1995). Recent development of collagen-based materials for medical applications and drug delivery system. *Biomaterial Science*, 7 (7), pp. 623 - 645.

Ramasamy, P., and Shanmugam, A. (2015). Characterization and wound healing property of collagen–chitosan film from sepia kobiensis (Hoyle, 1885). *International Journal of Biological Macromolecules*, 74, pp. 93 – 102.

Rinaudo, M. (2007). Seaweed Polysaccharide. *Comprehensive Glycoscience from Chemistry to System Biology*, 2, pp. 691 - 735.

Rochdi, A., Foucat, L., and Renou, J. (2000). NMR and DSC Studies during thermal denaturation of collagen. *Food Chemistry*, 69, pp. 295 - 299.

Rochdi, A., Foucat, L., and Renou, J. (2000). Systematic Revision of the Asian Catfish Species. *Proceedings of Academy of Natural Science Philadelphia*, 143, pp. 97 - 144.

Sang, L., Luo, D., Wang, X., & Li, X. (2011). Fabrication and evaluation of biomometic scaffolds by using collagen-alginate fibrillar gels for potential tissue engineering applications. Material Science and Engineering C, 31, pp. 262 -271.

Shrestha, A., Friedman, S., and Kishen, A. (2011). Photodynamically crosslinked and chitosan-incorporated dentin collagen. *Journal of Dental Research*, 90, pp. 1346 - 1351.

Simpson, B. K., Nollet, L. M. L., Toldrá, F., Benjakul, Paliyath, G., and Hui, Y. H. (2012). Fish Collagen. *Food Biochemistry and Food Processing*, pp. 365 – 387.

Singh, P., Benjakul, S., Maqsood, S., and Kishimura, H. (2011). Isolation and characterisation of collagen extracted from the skin of striped catfish (Pangasianodon hypopthalmus). *Food Chemistry*, 124, pp. 97 - 105.

Sionkowska, A., and Kozłowska, J. (2013). Properties and modification of porous 3-D collagen/hydroxyapatite composites. *International Journal of Biological Macromolecules*, 52, pp. 250 – 259.

Sionkowska, A., Kozłowska, J., Skorupska, M., and Michalska, M. (2015). Isolation and characterization of collagen from the skin of brama australi. *International Journal of Biological Macromolecules*, 80, pp. 605 – 609.

Surewicz, W.K., and Mantsch, H.H. (1988). New inside into protein secondary structure from resolution enhanced infrared spectra. *Biochimica et Biophysica Acta*, 952, pp. 115-130.

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

Tan, H., Gong, Y., Lao, L., Mao, Z., and Gao, C. (2007). Materials in Medicine. *Journal of Materials Science*, 18, pp. 1961 – 1968.

Tang, L., Chen, S., Su, W., Weng, W., Osako, K., and Tanaka ,M. (2015). Physiochemical properties and film-forming ability of fish skin collagen extracted from different freshwater species. *Process Biochemistry*, 50, pp. 148 - 155.

Tathe, A., Ghotdke, M., and Nikalja, A. P. (2010). A Brief Review: Biomaterials and Their Application. International Journal of Pharmacy and Pharmaceutical Sciences, 2, pp. 975 – 1491.

Torres, M. R., Sousa, A. P. A., Silva Filho, E. A., Melo, D.F., Feitosa, J. P. A., Paula, R. C. M., & Lima. (2007). Extraction and physiochemical characterization of Sargassum vulgare alginate from Brazil. *Carbohydrate Research*, 342, pp. 2067 - 2074.

Thomas, F., Lundqvist, L., Jam, M., Jeudy, A., Barbeyron, T., Sandstrom, C., Michel, G., Czjzek, M. (2013). Comparative Characterization of Two Marine Alginate Lyases from *Zobellia galactanivorans* Reveals Distinct Modes of Action and Exquisite Adaptation to Their Natural Substrate. *Journal of Biological Chemistry*, 32, pp. 23021 – 23037.

Vodjani, F. (1996). Methods of Testing Protein Functionality. *Handbook of Food Powder*, pp. 11 – 60.

Wang, L., Yang, B., & Du, X. (2008). Extraction of acid-soluble collagen from Grass Carp (*Ctenopharyngodon idella*) skin. *Journal of Food Process Engineering*, 32, pp. 743 - 751.

Wang, X., Sang, L., and Li, X. (2011). From collagen–chitosan blends to three-dimensional scaffolds: The influences of chitosan on collagen nanofibrillar structure and mechanical property. *Colloids and Surfaces B: Biointerfaces*, 82, pp. 233 – 240.

Wang, H., Chou, Y., Wen, Z., Wang, Z., Chen, C., and Ho, M. (2013). Novel Biodegradable Porous Scaffold Applied to Skin Regeneration. *Plos On*e, 8.

Wang, L., An, X., 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 mentella). *Food Chemistry*, 108, pp. 616 – 623.

Wang, L., and Rhim, J. (2015). Preparation and application of agar/alginate/collagen ternary blendfunctional food packaging films. *International Journal of Biological Macromolecules*, 80, pp. 460 – 468.

Wong, DWS. (1989). Mechanism and theory in food chemistry. *Food Biochemistry and Food Processing*, pp. 366.

Woo, J., Yua, S., Cho, S., Lee, Y., & Kim, S. (2008). Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids*, 22, pp. 879 – 887.

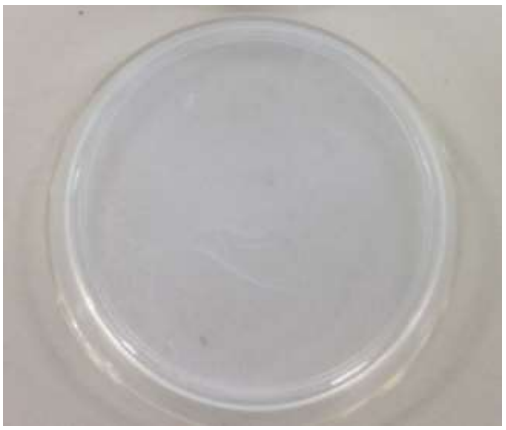
Yang, X., Guo, L., Fan, Y., and Zhang, X. (2013). Preparation and characterization of macromolecule cross-linked collagen hydrogels for chondrocyte delivery. *International Journal of Biological Macromolecules*, 61, pp. 487 - 493.

Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y., and Wu, X. (2007). Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp *(Ctenopharyngodon idella)*. *Food Chemistry*, 103 (3), pp. 906-912.

Zimmermannr, B., Timpl, U., and Kuhn, K. (1973). Intermolecular cross-links of collagen participation of the carboxy-terminal nonhelical region of the αl-chain. *European Journal of Biochemistry*, 35, pp. 216 - 221.

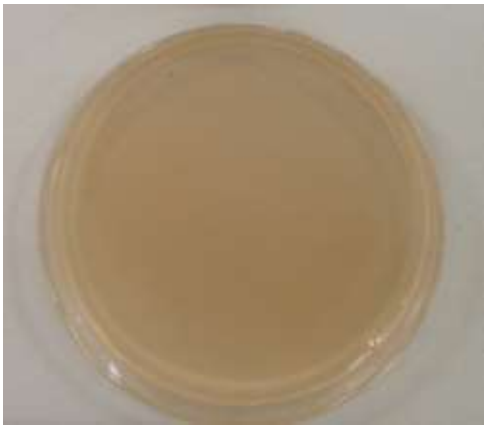
APPENDICES

APPENDIX A: Pictures of Hydrogel and CA Films



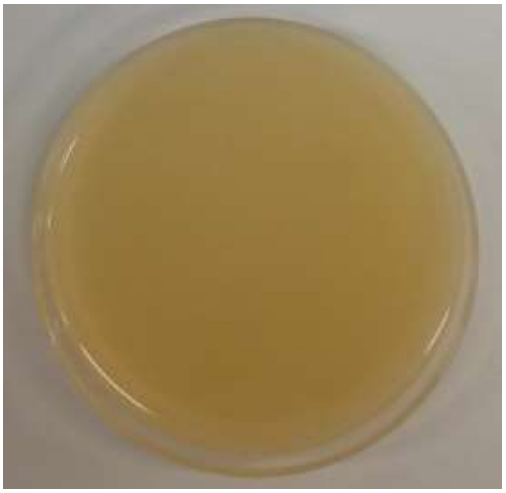
Pure Collagen (CA 100/0) Hydrogel

Pure Collagen (CA 100/0) Film



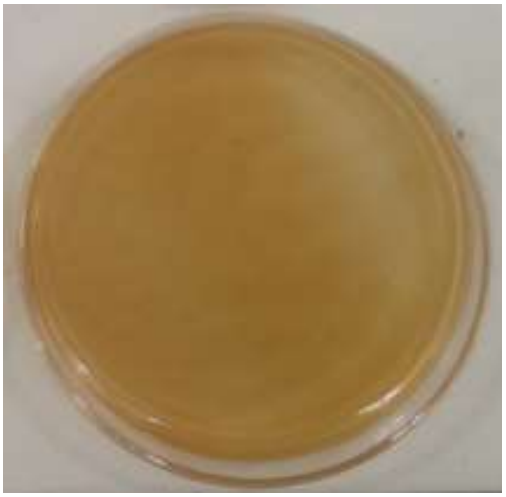
CA 80/20 Hydrogel

CA 80/20 Film

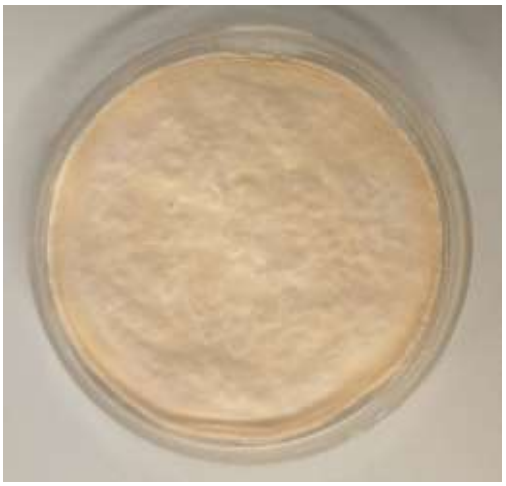


CA 70/30 Hydrogel

CA 70/30 Film

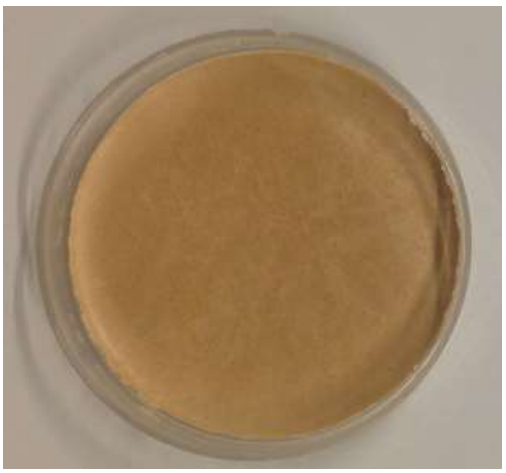


CA 50/50 Hydrogel

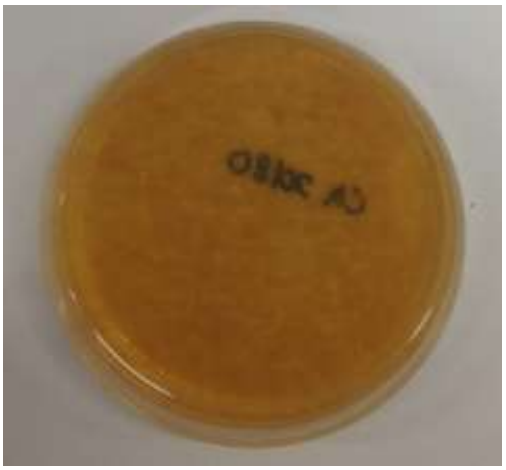


CA 50/50 Film

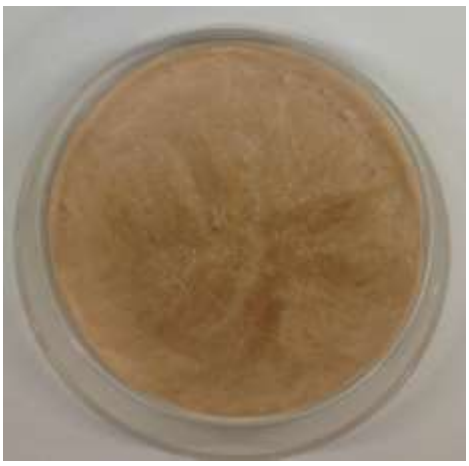
CA 30/70 Hydrogel



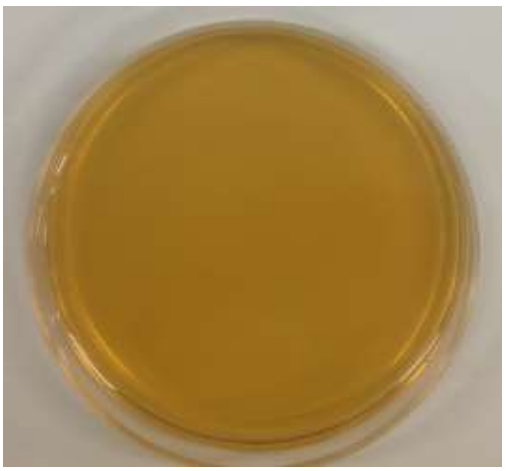
CA 30/70 Film



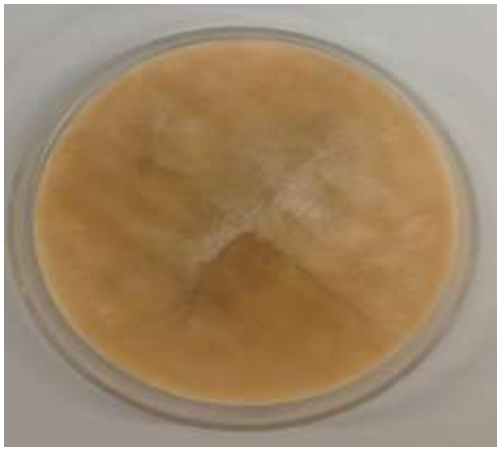
CA 20/80 Hydrogel



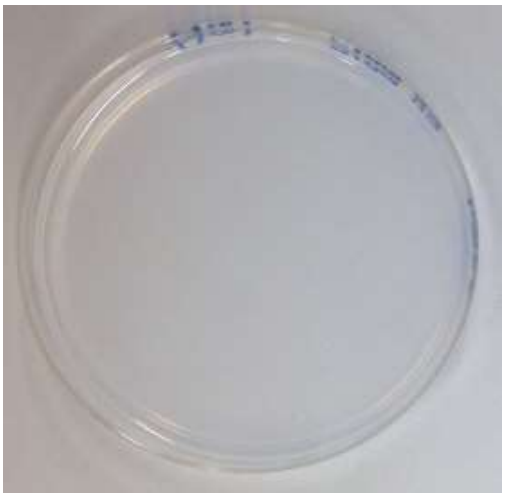
CA 20/80



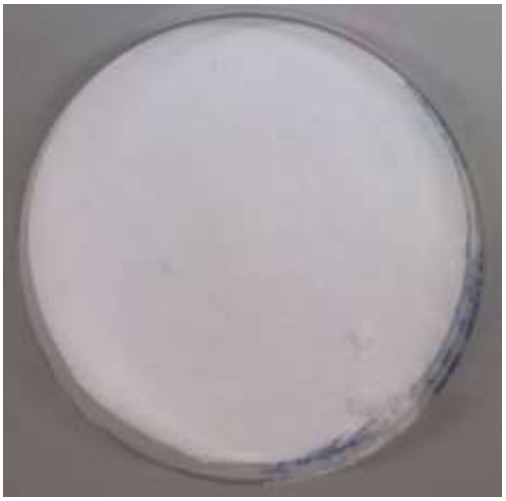
Pure Alginate (CA 0/100) Hydrogel



Pure Alginate (CA 0/100) Film



1% Pure Collagen (CA 100/0) Hydrogel



1% Pure Collagen (CA 100/0) Film

1.4% Pure Collagen (CA 100/0) Film

1.4% Pure Collagen (CA 100/0) Film

3% Pure Alginate (CA 0/100) Hydrogel

3% Pure Alginate (CA 0/100) Hydrogel

APPENDIX B: FTIR Spectra of CA Composite Films

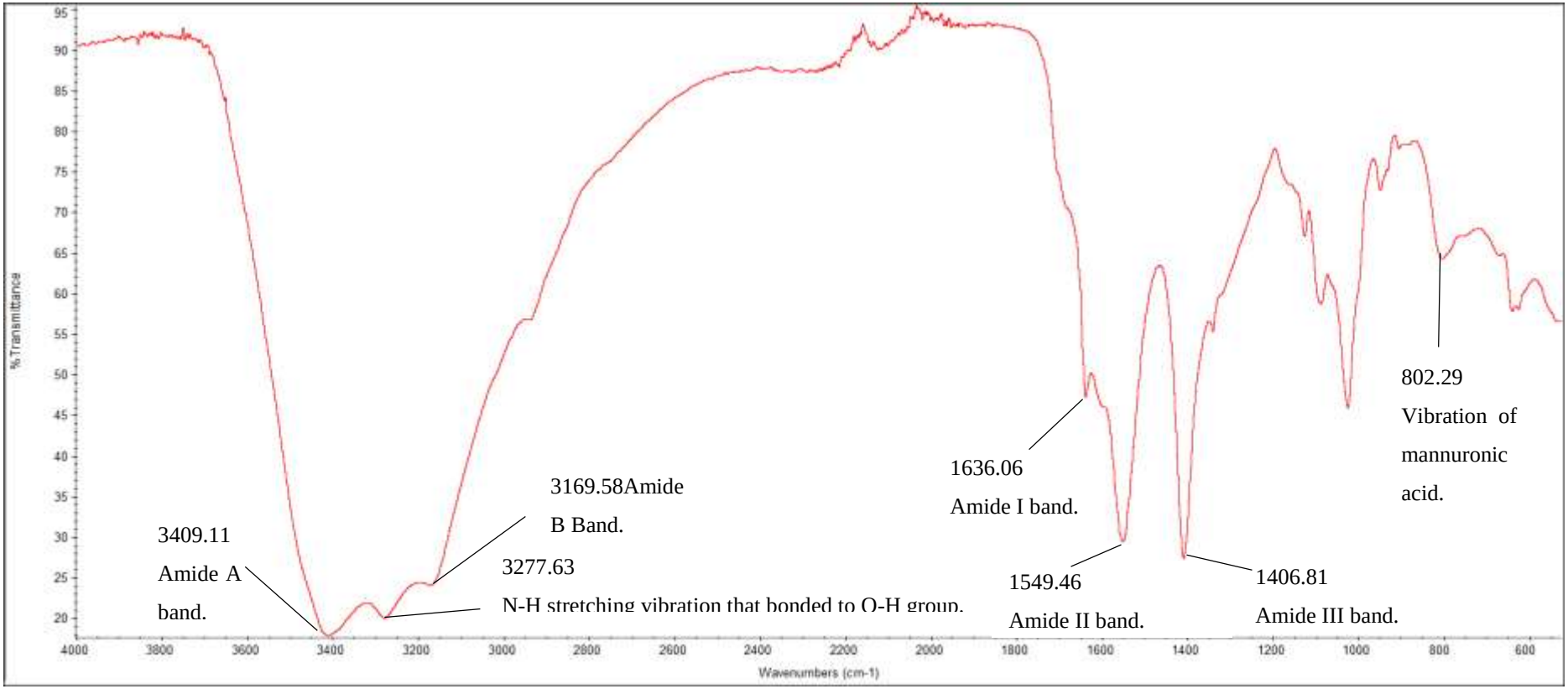


Figure B1: FTIR Spectra of CA 20/80.

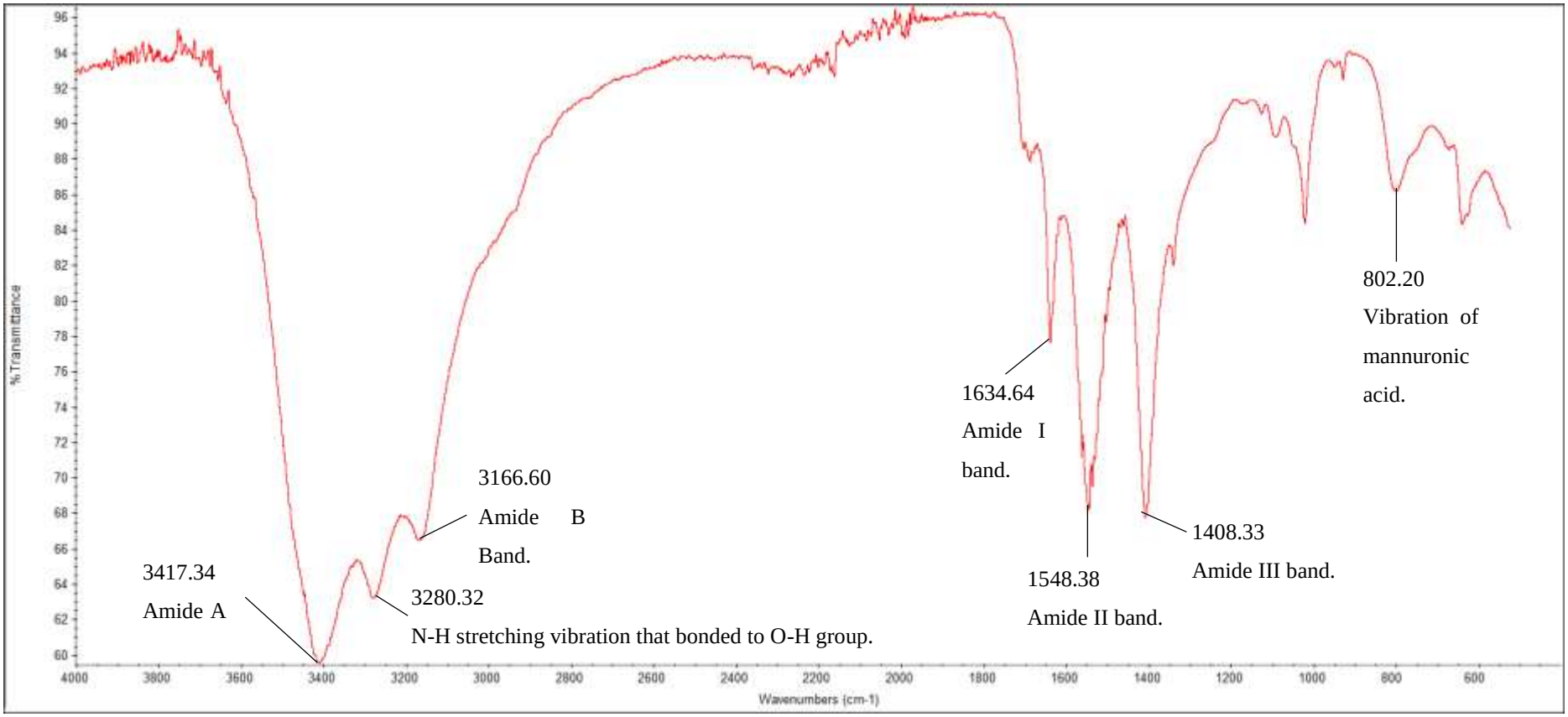


Figure B2: FTIR Spectra of CA 30/70.

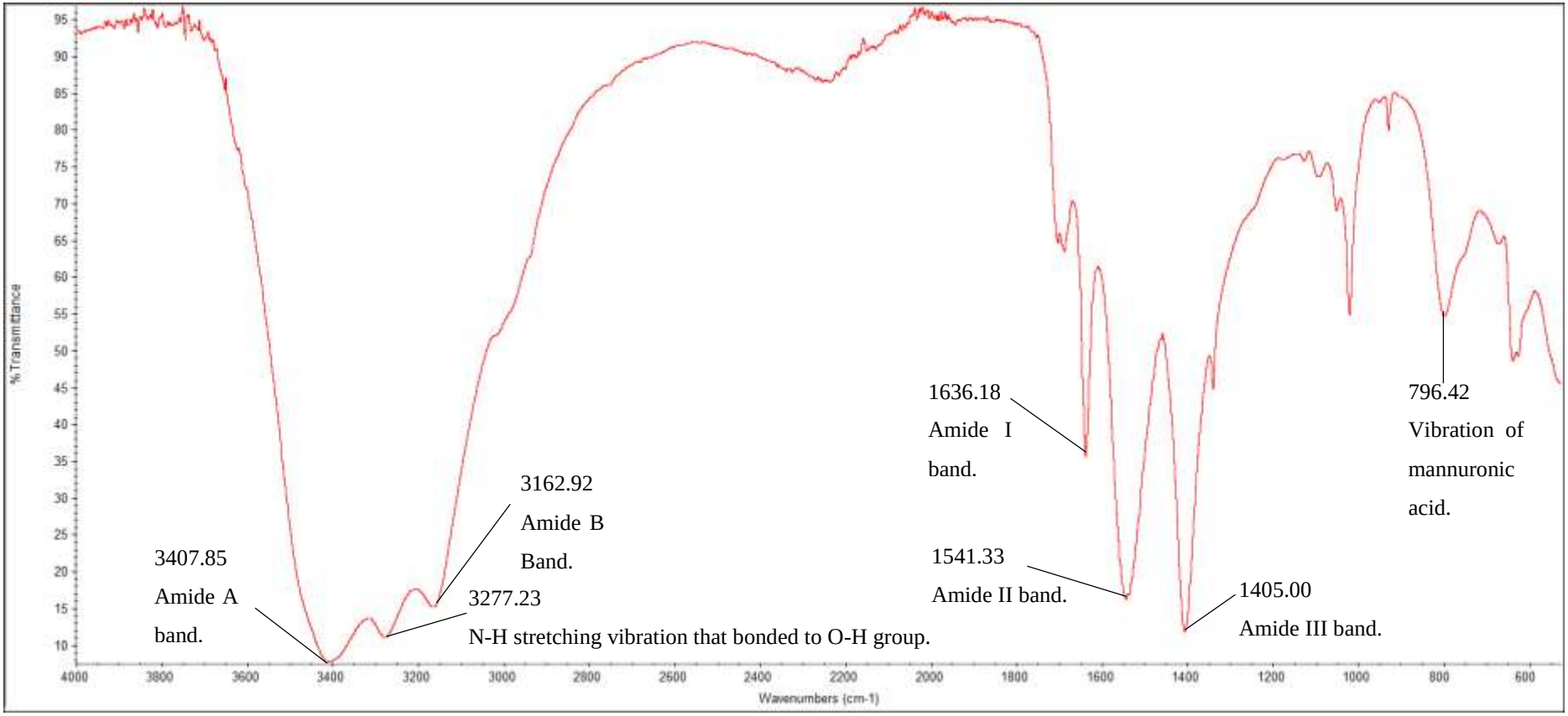


Figure B3: FTIR Spectra of CA 70/30.

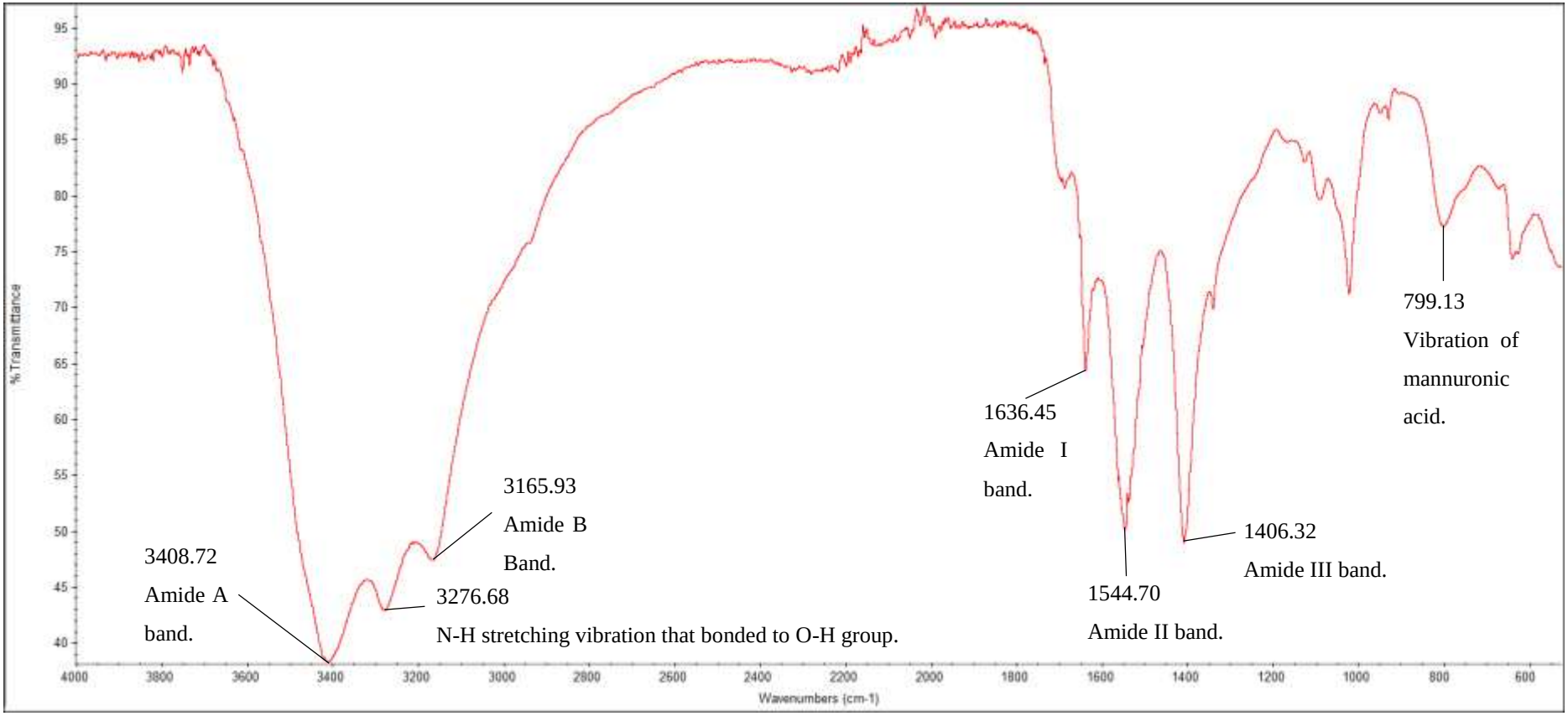


Figure B4: FTIR Spectra of CA 80/20.