การพัฒนาวัสดุรูพรุนจากแบคทีเรียเซลลูโลสสำหรับงานด้านชีวเวช

นางสาวนัดดา เชี่ยวประกอบกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

DEVELOPMENT OF POROUS MATERIAL FROM BACTERIAL CELLULOSE FOR BIOMEDICAL APPLICATIONS

Miss Nadda Chiaoprakobkij

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2007

Thesis Title	DEVELOPMENT OF POROUS MATERIAL FROM BACTERIAL CELLULOSE FOR BIOMEDICAL APPLICATIONS
By	Miss Nadda Chiaoprakobkij
Field of Study	Chemical Engineering
Thesis Advisor	Associate Professor Professor Muenduen Phisalaphong, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Engineering

(Associate Professor Boonsom Lerdhirunwong, Dr. Ing.)

THESIS COMMITTEE

..... Chairman

(Assistant Professor Artiwan Shotipruk, Ph,D.)

..... Thesis Advisor

(Associate Professor Muenduen Phisalaphong, Ph.D.)

..... Member

(Chada Phisalaphong, Ph.D.)

..... Member

(Assistant Professor Bunjerd Jongsomjit, Ph.D.)

..... Member

(Chanchana Tangwongsan, Ph.D.)

นัดดา เชี่ยวประกอบกิจ : การพัฒนาวัสดุรูพรุนจากแบคทีเรียเซลลูโลสสำหรับงานด้านชีววิศ. (DEVELOPMENT OF POROUS MATERIAL FROM BACTERIAL CELLULOSE FOR BIOMEDICAL APPLICATIONS) อ. ที่ปรึกษา: รศ.ดร. เหมือนเดือน พิศาลพงศ์ 105 หน้า.

พอลิเมอร์ชีวภาพผสมระหว่างแบคทีเรียเซลลูโลสและอัลจิเนตมีศักยภาพสำหรับนำมาใช้ผลิตเป็นวัสดุสำหรับใช้งานด้านชีววิศ คุณสมบัติทางกายภาพและชีวภาพของวัสดุที่ทำจากแบคทีเรียเซลลูโลสและอัลจิเนตยังไม่มีรายงานที่ชัดเจน ดังนั้นในงานวิจัยนี้จึงมุ่งเน้นที่จะศึกษาผลของอัตราส่วนระหว่างแบคทีเรียเซลลูโลสและอัลจิเนตต่อสมบัติทางกายภาพและชีวภาพของโครงพรุน(sponge)ที่พัฒนาจากแบคทีเรียเซลลูโลส โดยโครงพรุนที่ใช้ในการทดลองเตรียมโดยกระบวนการทำแห้งด้วยความเย็นของของผสม โครงพรุนถูกเตรียมโดยใช้อัตราส่วนการผสมต่างๆกัน ผลจาก Fourier transform infrared (FT-IR) spectroscopy พิสูจน์ให้เห็นว่าอันตรกิริยาระหว่างแบคทีเรียเซลลูโลสและอัลจิเนตเป็นแบบกายภาพในทุกสัดส่วนการผสม โดยไม่เกิดปฏิกิริยาทางเคมีในระดับโมเลกุล ในสภาพแห้งโครงพรุนที่ประกอบด้วยสัดส่วนอัลจิเนตเพิ่มขึ้นจะมีความแข็งแรงเชิงกลลดลง โครงพรุนจะมีค่าสัดส่วนการบวมน้ำประมาณ 11-14 เท่าของน้ำหนักวัสดุแห้ง จากการทดสอบการย่อยสลายทางชีวภาพโดยใช้เอนไซม์เซลลูเลสแสดงให้เห็นว่าการเติมอัลจิเนตทำให้อัตราในการย่อยสลายตัวของวัสดุช้าลง การทดลองการเลี้ยงเซลล์ผิวหนังชั้นในและเซลล์เยื่อบุช่องปากของมนุษย์ พบว่า โครงพรุนที่ทำจากแบคทีเรียเซลลูโลสล้วนและโครงพรุนที่มีสัดส่วนการผสมของอัลจิเนต 30 เปอร์เซ็นต์ สามารถกระตุ้นการเจริญเติบโตของเซลล์ทั้งสองชนิดอย่างมีประสิทธิภาพ อย่างไรก็ตามในสภาพเปียกโครงพรุนที่มีส่วนผสมของอัลจิเนต 30 เปอร์เซ็นต์เท่านั้นที่ทนต่อแรงเย็บเมื่อนำไปทดลองใช้ในผู้ป่วยที่มีแผลในช่องปากเพื่อเป็นวัสดุปิดบาดแผลในช่องปาก ผลการทดลองแสดงถึงความมีศักยภาพของการใช้พอลิเมอร์ชีวภาพผสมระหว่างแบคทีเรียเซลลูโลสและอัลจิเนตเพื่อพัฒนาเป็นวัสดุชนิดใหม่ในงานด้านชีววิศ

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....นัดดา เชี่ยวประกอบกิจ.....

สาขาวิชา.....วิศวกรรมเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....

4770325221: MAJOR CHEMICAL ENGINEERING

KEY WORD: BACTERIAL CELLULOSE/ ALGINATE / SCAFFOLD/ TISSUE ENGINEERING

NADDA CHIAOPRAKOBKIJ : DEVELOPMENT OF POROUS MATERIAL FROM BACTERIAL CELLULOSE FOR BIOMEDICAL APPLICATIONS. THESIS ADVISOR: ASSOC.PROF.MUENDUEN PHISALAPHONG, Ph.D., 105 pp.

Biopolymer blends between bacterial cellulose and alginate have good potentials for production of biomedical materials. The physical and biological properties of the blends of bacterial cellulose / alginate sponges have not been clearly elucidated. This study aims to investigate the effects of bacterial cellulose / alginate ratio on the physical and biological properties of developed sponges. The sponges were fabricated by freeze drying of the mixtures with various blending compositions. Results from Fourier transform infrared (FT-IR) spectroscopy proved that bacterial cellulose/alginate sponges in all blending compositions contained only physical but not chemical interaction in molecular level. The mechanical strength of dried sponges decreased with the increasing composition of alginate. Water uptake ratio of the dried sponges was approximately 11-14. The biodegradation test by cellulose enzyme demonstrated that the presence of alginate could prolong the biodegradation of bacterial cellulose/alginate sponges. *In vitro* culture of keratinocytes and gingival fibroblasts evidenced that pure bacterial cellulose sponges and sponges containing 30% of alginate were effective to promote and accelerate cell proliferation. However, in wet state, only the blend sponge with 30% alginate also had a good tear resistance from sewing. The results indicated that the blends of bacterial cellulose with alginate have high potential to be developed as an alternative material for biomedical approaches.

Department.....Chemical Engineering.....Student's signature.....

Field of study.....Chemical Engineering.....Advisor's signature.....

Academic year...2007.....

ACKNOWLEDGEMENTS

This research is completed with the aid and support of many people. The author would like to express her deepest gratitude to Associate Professor Dr. Muenduen Phisalaphong, her advisor, for her continuous guidance, helpful suggestions and warm encouragement. The author is also grateful to Assistant Professor Artiwan Shotipruk, Dr.Chada Phisalaphong, Assistant Professor Bunjerd Jongsomjit and Dr. Chanchana Tangwongsan for serving as the chairman and the members of the thesis committee, respectively, whose comments were constructively and especially helpful.

The author would like to thank Assistant Professor Dr. Neeracha Sanchavanakit and the Department of Anatomy, Faculty of Dentistry, Chulalongkorn University for her kind attentions and suggestions in cell culture as well as facilities.

The author would like to thank the staffs of Analytical Instrument Center and Laboratory for their helps with experiments. She would like to extend her grateful thanks to all members of Biochemical Engineering Research Group at the Department of Chemical Engineering as well as all members of Tissue Culture Laboratory at the Department of Dentistry, Chulalongkorn University.

Finally, the author expresses her sincere thanks to her parents and everyone in her family for their support, unfailing understanding and affectionate encouragement.

CONTENTS

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

PAGE

PAGE

CHAPTER I

INTRODUCTION

1.1 Background

For the past ten years, a number of natural polymers have been proposed as an new alternative biomaterials. Bacterial cellulose (BC), synthesized by *Acetobacter xylinum*, is known to be the appealing material in biomedical applications. Compare to collagen or gelatin, BC does not contain any component of animal origin and does not cause any allergic reactions.

BC posses significant advantages over plant cellulose with its structural and purity. BC has high mechanical strength, high swelling ability, high stability to pH variations and fine web-like network. BC has been applied as artificial skins for patients with burns and ulcers (Fontana *et al.*, 1990), temporary skin substitute for animals (Jonas and Farah, 1998) and artificial blood vessels for microsurgery (Klemn *et al.*, 2001). Svesson *et al.* (2005) suggested that BC is a promising material for potential scaffold for tissue engineering of cartilage. These suggested the potential used of BC in tissue engineering field due to its biocompatibility, non-toxicity, low cost and large agricultural availability.

One of the requirements of material to use in tissue engineering is to moisten dry areas and at the same time absorb unwanted fluids from the wound, there by maintaining and optimizing moist environment for wound healing. The polymer modification by means of blending with other polymers may be convenient and effective method to improve physical properties for practical utilization. For this

reason, the blending of biopolymers has been employed to produce desirable bacterial cellulose-based material.

Alginate, a linear polysaccharide (copolymer of (1-4)-linked β-mannuronic acid (M) and α-guluronic acid (G) monomers), has been widely used in biomedical applications due to its hydrogel properties. Alginate is soluble in water and becomes a hydrogel in the presence of divalent ion such as Ca^{2+}. The gellation process can be carried out under very mild conditions and in the absence of organic solvents. Alginate hydrogels have been wildely studied for the cartilage and bone regeneration application as a vehicles for biologically active molecules (Alberg *et al.*,2001). Several therapeutic agents, including antibiotics, enzymes, growth factors and DNA, have already been successfully incorporated into alginate gels, retaining a high percentage of biological activity (Smidsrod *et al*, 1996). Alginate-based products are currently popular in wound management. Alginate gel prevents the wound surface from drying out, discomfort during removal (Thomas, 1990). In addition, it is also reported that alginate-based wound dressing can enhance the rate of healing of skin wound (Jarvis *et al., 1987*).

However, the tissue compatibility and biological properties of bacterial cellulose (in a wet state)/alginate blend have been rarely reported. The aim of this work was to develop a new hybrid biomaterial fabricated from bacterial cellulose and alginate and also evaluate the possibility for the use of this material in tissue engineering. The structure, morphology, mechanical strength, biodegradability and tissue compatibility were investigated.

1.2 Objectives

To study the effect of blending composition on physical and biological properties of bacterial cellulose/alginate sponges.

1.3 Scope of work

1. Develop bacterial cellulose/alginate sponges.
2. Vary the blending composition ratio of bacterial cellulose to alginate: 100/0, 70/30, 50/50, 30/70 and 0/100.
3. Characterize the chemical and physical properties of bacterial cellulose/alginate polymeric sponges including:
   3.1 Fourier Transform Infrared (FT-IR) spectroscopy
   3.2 Tensile strength
   3.3 Swelling ratios
   3.4 Morphology
   3.5 Moisture content
   3.6 Shape retention test
4. Characterize the biological properties including:
   4.1 Biodegradation
   4.2 Cell Culture

CHAPTER II

RELEVANT THEORY

2.1 Cellulose

Cellulose is the most abundant naturally occurring polysaccharide formed out of glucose based repeat units, connected by 1,4-beta-glucosidic linkages. (Fig. 2.1).

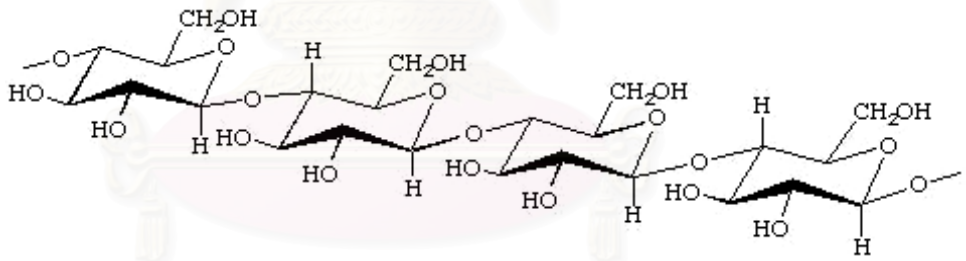
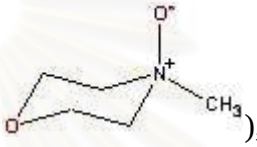


Figure 2.1 The cellulose molecule-$(C_6H_{10}O_5)_n$-in its chair configuration (Klemm *et al.*,2002)

Cellulose is found in plants as microfibrils (2-20 nm diameter and 100-40,000 nm long). These form the structurally strong framework in the cell walls. Cellulose is mostly prepared from wood pulp.

Cellulose is an insoluble molecule consisting of between 2000 - 14000 residues with some preparations being somewhat shorter. It forms crystals (cellulose

I_α) where intra-molecular (O3-H→O5' and O6→H-O2') and intra-strand (O6-H→O3') hydrogen bonds holds the network flat allowing the more hydrophobic ribbon faces to stack. Each residue is oriented 180° to the next with the chain synthesized two residues at a time. Although individual strand of cellulose are intrinsically no less hydrophilic, or no more hydrophobic, than some other soluble polysaccharides (such as amylose) this tendency to form crystals utilizing extensive intra- and intermolecular hydrogen bonding makes it completely insoluble in normal aqueous solutions (although it is soluble in more exotic solvents such as aqueous N-methylmorpholine-N-oxide (NMNO, ), CdO/ethylenediamine (cadoxen), LiCl/*N*,*N'*-dimethylacetamide or near supercritical water). It is thought that water molecule catalyze the formation of the natural cellulose crystals by helping to align the chains through hydrogen-bonded bridging.

When a cellulose molecule is completely extended, its chain resembles a flat ribbon, with hydroxyl groups extending laterally from the edges. This structure is capable of forming inter-and intra-molecular hydrogen bonds. The hydrogen atoms oriented above and below the plane of the ribbon and are thus hydrophilic. This structure allows for Van der Waals interactions between the hydrogen atoms (Klimentov *et al.*,1991).

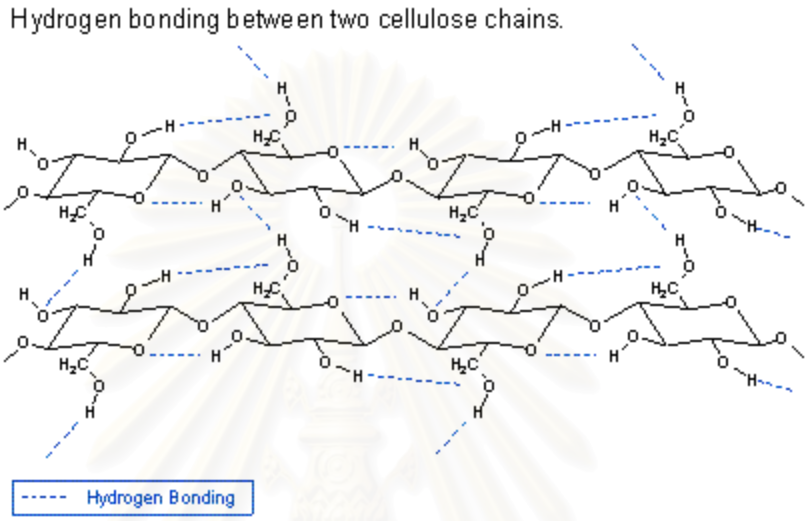


Figure 2.2 Hydrogen bond system of plant cellulose (http://www.bcnc.ac.th/html/biochemistry/biochem/carbohydrate2/sld048.htm)

The intermolecular hydrogen bonding in cellulose is considerable for the sheet-like of the native polymer. It is assumed that intermolecular hydrogen bonding between the hydroxyl group at the C-3' and C-6' positions of cellulose molecules adjacently occurred along the same lattice planes (Kolpack & Blackwell,1976).

Cellulose fibrils are highly inelastic and insoluble (Ross *et al.*,1991). The β-1,4 linkage results in a stiff, ribbon-like molecule that is ideally suited for forming fibrils via hydrogen bonding.

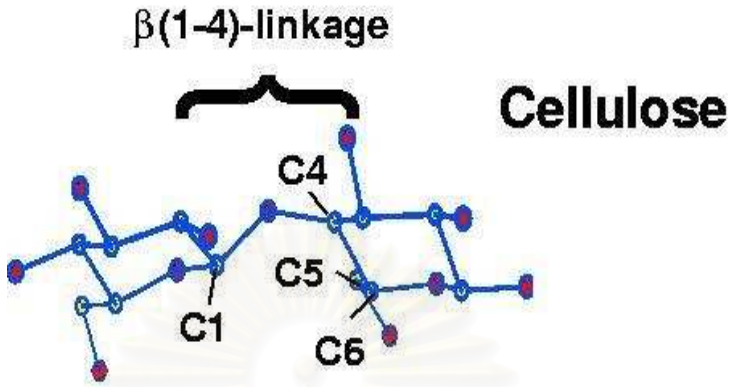


Figure 2.3 Location of carbon atom in cellulose molecules

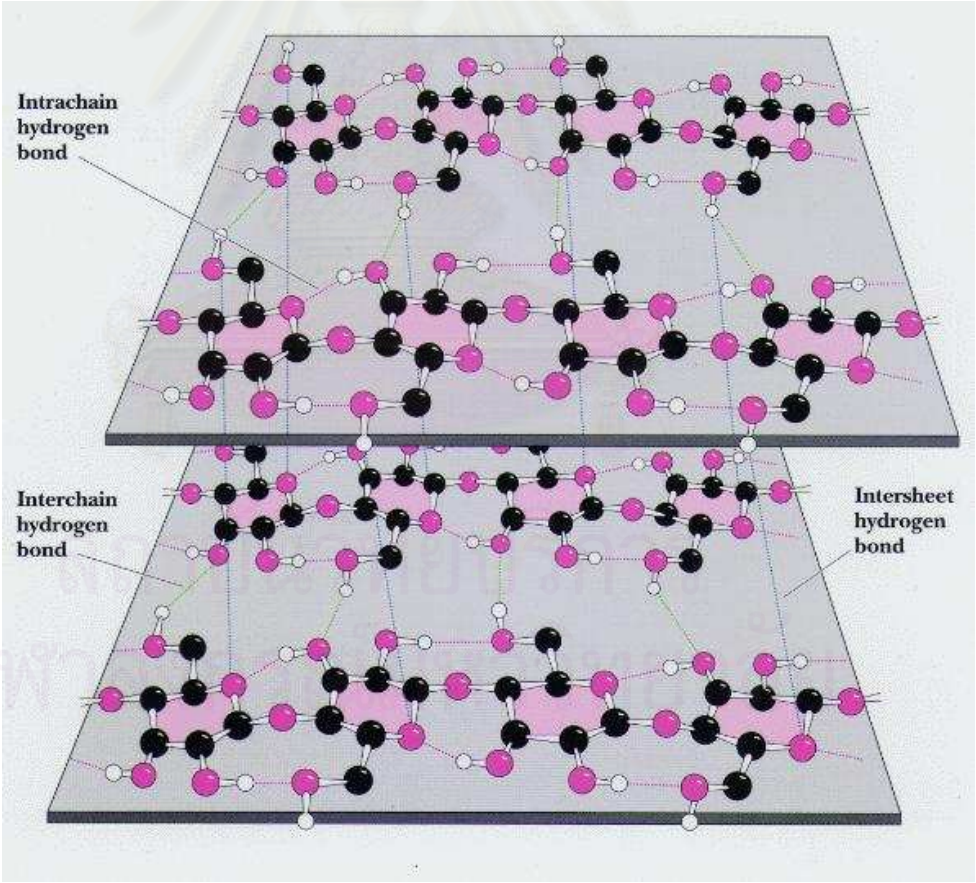


Figure 2.4 Interchain and intersheet hydrogen bond of cellulose (http://nd.edu/~aseriann/cellulose.html)

2.2 Bacterial cellulose

Cellulose produced by plants is the main source of this biopolymer but it is accompanied by many copolymers of biogenic origin, e.g. lignins, hemicelluloses, proteins. These impurities make the production of pure cellulose costly. Microorganisms can be produced cellulose as well as plants. Bacterial cellulose (BC) has the same basic molecular structure as plants.

Several different techniques for bacterial cellulose production have been reported. Some of which demonstrate a potential tool for economic and commercial BC production: stationary culture, agitated culture, cultivation in the horizontal fermentors or cultivation in the internal-loop airlift reactors. The choice of cultivation technique is strictly dependent on further biopolymer commercial destination. In the stationary culture conditions a thick, gelatinous membrane of bacterial cellulose is accumulated on the surface of a culture medium, whereas under agitated culture conditions cellulose can be produced in the forms of a fibrous suspension, irregular masses, pellets or spheres. While stationary culture has been quite widely investigated and applied for production of some successful commercial cellulose products (*Nata de Coco*, transducer diaphragms, wound care dressing material, etc.), agitated culture is still considered as a cultivation technique which is more suitable for the commercial production of BC mainly due to the higher production rates which potentially can be achieved.

Figure 2.5 (left) Cultivation of bacterial cellulose (right) Processed bacterial cellulose (Evans *et al*., 2003)

Swelled bacterial cellulose (ex. *Acetobacter xylinum*), in its never-dried state with much smaller fibrils (~1%) than from plants, exhibits pseudoplastic viscosity like gels but this viscosity is not lost at high temperatures and low shear rates as the cellulose can retain its structure. Bacterial cells can be removed by hot alkali and the clean wet cellulose used as a substrate for immobilizing biomolecules or for covering wounds. On drying, bacterial cellulose irreversibly looses their hydrated properties and tend to become like those of plant cellulose.

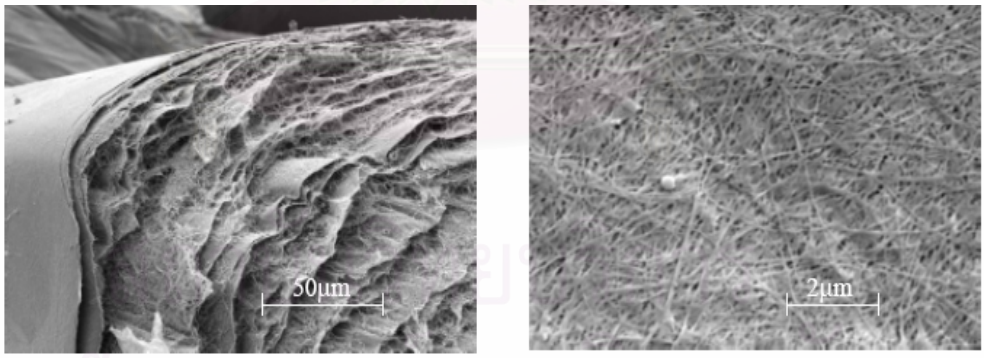


Figure 2.6 SEM micrographs of freeze-dried bacterial cellulose

Based on the its main properties, e.g. high purity, high water absorption capacity, high mechanical strength or molding capabilities during the biosynthesis, there is an increasing interest on the development for a large number of applications.

Bacterial cellulose displays unique physical, chemical and mechanical properties including high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility.

According to the unpurified-structure of plant cellulose, plant cellulose must endure a many stage pulping process to remove lignin, hemicellulose and other compounds. This procedure is costly for the delignification step which is necessary for the paper manufacturing. Besides, this process requires energy and chemicals that are often harmful to the environment.

Bacterial cellulose has an ultra-fine and highly pure fiber network structure. It is extremely hydrophilic, absorbing 60-700 times of its weight in water. Plant-derived cellulose, wood or cotton, must be physically disintegrated to make them hydrophilic (Brown, 1991). Bacterial cellulose will retain its long fibrils and exceptional strength because it is formed in a hydrophilic matrix and needs no treatment.

Cellulose is a linear β-(1,4)-D-glucan polymer synthesized by plants or bacteria. Bacterial cellulose is chemically identical to plant-derived cellulose, but both macromolecular structure and properties dissimilar from plant-derived cellulose. The subfibrils of bacterial cellulose are crystallized into microfibrils (Jonas and Farah, 1998), these into bundles, and the latter into ribbons (Yamanaka *et al.*,2000).

The diameter of bacterial cellulose fibril is about 1/100 smaller than of that of plant cellulose as shown in figure 2.7 and Young's modulus of bacterial cellulose is approximately 4 times greater than any organic fiber (Yamanaka *et al.*,2000). Compared with cellulose from plants, bacterial cellulose possesses higher water holding capacity, higher crytallinity, higher tensile strength, and a finer web-like network. Bacteria produce bacterial cellulose in a few days so it is expected to be a new biomaterial and the replacement for cellulose from plants.

Figure 2.7 (left) Bacterial cellulose (×20000) and (right) Plant cellulose (×200) fiber

2.3 Alginate

Alginates are naturally occurring polysaccharides that have been finding increasing applications in the biotechnology field. They belong to a family of linear copolymers of (1-4)-linked β-D mannuronic acid (M) and α-L-guluronic acid (G) residues, derived primarily from brown seaweed and also from bacteria. Within the alginate polymer, the M and G monomers are sequentially assembled in either repeating (MM or GG) or alternating (MG) blocks. The amount and distribution of each monomer depends on the species, section and age of seaweed from which the alginate is isolated.

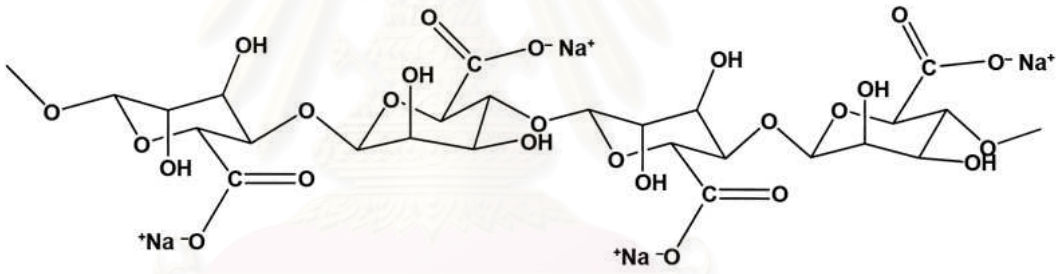


Figure 2.8 Chemical structure of alginate
(http://www.aapspharmscitech.org/view.asp?art=pt0803072)

Since alginic acid is insoluble, to convert to a water-soluble form, alginic acid is transformed into commercial alginate through the incorporation of different salts. The most widely used alginic acid salt is sodium alginate (Na-alginate) which promptly dissolved in cold or hot water.

The primary function of alginate in tissue engineering application is to provide mechanical integrity, while simultaneously transmitting initial mechanical signals to the cells and developing tissue. Among the various fibrous and hydrogel products, alginate-based products are currently the most popular ones used in wound management, since they offer many advantages over traditional cotton and gauzes They are biocompatible and form a gel on absorption of wound exudates. This eliminates fibre entrapment in the wound which is a major cause of patient discomfort during dressing removal. Such gelation prevents the wound surface from drying out, which is beneficial since a moist wound environment promotes healing and leads to a better cosmetic repair of wound (Winter, 1962). Performance requirements for such gelled dressings (which often aim to replicate the inherent permeability/water content of natural skin) are obviously higher than mere absorbent coverings in order to remain moist for the wound during the contact period (which is could be more than several days) (Thomas, 1990).

Sodium alginate and most other alginates from monovalent metals are soluble in water, forming solutions of considerable viscosity. Due to their suitable rheological properties, alginates have been used in the pharmaceutical industry as thickening or gelling agents, as colloidal stabilizers and as blood expanders (Smidsrod , 1996).

Sodium alginate forms relatively stable hydrogels through ionotropic gelation in the presence of many multivalent ions, being Ca^{2+} the most widely used. The crosslinking process can be carried out under very mild conditions, at low temperature

and in the absence of organic solvents. Several therapeutic agents, including antibiotics, enzymes, growth factors and DNA, have already been successfully incorporated in alginate gels, retaining a high percentage of biological activity. Moreover, alginate hydrogels have been widely studied for cartilage and bone regeneration applications as scaffolds for cells (Alsberg *et al.*, 2001)

2.3 Applications of bacterial cellulose

Bacterial cellulose can be altered to suite many potential commercial applications due to its physical properties such as hydrophilicity, tensile strength and high purity. Many researches on the production of bacterial cellulose have been studied in many years but applications of the material are mostly current. This is because it could be change in mentality towards the value of biodegradable material.

One of the first commercially available products of bacterial cellulose is Nata de coco, a food product of the Philippines. It was only in the last ten years that other possible commercial application of bacterial cellulose was widely investigated.

The specific application of bacterial cellulose as a dialysis membrane was examined by Shibazaki *et al.* (1993). Bacterial cellulose film showed a significantly higher permeation rate and a greater molecular weight cut-off when compared to a commercial dialysis membrane (regenerated cellulose membrane). Also, The additional benefit of the bacterial cellulose film compared to the regenerated cellulose membrane was that the added mechanical property allowed the use of a thinner material.

It is well known that the potential and current applications of bacterial cellulose are used in many fields. The first listed application is acoustic speaker diaphragms. Sony Corporation worked with Ajinomoto and Japanese Textile Research Institute to develop the first audio speaker diaphragms using bacterial cellulose (Yamanaka *et al.*, 1994). The unique characteristic of bacterial cellulose paper is used to create a sound

transducing membrane which is about the best material available to meet the strict requirements for optimal sound reproduction. In the field of paper, It is found that adding disintegrated bacterial cellulose to paper pulp was able to create a strong paper.

In the filed of paper, it is well known that bacterial cellulose has several advantages over synthetic paper (Brown, 1989):

a) unique nanostructure
b) complete purity
c) higher dimensional stability
d) greater mechanical strength
e) greater capacity to hold water

It is synthesized in a wet state from culture and is never dried during synthesis. In its dry state, bacterial cellulose has extraordinary absorptivity, which leads to many uses in fields such as wound care and fuel cell membranes (Evan *et al*, 2003), where it can be manipulated to absorb external materials to impart functionalities.

In a series of papers published in by Okiyama *et al.* (1992) dealt with applications of bacterial cellulose in the food industry. It has been known that in its never dried state, bacterial cellulose is too tough to bite. When treated with ethanol and calcium chloride, it becomes softer and has a texture similar to fruit and other foods. Applications of bacterial cellulose as food products are such as low-calorie desserts, salads and fabricated foods. Also, this material is used as a food additive for a chocolate drink in place of xanthan gum (Okiyama *et al.*, 1993). Besides it can be used as a food thickener and coater.

The specific application of bacterial cellulose as a dialysis membrane was examine by Shibazaki *et al*. (1993). Bacterial cellulose film showed a significantly higher permeation rate and a greater molecular weight cut-off when compared to a commercial dialysis membrane (regenerated cellulose membrane). Also, the additional benefit of the bacterial cellulose film compared to the regenerated cellulose

membrane was that the added mechanical property allowed the used of the thinner material.

Yoshino and coworkers (1990) were prepared a graphite film by pyrolysis of bacterial cellulose. It has been concluded that the membranes made from bacterial cellulose were stable 6 to7 times longer than those that were made from wood. Also, bacterial cellulose has also been used as a substrate for mammalian cell cultures (Watanabe *et al*., 1993).

Industry	Application
Cosmetics	Stabilizer of emulsion such as creams, tonics, nail conditioners an polishes, artificial nail component
Textile	Artificial skin and textiles, highly adsorptive materials
Tourism and sports	Sport clothes, tents and camping equipment
Mining and refinery	Split oils collecting sponges, materials for toxins adsorption
Waste treatment	Recycling of minerals and oils
Sewage purification	Municipal sewage purification, water ultrafiltration
Broadcasting	Sensitive diaphragms for microphones and stereo headphones
Forestry	Artificial wood replacer, multi-layer plywood, heavy duty containers
Paper Industry	Specialty papers, archival documents repairing, more durable banknotes, diapers, napkins
Machine Industry	Car bodies, aeroplane parts, sealing of cracks in rocket casings
Food production	Edible cellulose
Medicine	Temporary artificial skin for therapy of burns and ulcers, component of dental implants
Laboratory	Immobilization of proteins, cells; chromatographic techniques; Medium for tissue cultures

Table2.1 Bacterial Cellulose applications
(www.biotechnologypl.com/science/krystynowicz.htm)

2.5 Applications of alginate

Alginate absorbs water quickly, which makes it useful as additive in dehydrated products such as slimming aids and in the manufacture of paper and textiles. It is also used for fireproofing fabric, for thickening drinks, ice cream and cosmetics and as a detoxifier that can absorb poisonous metals from the blood.

The most alginate applications are utilized in the food industry. Dressing, sauces and beverages are important propylene glycol alginate applications. For sodium alginate, the application include cheese sauces, fruit fillings, instant flans and mousses, icings and glazes and restructured foods such as onion rings and meats. The important and useful property of alginates in the food industry is the ability to form eatable gels by cross linking with calcium salts. Alginate gel is of interest since alginate is able to use for viscosity tuning. The gel can be formed without any heating and the gel network will remain through freeze and thaw cycles. Its gel is thermally stable and therefore continues to provide functionality even when to food is heated. It is also effective at both highly acidic and neutral pH levels and it is not thermoreversible as gelatin.

One of interesting properties of soluble alginate solution found useful in the food industry is film formation. Alginate film is prepared by drying a thin layer of soluble alginate solution or by treating a soluble film with di- or trivalent metal (Glicksman, 1985). Calcium has found to be the most favorable as the divalent ion for gel formation because its salts are cheap, readily available and non-toxic. Calcium ions are reported to be more effective than magnesium, alumina, manganese, ferrous and a ferric ions, in bridging alginate chains together ionic interactions followed by interchain hydrogen (Gannadios, 1997). Stronger alginate coatings will be obtained from calcium chloride rather than calcium gluconate, nitrate and propionate. Besides, there are the applications of the alginate film in membrane separation process such as pervaporation. Alginate is almost widely used as thickener for the reactive dyes used for cellulose fibers. The great advantages of alginate are that it does not react with the dye or the fiber, so it results in clear colors and soft fabrics.

Owing to alginates biocompatibility and simple gelation with divalent cations, it is utilized for medical applications, such as encapsulation of cells. One example of this application is the use of alginate for the encapsulation of insulin-producing. Cells for better treatment of patients suffering from diabetes (Gimmestad *et al.,* 2003). Alginate is used extensively as a mold-making material in dentistry and prosthetics.

Besides, calcium alginate is also used in burn dressings to promote healing and can be remove painlessly.

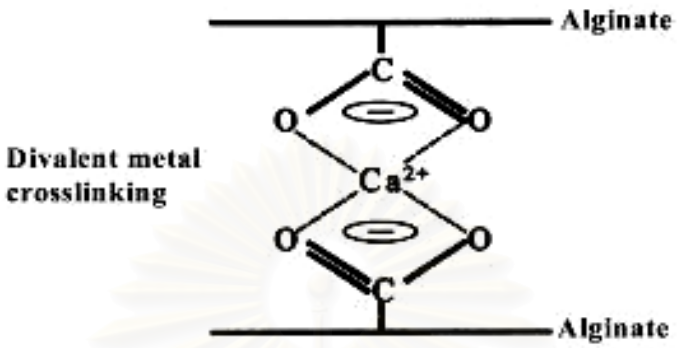


Figure 2.9 Alginate gel network formation by crosslinking with Ca^{2+} ion (Yang *et al.*, 2000)

2.6 Skin

Anatomically and functionally, skin has two layers. The superficial epidermal layer provides a barrier against infection and moisture loss. The deeper dermal layer is responsible for the elasticity and mechanical integrity of the skin and contains the blood vessels that are responsible for the nutrition of the epidermal layer. Appendages such as hair follicles or sweat glands breach the epidermal and dermal layers. Cutaneous sensory nerves pass through the dermal tissue into the epidermal tissue. Regeneration of the epidermis relies on residues of epidermal cells that lie deep within dermal structures.

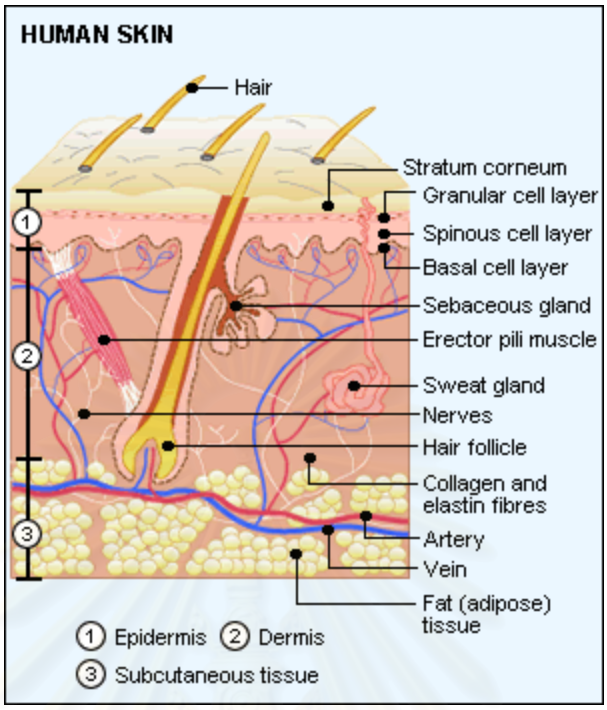


Figure 2.10 Layers & structures of the skin (Sherratta and Dallon , 2002**)**

2.6.1 Function of skin

2.6.1.1 Function of epidermis

The epidermis protects the organism against dehydration and acts as a physical barrier against invasion by microorganisms. It also protects against diverse insults, including those of mechanical, thermal, chemical, and ultraviolet origin.

2.6.1.2 Function of dermis

The dermis supports the epidermis in two vital ways. First, it provides a tough base that can repeatedly absorb substantial mechanical forces of various types, including shear, tensile, and compressive forces. Second, it incorporates a rich vascular system that is required for the metabolic support of the avascular epidermis.

The blood supply of the dermis becomes intimately available to the epidermis at the papillary layer. In addition, the dermis is the largest sensory organ in the body and contains receptors for touch, pressure, pain, and temperature. It also thermoregulates the body by the presence of hair and subcutaneous fat. Heat loss is facilitated by the evaporation of sweat from the skin surface and by an increase in blood flow through the rich vascular network of the dermis.

2.6.2 Regeneration of skin

Epidermis, the regenerative tissue, is spontaneously synthesized by keratinocytes. In contrast, the dermis does not spontaneously regenerate. This can be observed in the response to a severe injury, such as full-thickness wound. The resulting wound closes spontaneously by contraction of edges and synthesis of epithelialized scar. The epidermis of scar is thinner and there are few undulations in basement membrane. In the subepidermal region of scar, collagen fibers with their axes oriented in a relatively random array, are absent. The connective tissue layer of scar or dermal scar is largely avascular, rarely has nerve endings, and the collagen fibers are packed tightly with their axes oriented largely in the plane of the epidermis. The characteristics of non-preferable scar tissue can be concluded as follows.

a) Fully crosslinked, having only 70% of the tensile strength of the tissue it replaces.
b) It is not fully functional and is undifferentiated.
c) Scar tissue is aesthetically disfiguring.

2.7 Wounds

2.7.1 Types of burn

Burns are classified by severity as first, second or third-degree categories.

2.7.1.1 First-degree burns, the results of mild to moderate sunburn, only affect the epidermis as shown in Figure 2.11. They can be healed rapidly, and generally do not require medical attention.

Figure 2.11 First-degree burn (www.burnsurgery.org/BurnWound/part_iv.htm)

2.7.1.2 Second-degree burns, the results of direct contact with flames, cause damage to both the epidermis and the dermis that can be dangerous to health as shown in Figure 2.12.

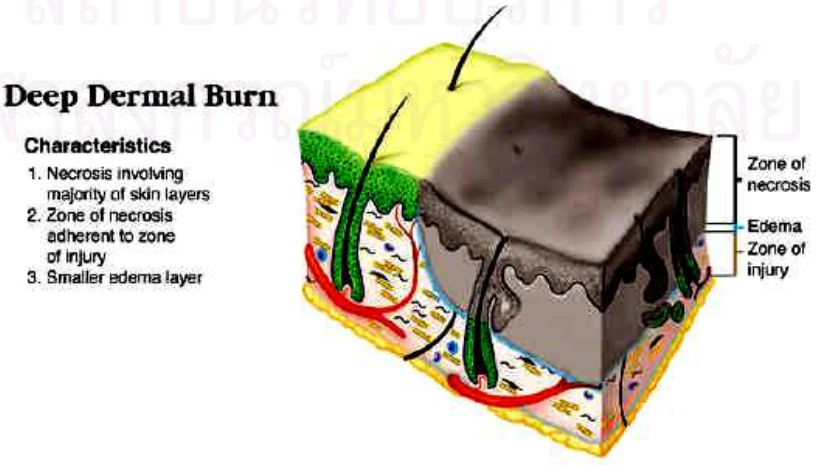


Figure 2.12 Second-degree burn (www.burnsurgery.org/BurnWound/part_iv.htm)

2.7.1.3 Third-degree, or Full thickness burns, the results of flames or explosion with very high temperature, are burns for which the skin has been damaged or destroyed to its full depth, and may also involve damage to underlying tissues such as fat, muscle, and connective tissues as shown in Figure 2.13.

Figure 2.13 Third-degree burn (www.burnsurgery.org/BurnWound/part_iv.htm)

2.8 The role of skin substitute

Wound closure requires a material to restore the epidermal barrier function and become incorporated into the healing wound, whereas materials used for wound cover rely on the ingrowth of granulation tissue for adhesion. Materials for wound cover are most suited to superficial burns, where they create an improved environment for epidermal regeneration by providing a barrier against infection and controlling water loss.

The increasing of severe burn patients, who have insufficient sources of autologous skin for grafting, brings a high cost for hospitalizations. In addition, there are many disadvantages of some traditional treatments. These treatments bring with them a viral infection. Furthermore, they are very expensive and time-consuming.

Therefore, the synthesis of skin substitute from natural and synthetic materials a novel method, has been developed instead of the traditional treatments.

What is required from the skin substitute will be different according the nature of the wounds treated. Perhaps the general functions of individual substitutes can be identified as following.

Protection

Many substitutes can be used to provided an impermeable barrier at the wound surface. They have a benefit in interesting fluid loss and providing a barrier to wound colonization. Generally, an impermeable dressing may not be wholly desirable because the difficult management of exudates is then encultured. Impermeable membranes are often designed to have a measurable rate of vapor transmission although they will restrict access to the wound by microorganisms. If pooling of exudates occurs and accesses from the edge of dressing, these dressing may cause problems in the management of subsequent wound colonization and infection. Skin substitute must be taken to protect the wound bed until the wound can be finally closed.

Promotion

Skin substitute should have the ability to provide a suitable environment to promote biological activities involved in the replacement of lost tissue and the salvage of damaged tissue. Substitutes may supply matrix components, cell growth and growth factors to promote the rate of wound healing.

Provision

Provision of new elements should be incorporated into the healed wound. A skin substitute should supply an organized structure that forms the basis of a stable wound. Cells may be seeded to accelerate the process of tissue replacement or repair. Materials supplied may be designed to persist in the healed wound.

2.9 Extracellular matrix scaffold as a skin substitute

2.9.1 Scaffold: Skin regeneration template

A scaffold is made to mimic the structure and composition of the natural ECM in dermis, which is mainly composed of type I collagen and glycosaminoglycan (GAG). The first skin regeneration template is called INTEGRA by Yannas.

Figure 2.14 Integra artificial skin (www.integra-ls.com/products/?product=201)

The synthetic scaffold was designed to approximate the supporting layer of protein and carbohydrate normally secreted by dermal cells. Unexpectedly, Burke and Yannas found that when the synthetic scaffold was applied to a wound site, dermal

cells around the wound site migrate into to the artificial matrix and attach to the collagen fibers. The bovine collagen is slowly degraded and replaced with authentic human collagen which synthesized by the dermal cells. Blood vessels grow into the wound to vascularize the new tissue. After the dermal layer has had a chance to repair itself, the outer membrane can be removed and replaced with very thin epidermal transplant that providing a natural moisture seal.

To have a collagen matrix that will attract dermal cells to colonize it, the matrix must have a specific surface which involves the density of attachment sites for cells. The rate at which the matrix degrades is also very important. The matrix must persist while the inflammation rages on, but ultimately it must be degraded so that it can be replaced by authentic human collagen.

For a severe burn, the protein matrix itself is severely damaged, and the original cells in the wound have died. The burn site itself is temporarily occupied with immune system cells, like macrophages and lymphocytes, which keep infection from spreading. There are no appropriate cells left to replace the matrix correctly, and there is no matrix left to organize the tissue. Skin regeneration template solves the problem by providing a synthetic matrix, or scaffold, on which new tissue can arrange itself. Integra claims to have excellent results at healing burns, even in older patients, whose skin is already thin and brittle with age. Integra is now conducting clinical trials to expand its use to cosmetic plastic surgery to treat scarring caused by previous wounds or burns. This is a larger market than the original indication.

2.9.2 Characteristics of extracellular matrix scaffold

Growing cells in three-dimensional scaffold has been of great interest. In this approach, scaffold plays an important role. It guides cells to grow, synthesizes

extracellular matrix and other biological molecules, and facilitates the formation of functional tissues.

Ideally a scaffold should possess the following characteristics to bring about the desired biologic responses (Buckley and O'Kelly, 2004):

a) Three-dimensional and highly porous with an interconnected pore network for cell/tissue growth and flow transport of nutrients and metabolic waste.

b) Biodegradable or bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*

c) Suitable surface chemistry for cell attachment, proliferation and differentiation

d) Mechanical properties to match those of tissues at the site of implantation

e) Easily processed to form a variety of shapes and sizes

2.10 Biomaterials for scaffold fabrication

The three main material types which have been successfully used in developing scaffolds include:

a) Natural polymers, such as collagen, gelatin, glycosaminoglycan, cellulose, alginate and chitosan.

b) Synthetic polymers, based on polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers (PLGA).

c) Ceramics, such as hydroxyapatite(HA) and β-tricalcium phosphate (β-TCP).

Synthetic materials can be easily processed into various structures and can be produced cheaply and reproducibly. Moreover, it is possible to tightly control

various properties such as the mechanical strength, hydrophobicity, and degradation rate. Natural biomaterials must be isolated from plant, animal, or human tissue. They are typically expensive and suffer from large batch-to-batch variations. Furthermore natural materials sometimes exhibit a limited range of physical properties and can be difficult to isolate and process. Although they have some limitations, there are some important advantages of using natural materials, for example, they do have specific biological activity and generally do not elicit unfavorable host tissue response. Some synthetic polymers, in contrast, can elicit a long-term inflammatory response from the host tissue. Ceramics have also been widely used, due to their high biocompatibility and resemblance to the natural inorganic component of bone and teeth. Ceramics are inherently brittle so that their applicability in soft tissue engineering is limited. Beside of the materials, fabrication techniques are also another factor that presents many challenges in obtaining specific physical and biological properties of the scaffolds. Many choices of fabrication techniques lead to the desired scaffold properties.

2.11 Scaffold fabrication techniques

Several techniques have been developed to fabricate scaffolds. These include solvent casting and particulate leaching, gas foaming, fiber meshes/fiber bonding, phase separation, melt molding, emulsion freeze drying, and freeze drying as shown in Table 2.2.

Process	Advantages	Disadvantages
Solvent casting and particulate leaching	Large range of pore sizes	Limited membrane thickness (3mm)
	Independent control of porosity and pore size	Limited interconnectivity
	Crystallinity can be tailored	Residual porogens
	Highly porous structures	Poor control over internal architecture
Fibre bonding	High porosity	Limited range of polymers
		Residual solvents
		Lack of mechanical strength
Phase separation	Highly porous structures	Poor control over internal architecture
	Permits incorporation of bioactive agents	Limited range of pore sizes
Melt moulding	Independent control of porosity and pore size	High temperature required for nonamorphous polymer
	Macro shape control	Residual porogens
Membrane Lamination	Macro shape control	Lack of mechanical strength
	Independent control of porosity and pore size	Limited interconnectivity
Polymer/ceramic fibre composite foam	Independent control of porosity and pore size	Problems with residual solvent
	Superior compressive strength	Residual porogens
High-pressure processing	No organic solvents	Nonporous external surface
		Closed-pore structure
Freeze drying	Highly porous structures	Limited to small pore sizes
	High pore interconnectivity	
Hydrocarbon templating	No thickness limitation	Residual solvents
	Independent control of porosity and pore size	Residual porogens

Table 2.2 Conventional scaffold processing techniques for tissue engineering (Buckley and O'Kelly, 2004)

2.11.1 Procedure and Theory (Snowman, 1998)

Freeze drying or lyophilization is an ideal method of preserving products, and active ingredients such as proteins, enzymes, microorganisms, chemical and natural products. Freeze drying is the only way that water can be successfully removed from an organic substance or material without damaging the cell structure and losing of volatile components.

Freeze drying has been used in a number of applications for many years, most commonly in food and pharmaceutical industries. There are also some other uses for the processes of whole animal specimens for museum display, restoration of books and other items damaged by water and recovery of reaction products. Freeze drying involves the removal of water or other solvent from a frozen sample by a process called sublimation. Sublimation occurs when a frozen liquid goes directly to gas phase without passing through liquid phase. In contrast, drying at ambient temperatures from the liquid phase usually results in changes in the product and may be suitable only for some materials. The scaffolds used in this research were fabricated using a freeze drying (lyophilization) process where a suspension of bacterial cellulose/alginate coprecipitating between the growing ice crystals. The process produces a continuous, interpenetrating network of ice and the coprecipitate. Sublimation of ice crystals forms the highly porous scaffold structure. The pore volume fraction and the size of the pores can be controlled by the volume fraction of the precipitate in the suspension and by the underlying freezing processes with typical pore volume fractions above 99%.

The freeze drying process consists of three stages involving prefreezing, primary drying and secondary drying.

2.11.1.1 Pre-Freezing

Since freeze drying is a state change from solid to gas phase, material to be freeze dried must first be adequately frozen. The method of prefreezing and the final temperature of the frozen sample have a direct effect on freeze dried material. Rapid cooling rate results in small ice crystals, useful in preserving structures to be examined microscopically, but more difficult to freeze dry. Slower cooling rate results in larger ice crystals and lesser restrictive channels in the matrix during the drying process. A low final freezing temperature can increase the rate of cooling as well as the rate of ice crystal nucleation and decreases the rate of heat and protein diffusion, leading to small ice crystals. Samples that are subjected to freeze drying consist primarily of water or solvent, materials dissolved or suspended in water or solvent. Most samples used to be freeze dried are eutectics which are a mixture of substances that freeze at lower temperature than surrounding water. When the aqueous suspension is cooled, changes occur in the solute concentrations of the matrix. As cooling proceeds, water is separated from solutes as it changes to ice, creating more concentrated areas of solute. These pockets of concentrated materials have a lower freezing temperature than water. Although a product may be frozen because of all the ice presented, it is not completely frozen until all of the solute in the suspension is frozen. It is very important in freeze drying to freeze the sample to below the eutectic temperature before beginning the freeze drying process. Small pockets of unfrozen material remaining in the sample expand and compromise the structural stability of the freeze dried sample.

2.11.1.2 Primary drying

Several factors can affect the ability to freeze dry a frozen suspension. While these factors can be discussed independently, it must be remembered that they interact in a dynamic system, and it is delicate balance between these factors that results in a properly freeze dried sample. After prefreezing the product, conditions

must be established in which ice can be removed from the frozen sample via sublimation, resulting in a dry, structurally intact sample. This requires very careful control of two parameters, temperature and pressure. The rate of sublimation of ice from a frozen sample depends upon a difference in the vapor pressure of the product compared to the vapor pressure area. Since vapor pressure is related to temperature, it is necessary that the sample temperature is warmer than the cold trap (ice collector) temperature. The balance between the temperature that maintains the frozen integrity and the temperature that maximizes the vapor pressure of the sample is extremely important, since it is the key to optimum drying. The typical phase diagram show in Figure 2.15 illustrates this point. Most samples are frozen well below their eutectic temperature, and then the temperature is raised to just below this critical temperature and they are subjected to a reduced pressure. At this point the freeze drying process is started.

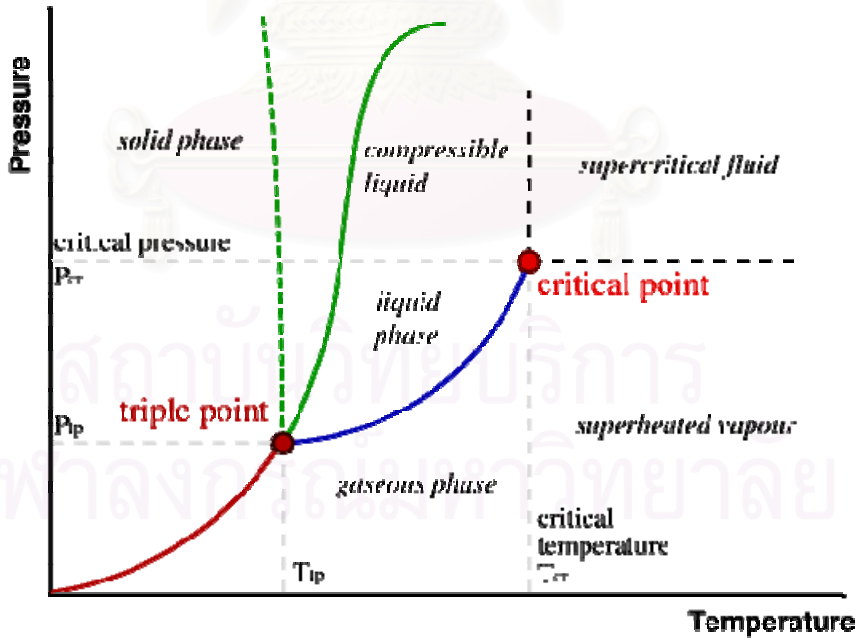


Figure 2.15 A typical phase diagram
(http://en.wikipedia.org/wiki/Phase_diagram)

Freeze drying conditions must be created to encourage the free flow of water molecules from the sample. Therefore, a vacuum part is essentially needed to lower the pressure of the environment (to point C). Another essential part is a collecting system, which is a cold trap used to collect moisture that leaves the frozen sample. The collector condenses all condensable gases, i.e. water, and the vacuum part removes all non-condensable gases. It is important to understand that the vapor pressure of the sample forces the sublimation of the water vapor molecules from the frozen sample matrix to the collector. The molecules have a natural affinity to move toward the collector because its vapor pressure is lower than that of the sample. Therefore, the collector temperature (Point D) must be significantly lower than the sample temperature. In addition, raising the sample temperature has more effect on the vapor pressure than lowering the collector temperature.

The last essential part in a freeze drying system is energy. Energy is supplied in form of heat. Energy required to sublime a gram of water from the frozen to gas phase is almost 10 times greater than that required to freeze a gram of water. Therefore, with all other conditions being adequate, heat must be applied to the sample to encourage the removal of water in the from of vapor from the frozen sample. The heat must be very carefully controlled, since applying more heat than the evaporative cooling in the system can warm the sample above its eutectic temperature. Heat can be applied by several methods. One is to apply heat directly through a thermal conductor shelf such as in tray drying. Another method is to used ambient heat as in manifold drying.

2.11.1.3 Secondary drying

After primary freeze drying is completed and all ice has sublimed, bound moisture is still presented in the sample. The residual moisture content may be as high as 7 -8%. Continued drying is necessary at a higher temperature to reduce the residual moisture content to optimum values. This process is called isothermal desorption as the bound water is desorbed from the sample.

Secondary drying is normally operated at a sample temperature higher than ambient but compatible with the sensitivity of the sample. All other conditions, such as pressure and collector temperature, remain the same. Secondary drying is usually carried out for approximately 1/3 to 1/2 the time required for primary drying.

The key components of a lyophilizer and each component's function are along with the five significant stages of processing. Figure 2.16 summarizes the procedures of lyophilization.

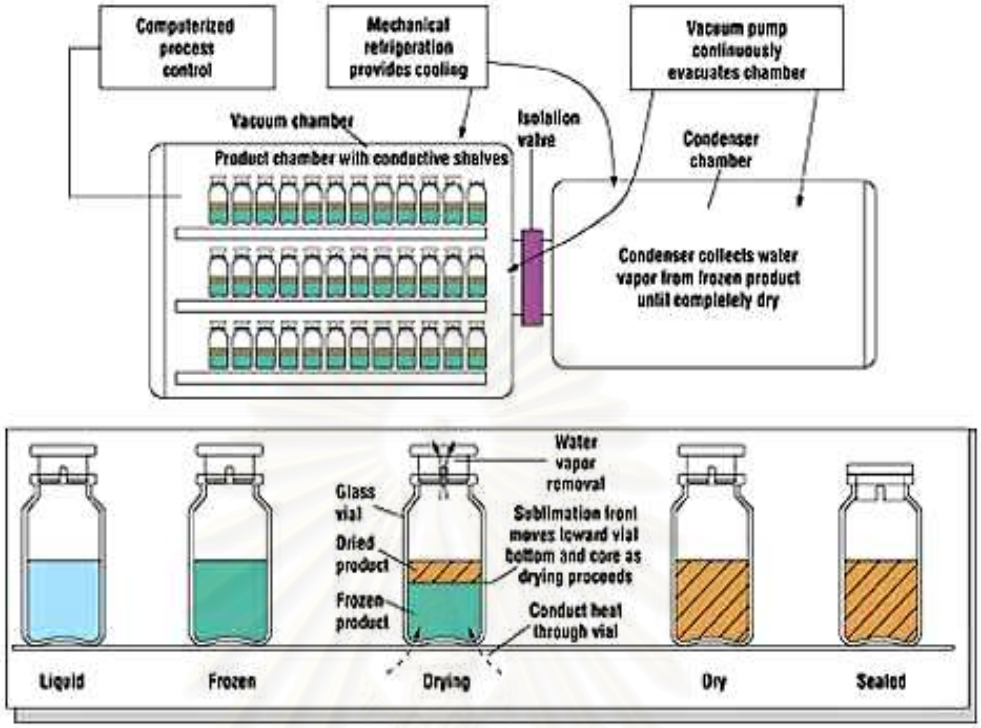


Figure 2.16 Steps of lyophilization (Timothy, 2002)

2.12 Fourier transform infrared infrared (FT-IR) spectroscopy (Stuart, 2002)

Infrared spectroscopy is a widely used technique providing the specific information of molecular level. When infrared interacts with a matter, it can be absorbed and caused the vibrations of chemical bonds consisted in the material. There are two kinds of fundamental vibrations for a molecular: stretching and bending. Stretching is the movement along the bond axis (i.e., symmetric and a symmetric types). In the contrary, bending vibrations arise from changes in bond angle between two atoms and movement of an atomic group. It relates to the remainder of the molecule. As a result, molecule adsorbs a certain energy that is equivalent to raise the vibrational energy and additional exchange its electronic dipole moment. Absorption peaks can be noticed at the wavenumber of IR absorbed. Various stretching and bending vibrations occur at specific frequencies. Bending variations normally require less energy and occur at longer wavelength than those with stretching vibrations. Vibrational frequencies are not only affected by the structures of specific atomic groups, but also shifted by either greater or lesser degree by steric hindrance effects by other parts of molecules. Therefore, different molecules represent different vibration patterns due to different compositions in molecular structure.

Since molecular vibrations readily reflect chemical feature of molecule, such as a rearrangement of nuclei and chemical bonds within the molecule, infrared spectroscopy contributes considerably to not only molecular identification but also studies of the molecular structure. As a consequence, infrared spectroscopy has become a useful technique to determine molecular interactions. The infrared spectrum can be divided into three categories, i.e. the far-infrared (<400 cm^{-1}), the mid-infrared (400-4000 cm^{-1}), and the near-infrared (4000-13000 cm^{-1}) regions. Most infrared applications employ the mid-infrared region since it contains fundamental mode of vibration of molecules. It is used to establish the identity of a compound. However, the near- and far-infrared regions can also provide specific information

about materials. The near-infrared regions consist mostly of overtone or combination bands of fundamental modes appearing in the mid-infrared region. The far-infrared region can provide such information regarding lattice vibrations.

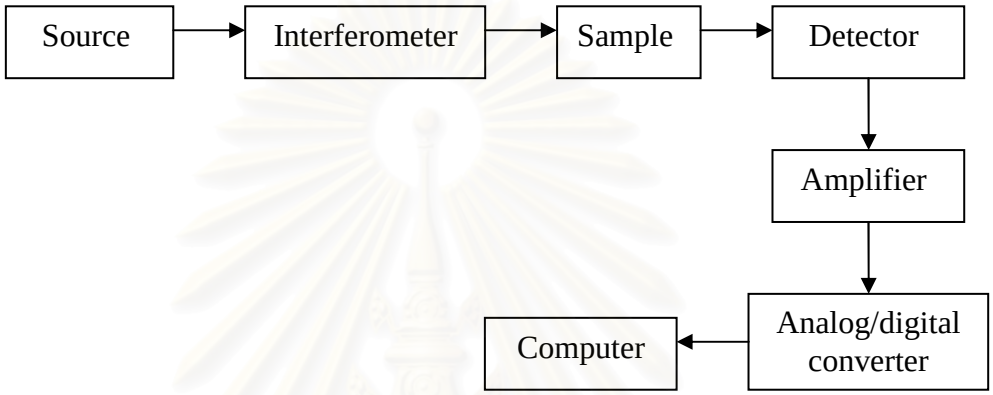


Figure 2.17 Schematic of a typical FT-IR converter spectrometer

FT-IR spectrometer is based on the interference of two beams, producing an interferogram. Time and frequency domains are interconvertible by the Fourier transform method. The basic components of FT-IR spectrometer are shown schematically in Figure 2.17. The radiation generated by the IR source passes through an interferometer. Beam splitter separates an incident beam into two paths and then recombines them. The intensity variations of the exit beam can be measured by a detector as a function of travel path differences. The signal produced as a function of changes between the two beams. The exit beam is absorbed by a sample before reaching a detector. Upon the signal amplification, in which high-frequency contributions have been eliminated by a filter, the data are converted into a digital format by an analog-to-digital converter. Then they are transferred to the computer for Fourier transformation.

The finally FT-IR spectrum is obtained from the ratio of a single beam of the sample against that of the reference.

2.13 *in vitro* Cell culture

Until animal cell culture emerged as a valuable research tool, experimental approaches to the study of cellular physiology were limited to either studies in the whole animal or short term studies with isolated cells or slices from various tissues. The development of cell culture led to a new experimental approach to cellular physiology. Functionally differentiated cells could be maintained in culture under conditions that allowed direct manipulations of the environment and measurement of the resulting changes in the function of a single cell type. Today, many aspects of research and development involve the use of animal cell as *in vitro* model system, substrates for virus replication, and in the production of diagnostic and therapeutic products.

2.13.1 Primary cell cultures (Davey, 2003)

The *in vitro* cultivation of animal tissues was first shown to be possible in 1907 and the first human cell line was obtained in 1952. However, it is only in the last 20-25 years that reproducible and reliable large scale culture of mammalian cells has been achieved. The process of initiating a culture from cells, tissues or organs taken directly from an animal and before the first subculture in know as primary culture. Primary culture is the source of all cells in the culture. There are a number of advantages and applications associated with primary culture:

► To provide tissue for surgery, e.g. primary culture of skin samples in skin grafting and plastic surgery.

► To optimize drug treatment, especially anti-cancer drug treatments, using samples from each individual patient.

► To produce cells that have the potential to become cell lines. This allows animals to be replaced as a model and improves the reproducibility of experiments.

2.13.2 MTT cell proliferation assay (Mosmann, 1983)

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared I media or salt solutions lacking phenol red. Dissoloved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzyme from mitochondria. The yellow tetrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by mebolically active cells (see Figure 2.18), in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formanzan can be solubilized using isopropanol or other solvents such as dimethyl sulfoxide (DMSO) and the dissolved material is measured spectrophotochemically yielding absorbance as a function of concentration of converted dye.

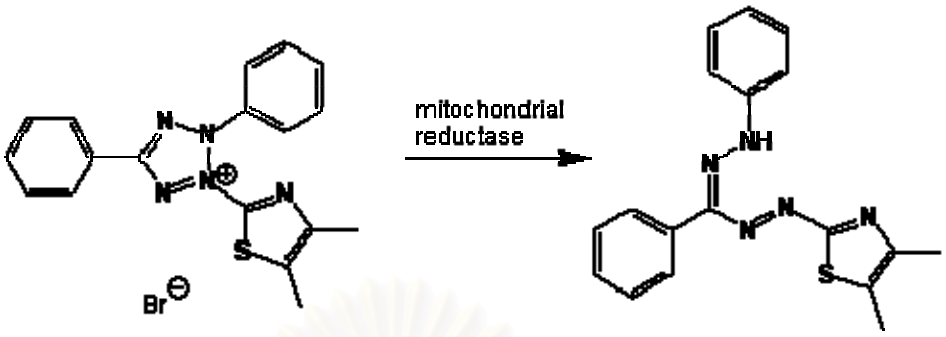


Figure 2.18 Reduction reaction of MTT (Mosmann, 1983)

The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop as assay system alternative to the conventional H-thymidine uptake and other assays for measurement of cell proliferation. Active mitochondril dehydrogenases of living cells will cause this conversion. Dead cells do not cause this change. This has been mainly applied to measure the cytotoxicity and the number of cells. The MTT cell proliferation assay is also used to measure the cell proliferation rate as well as cell viability. The number of assay steps has been minimized as much as possible to expetite sample processing. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

Procedures

1) Make a solution of 5 mg/ml MTT dissolved in DMEM without phenol red.

2) After 5 hours of the cell incubation, remove media and then wash with PBS.

3) Add MTT solution from step one to each well containing cells.

4) Incubate the plate in a CO_2 incubator at 37ºC for 30 min.

5) Remove MTT solution.

6) Add DMSO to each well and pipette up and down to dissolve crystals.

7) Transfer to the cuvettes and measure the absorbance at 570nm.

2.14 cellulolysis

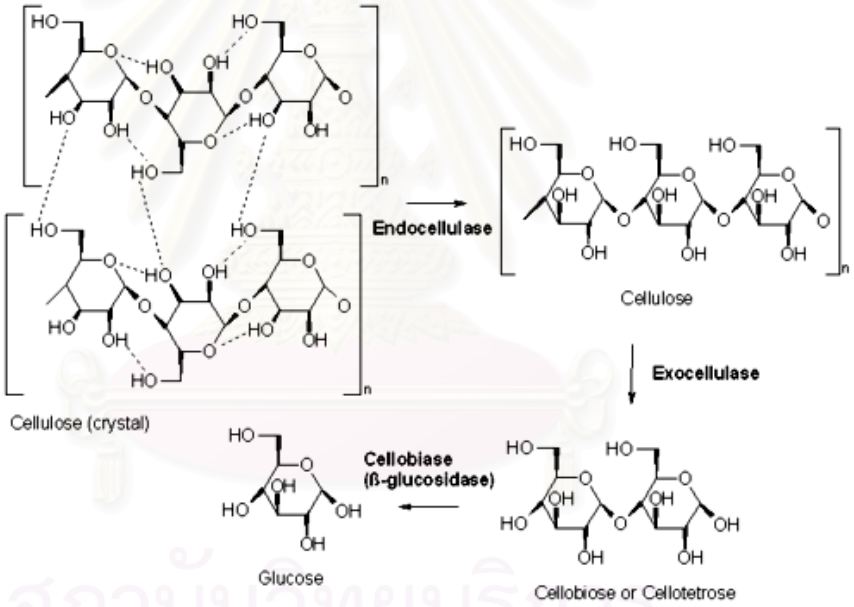


Figure 2.19 The three types of reaction catalyzed by cellulases (Chapin, *et al*.,2002)

The term cellulose refers to a group of enzymes that act synergistically to hydrolyze cellulose. Cellulases perform a specific catalytic activity on the 1,4-β-glucosidic bonds of the cellulose molecule. The hydrolysis of this bond cleaves the molecule into smaller parts that may be further reduced. Commercial cellulases, which are usually produced by submerged fermentation of *Trichoderma reesi*, are multi-component enzyme systems typically containing one or more exo-cellulase activities known as exo-cellobiohydrolases, multiple endo-glucanases, and beta-glucosidases. Exo-cellulases act on cellulose polymer chain ends and produce primarily cellobiose. Endo-cellulases act randomly along the cellulose polymer chains breaking very long polymers into shorter chains. Beta-glucosidases act on short, soluble oligosaccharides to produce primarily glucose (Karmakar, 1998)

The complementary activities of the different enzymes is thought to be responsible for synergistic effects, whereby the enzymatic activity of a mixture of tow or several enzymes is substantially higher than the sum of the activities of the individual enzyme.

Synergism between the different components in the cellulose system has been documented, but detailed explanation of their mechanism and kinetics is not completely understood. The most widely proposed mechanism of hydrolysis of cellulose can be conveniently divided into the following stages (Lee and Fan, 1982).

a) Transfer of enzyme molecules from the aqueous phase to the surface of cellulose molecules

b) Adsorption of the enzyme molecules onto the surface of cellulose resulting in the formation of an enzyme-substrate (E-S) complex

c) Transfer of molecules of water to the active sites of the E-S complex

d) Surface reaction between water and cellulose catalyzed by the E-S complex

e) Transfer of the products of the reaction of cellobiose and glucose to the aqueous phase

Since cellulase is normally absent in mammals, biocompatibility could be studied to support the use of this enzyme in *in vivo* applications. In 2004, Emilia and coworkers studied the cellulase compatibility with cardiac cells. Cardiac myocytes could resist to the action of enzyme and showed no apparent differences from control samples in cytoskeletal structure and macroscopic organization. Further studies are needed to confirm *in vivo* application of cellulose and its long-term effect on the cell growth.

CHAPTER III

LITERATURE REVIEWS

3.1 Skin substitutes and wound healing

In 2002, Jones and coworkers reviewed the function of normal skin and a large number of new approaches and products that are emerging in the quest to develop biologically based skin substitutes for clinical use. Additionally, examination of the range of different products that are available and the current evidence for their effectiveness and their relative costs were shown. Integra, the most widely accepted synthetic skin substitute to be developed for use in burns by Yannas, was described in details. The pore size of Integra has been designed at 70-200 μm in order to allow migration of the patient's own endothelial cells and fibroblasts. Smaller pores delayed biointegration, whereas larger pores provided an insufficient attachment area for invading host cells.

There were disadvantages to the use of this product. It was relatively expensive when compared with cadaveric allograft skin from skin banks, and the learning curve was reported to be steep, with high failure rates initially. The advantages were that it provided improved elasticity and cosmesis compared with an ultra-thin allograft, with reduced donor-site morbidity compared with a standard-thickness allograft, as it healed faster with less scarring. It avoided the risks of cross infection inherent with allografts. Integra had an important role in providing immediate wound cover following early excision in patients with insufficient autograft.

In 2005, Dorothy and coworkers reviewed the structure and function of human skin and the available skin substitutes. There were several skin substitutes that had been useful for replacement or reconstruction of one or both layers of the skin, facilitating wound healing in several different clinical settings. These skin substitutes acted as temporary wound covers or permanent skin replacements, depending on their design and composition. The most basic of the skin substitutes were synthetic, acellular materials designed to act primarily as barriers to fluid loss and microbial contamination. One example that had been widely used for coverage of excised burn wounds was Integra. Integra Dermal Regeneration Template had proved to be particularly valuable in patients with large burns and limited autograft donor sites. Integra consisted of two layers. The dermal layer served as a matrix for infiltration by fibroblasts and other cells from the wound bed. As the collagen matrix was populated by these cells, it was gradually degraded and replaced by newly synthesized collagen. The silicone layer provided a functional barrier that was removed upon vascularization of the dermis, to be replaced by a thin layer of autograft. Other examples, such as Biobrane, Alloderm, Dermagraft etc., were also mentioned in this work.

3.2 Scaffold fabrication

A number of methods and materials have been continuously developed for preparing porous materials for cell scaffolding. In 1999, Kang and coworkers studied the use of freeze-drying technique to produce highly porous scaffold from biodegradable gelatin. The micro structure of the gelatin scaffold could be modified by changing the freezing rate. Furthermore, it was concluded that the present freeze drying procedure was a bio-clean method for formulating biodegradable sponges with different pore structures without use of any additives and organic solvents. This method is applicable to any hydrogel, irrespective of the type of polymers and copolymers.

In 2002, Salgado and coworkers has studied a new scaffold based on a blend of starch with cellulose acetate (SCA) processed by a novel extrusion technique. The scaffolds present a range of properties that make them adequate to be used in tissue engineering. Cells remain viable after 3 weeks in culture, showing that the scaffolds have the adequate porosity. This starch-based scaffold processed by extrusion in combination with blowing agent might be very useful in tissue engineering.

3.3 Properties of scaffolds

There are studies on the production and efficiency of bacterial cellulose as a temporary skin substitute called Biofill. This artificial skin for burn and skin injuries treatment shows dramatic clinical results (Fontana *et al*., 1990). Positive indications are immediate pain relief, diminished post-surgery discomfort, faster healing, reduced infection rate and reduced treatment time and cost. These products of bacterial cellulose that now had wide applications in surgery and dental implants. Besides, the bacterial cellulose can also be applied for temporary substitute for animal skin (Jonas and Farah, 1998) and artificial blood vessels for the microsurgery (Klemm, 2001).

In 2002, Hart and coworkers described a new wound treatment comprising oxidized regenerated cellulose and collagen (ORC/collagen). This new wound healing was found to promote fibroblast migration and proliferation in vitro and accelerate wound repair in the diabetic mouse. The results also indicated that ORC/collagen may have the ability to limit proteolytic and free-radical damage and promote tissue formation when introduced into chronic wound environment. This work supports the further evaluation and future used of ORC/collagen in the clinical setting.

In order to develop effective sponges for biomedical application, it is essential to consider their properties. In 2002, Lai and coworkers studied sponges composed of sodium alginate and chitosan prepared via freeze drying process. This study has indicated that the mechanical, drug release and morphological properties of alginate/chitosan sponges are highly dependent to composition. It was noted that the sponges had a flexible yet strong texture. The mixed system had more randomly ordered network than the single component sponges. The resistance compression was greatest for the chitosan alone and was markedly lower for the mixed systems and alginate alone. The dissolution of a model drug (paracetamol) studies showed that systems containing chitosan alone showed the slowest release profile, with the mix systems showing a relatively rapid dissolution profile. Therefore, it is possible to manipulate both of mechanic and drug release properties of the sponges by altering the polysaccharide composition.

In 2004, Wu and coworkers studied polysaccharide-based membrane of chitosan and cellulose blends, prepared by using trifluoroacetic acid as a co-solvent. The studies of water vapor transpiration through the chitosan/cellulose membranes and the antimicrobial capability against *E. coli* and *S. aureus* indicates that the membranes any be suitable to be used as a wound dressing because they may prevent wound from dehydration and infection.

The bacterial cellulose secreted by *Acetobacter xylinum* was explored as a novel scaffold material due to is unusual material properties and degradability. The results of the study of Svesson *et al.(*2004) indicated that the unmodified BC supports chondrocyte proliferation at levels of approximately 50% of the native tissue substrate, collagen type II. However, compared to tissue culture plastic and alginate, unmodified BC showed significantly highter levels of chondrocyte growth at smilar levels of *in vitro* immune system.

Skin is permeable to vapor and protects the deeper layer tissue against mechanical injuries and infection. In order to heal effectively, a wound must be maintained in a wet condition. In 2004, Danuta and coworkers have modified bacterial cellulose with chitosan during its biosynthesis results in a composite material with glucosamine and N-acetylglucosamine units incorporated into the cellulose chain, which is characterized by a number of valuable features: good mechanical properties in wet state, high moisture. Keeping properties, release mono-and oligosaccharides under lysozyme action, bacteriostatic activity against Gram (-) and Gram (+) bacterial and bactericidal activity against Gram (+) bacteria. These features make modified bacterial cellulose an excellent dressing material for treating different kinds of wounds and burns.

In 2004, the development of a biodegradable porous scaffold made from naturally derived chitosan and alginate polymer has been studied by Li and coworkers. Significantly improved mechanical strength is structurally stable due to the strong ionic bonding between the amine groups of chitosan and the carboxyl groups of alginate. The biological properties of scaffold were evaluated by seeding with osteoblast cells. The cell-material interaction study indicated that cells seeded on the chitosan alginate scaffold appeared to attach and proliferate well. The *in vivo* study showed that the hybrid scaffold had a high degree of tissue compatibility. The scaffold also provided a high porosity. These encouraging results support the

potential uses of chitosan-alginate scaffold as alternative natural polymer-based scaffolds for clinical trials.

In 2006, Wan and coworkers proposed a new class of biomaterial with high mechanical performance and good osteo conductivity and biodegradation for tissue engineering and orthopaedic surgery. Biomimic hydroxyapatite HAp/bacterial cellulose (BC) nanocomposites were synthesized via biological route by soaking both bacterial cellulose and phosphorelated bacterial cellulose in simulated body fluid (SBF). The preliminary results suggested that HAp/BC nonocomposites are promising for applications in tissue engineering. The further investigation of the mechanical and biological properties is on the way.

CHAPTER IV

EXPERIMENTAL WORK

The experimental work can be divided into three main parts:

1. Materials and chemicals
2. Equipments
3. Experimental procedures

All experiments procedures are summarized in Figure 4.1

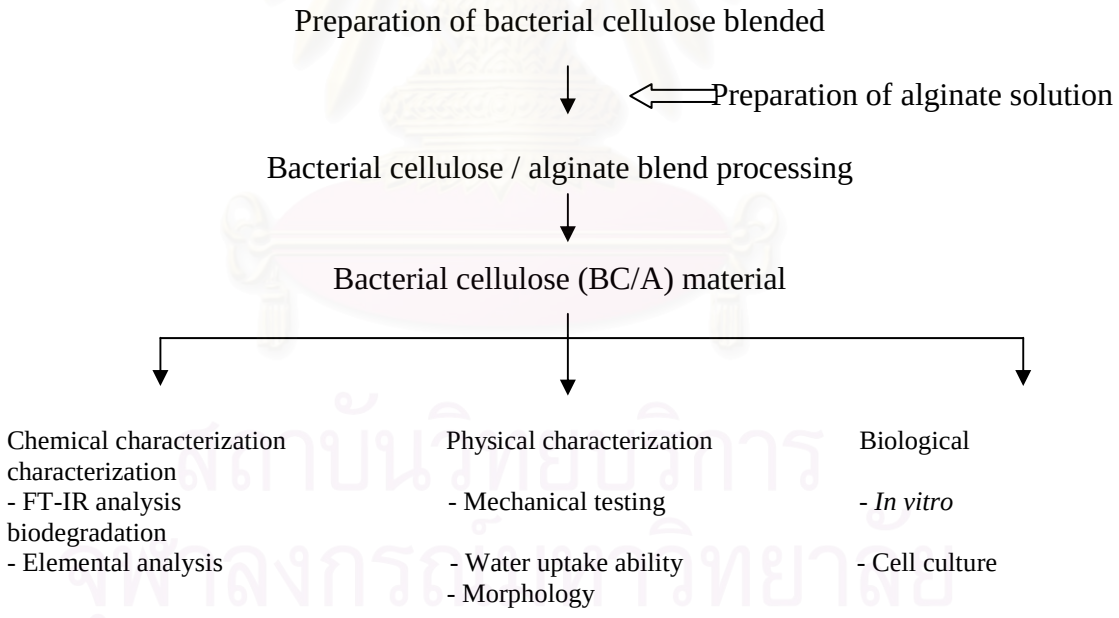


Figure 4.1 The schematic diagram of experimental procedures

4.1 Materials and Chemicals

1. Bacterial cellulose

Bacterial cellulose used in this work provided by the Institute of Research and Development of Food Products, Kasertsart University. Bacterial cellulose was purified by treatment of 10 wt% (w/v) of NaOH solution at room temperature for deproteination for 24 hours and then was rinsed several times with distilled water for neutralization (pH=7).

2. Calcium chloride was supplied by Italmar (France).

3. Sodium alginate (Na-alginate) and Sodium hydroxide (NaOH) were supplied by Carlo Erba, Italy.

Please noted that, all of the chemicals used in this work were analytical grade, and were purchased from commercial sources in Thailand.

4.2 Equipments

1. Blender (Philips Cusina)
2. Fourier transform infrared (FT-IR) spectroscopy from Perkin from Perkin Elmer
3. Lyophilizer and -40°C freezer from Thermo Electron Corporatin (Heto PowerDry LL30000, USA)
4. Universal mechanical testing machine from Instron (Instron 5567, USA)
5. Scanning electron microscopy (SEM) from Joel (JSM 5400, Japan)
6. CHN-Elemental analyzer (LECO CHN 2000 Analyzer, USA)

Figure 4.2 Example of lyophilizer (Heto Powerdry LL3000)

4.3 Experimental Procedures

4.3.1 **Blended bacterial cellulose preparation**

The neutral bacterial cellulose was blended by blender at room temperature until the homogeneous mixture was formed.

4.3.2 Alginate solution preparation

To prepare 1.5% (w/v) of alginate solution, sodium alginate powder was dissolved in distilled water at room temperature. The alginate was vigorously stirred overnight to ensure homogeneous and uniform solution.

4.3.3 Preparation of bacterial cellulose/alginate blend sponge

The bacterial cellulose was mixed with alginate solution at different proportions to form bacterial cellulose/alginate (BC/A) mixture at the weight ratios of 100/0, 70/30, 50/50, 30/70, and 0/100. BC/A mixtures were stirred at room temperature for 24 hours. The mixtures were cast in Petri dish with 50g of mixture per plate before cross linking in a 1.5 wt% (w/v) $CaCl_2$ aqueous solution for 3 hours, and then rinsed with distilled water to remove the excess chlorides. After that, the cross linked mixture was frozen at -40°C refrigerator for 24 hours prior to freeze drying under vacumm pressure (<100 mTorr) at the condenser temperature of -40°C for 24 hours.

Figure 4.3 Bacterial Cellulose/alginate at various blending composition

4.4 Chemical Characterization

4.4.1 Fourier transform infrared (FT-IR) spectroscopic analysis

Blending mixture of bacterial cellulose and alginate was prepared as mention in section 4.3.3. To obtain the films of polymer blends for FT-IR analysis, the mixture was cast on Teflon plate. After solvent evaporation for 24 hours at room temperature, the films were further cross linked by 1.5 wt% (w/v) $CaCl_2$ aqueous solution

The information on structural contribution was collected in the FT-IR analysis using Perkin Elmer Spectrum GX model (FT-IR system). All spectra were recorded in the wave number range from 4000 to 400 cm^{-1} at the resolution of 4 cm^{-1}. The FT-IR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups presented in the samples.

4.4.2 Elemental analysis

Samples were cut into particle-like size and vaccum dried for 24 hours before the measurement of elemental analysis. The content of nitrogen in the samples were determined by elemental analyzer (LECO CHN-2000 Analyzer). The contents of calcium and sodium in the dried samples were determined with an X-ray Fluorescence (XRF) Spectrometer (OXFORD ED 2000).

4.5 Physical characterization

4.5.1 Morphology

The morphology of bacterial cellulose, bacterial cellulose/alginate and alginate sponges were analyzed by scanning electron microscope (SEM, Joel JSM5400) at an accelerating voltage of 12-15 kV. Dry samples were coated with gold) Ion (sputtering device, JFC 1100) at 40mA prior to observing under SEM.

4.5.2 Mechanical testing

A universal testing machine (INSTRON 5567, NY, USA) was used to determine tensile strength of the samples. The determination was done under the following conditions: sample width, 10mm; length, 10cm (50mm between the grips); stretch rate, 2mm/min. The reported values of each blend composition are the mean of 5 specimens.

4.5.3 Equilibrium water content

To measure the equilibrium water content of the sponge, pre-weighed dry sample was immersed in distilled water for 20s. After the bulk water was removed by placing the wet sponge on the Petri-dish for 1 min, the weight of wet sample was measured. Each experiment was repeated three times and the average value was taken as the water uptake ability. Then, the water uptake ability was determined according to the following equation

$$\text{Equilibrium water content} = \left(\frac{W_w - W_d}{W_d} \right)$$

where W_w and W_d represent the weight of wet and dry samples, respectively.

4.5.4 Moisture content determination

Moisture content (M_n) was measured by the oven drying method. The samples were preweighed and then heated at 50°C in oven. The samples were dried until they reached constant mass. All experiments were run in triplicate. The loss of mass is used to calculate the moisture content of samples. M_n was calculated using the following equation:

$$\text{Moisture content (\%)} = \left(\frac{W_w - W_d}{W_w} \right) \times 100$$

Where W_w and W_d represent the weight of wet and dry samples, respectively.

4.5.5 Shape retention test

The shape retention of the swollen samples was investigated by exposing them to different media: distilled water and PBS solution.

4.6 Biological characterization

4.6.1 *In vitro* biodegradation

The bacterial cellulose, bacterial cellulose/alginate and alginate sponges of know dry weights were sterilized by immersing in 70% ethanol for 5 minutes and were placed in a pH=7.4 PBS solution with 70 units cellulases. The samples were maintained at 37°C. Samples were stored for 21 days and then were removed from the medium, rinsed with distilled water, frozen, freeze dried and weighed. The remaining weights of the sponges were measured. The remaining weight (%) of the sponge was calculated by using the following equation:

$$\text{Weight remained (\%)} = \frac{W_o - W_t}{W_o} \times 100$$

Where W_o represents the initial weight of sponges and W_t represents the weight of digested sponges.

4.6.2 Cell culture (*In vitro* studies)

L929 cells, mouse connective tissue keratinocytes and gingival fibroblasts were selected to evaluate adhesion and proliferation as a direct contact test. Cells were cultured in 6 mm diameter culture dish (NUNC, Roskilde, Denmark) using growth medium composed of Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, 100 IU⁄ml penicillin, 100 µg⁄ml streptomycin, and 0.25 µg⁄ml amphotericin-B. They were incubated in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. At confluence, keratinocytes and gingival fibroblasts cells were harvested using a suspension of 0.25% trypsin and subcultivated in the same medium with 12 and 4 dilutions, respectively.

4.6.3 Cell adhesion and proliferation tests

The sponges (13 mm in diameter and 2 mm in thickness) were immersed in 70% ethanol for 5 mins for sterilization, followed with 4 times of solvent exchange by deionized water. The sponges were then placed on a 24-well polystyrene plate and 350 µl of culture medium was added to each well before cell seeding, 6×10^4 cells⁄well. The number of cells seeded onto sponges was similar in case of either L929 keratinocytes or gingival fibroblasts cells. Cells were allowed to initially attached or 5 h. For proliferation test, 2×10^4 cells were seeded onto each of the matrices and cultures were harvested after 5, 24 and 72 h. The similar procedure was performed in case of either L929 keratinocytes or gingival fibroblasts cells. The attached or proliferated cells were then quantified by the 3-(4,5-dimethythiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Three hundreds and fifty microliters

of MTT solution (0.5 mg⁄ml in DMEM without phenol red, filter-sterilized) were added to each culture well. After incubation for 5 h, The MTT reaction medium was removed, and 900 µl of dimethylsulfoxide and 100 µl of glycine buffer (0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) were added. Optical densities were determined by spectrophotometer (Genesis 10 UV scanning, NY USA) at the wavelength of 570 nm.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Chemical characterization of sponges

5.1.1 Fourier transform infrared (FT-IR) spectrophotometric analysis

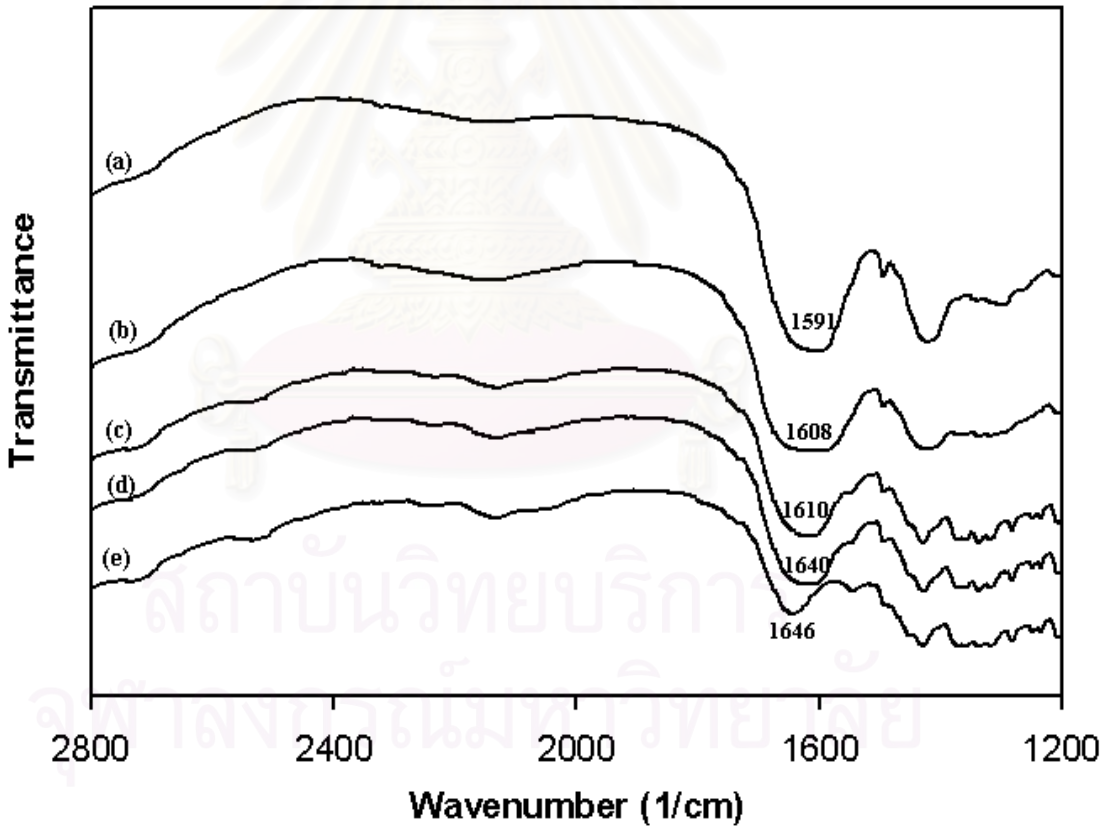


Figure 5.1 FTIR spectra of various blends between bacterial cellulose (BC) and alginate (A): (a) pure A, (b) BC/A; 30/70, (c) BC/A; 50/50, (d) BC/A; 70/30 and (e) pure BC

The FT-IR spectra of bacterial cellulose/alginate blends at various blending composition measured at wave number ranging from 2800-1200 cm^{-1}, shown in Figure 5.1. The presence of a peak at a specific wave number would indicate the presence of a specific chemical bond. The bacterial cellulose showed a band at 1646 cm^{-1}, which was attributed to glucose carbonyl of cellulose. In addition, the interaction between bacterial cellulose and alginate could be identified by carbonyl group bands and carboxyl group bands presented in the range of 1900-1500 cm^{-1}. The carboxyl group bans for blends sponges were shifted from 1591 cm^{-1} to 1608, 1610 and 1640 cm^{-1}, respectively, when compared to that of pure alginate sponge. The small shifts could be observed on carboxyl band between the results from bacterial cellulose/alginate blends which might be attributed to the specified intermolecular hydrogen bonds. The hydrogen bonds might lead to small variations of the rotation and vibration frequencies.

In conclusion, the results showed that the blends between bacterial cellulose and alginate had the same characteristic absorption bands without any occurrence of new peaks and disappearance of the original bands of their parent molecules. Therefore, these results suggested that both polymers could exert their characteristic individually. So that, we could get the beneficial aspects from the properties of both polymers.

5.1.2 Elemental analysis

Elemental analysis results analyzed by CHNS/O Analyzer revealed the absence of nitrogen and sulfur in the sponges which mean bacteria was completely removed from cellulose during washing.

The X-Ray Fluorescence Spectroscopy (XRF) results showed no Sodium (Na) Peak. Therefore NaOH were also totally removed from the developed sponges. The sponges compositions analyzed by XRF are summarized in Table 5.1.

Blending composition (Bacterial cellulose/alginate)	Elemental content (%) by XRF	
	Na	Ca
100/0	-	-
70/30	-	0.88
50/50	-	2.49
30/70	-	2.96
0/100	-	5.57

Table 5.1 The sponge elemental content analyzed by X-Ray fluorescence Spectroscopy (XRF)

5.2 Physical Characterization of sponges

5.2.1 Tensile strength

Tensile strength of dried bacterial cellulose/alginate sponges was shown in Figure 5.2. In the case of pure component, the tensile strength of pure bacterial cellulose was greater than those of pure alginate.

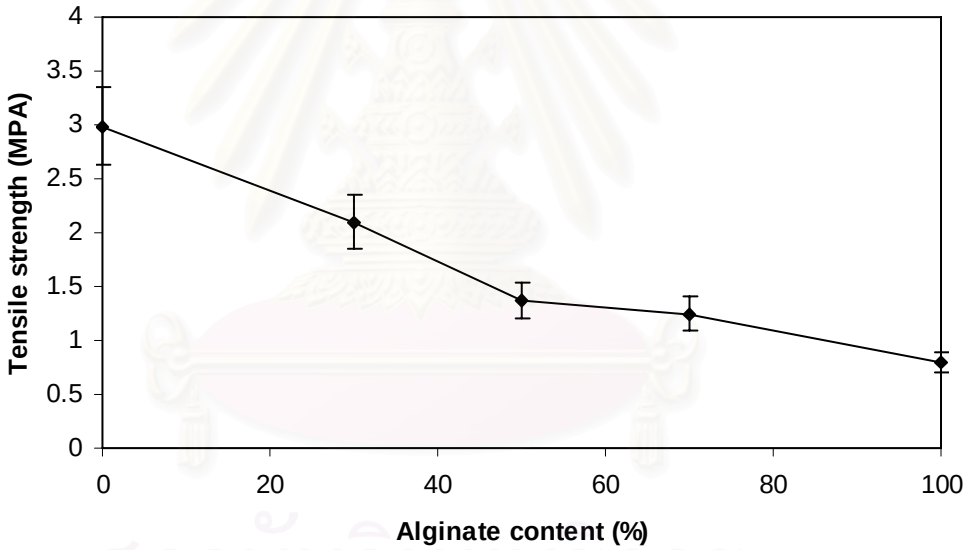


Figure 5.2 The tensile strength of the bacterial cellulose/alginate sponges as a function of alginate content

Considering bacterial cellulose/alginate sponges, the tensile strength decreased with an increase of alginate content.

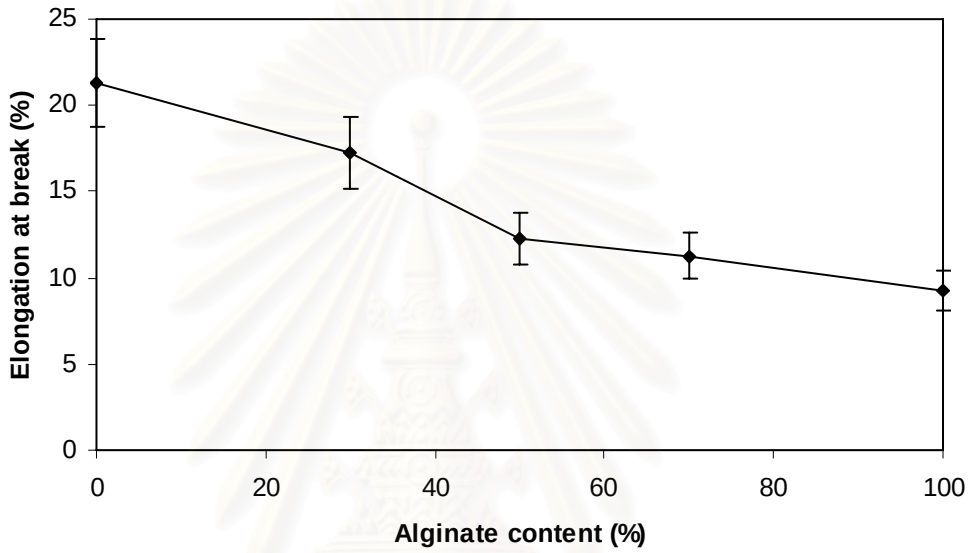


Figure 5.3 The elongation at break of the bacterial cellulose/alginate sponges as a function of alginate content

Similarly, the pure alginate sponges gave lower elongation at break as compared to the pure bacterial cellulose and the bacterial cellulose/alginate blends. This was similar to the work of Phisalaphong *et al.* (2007) reported on the mechanical properties of bacterial cellulose and alginate blend membrane. They showed that the presence of alginate decrease the mechanical properties of bacterial cellulose film. This may be attributed to the occurrence of some specific interaction between cellulose and alginate molecules. Wu and coworkers (2004) suggested that the intermolecular hydrogen bonding of cellulose was supposed to break down to form cellulose alginate hydrogen bonding. The new intermolecular interaction reduced the high crystallinity of bacterial cellulose.

5.2.2 Swelling properties

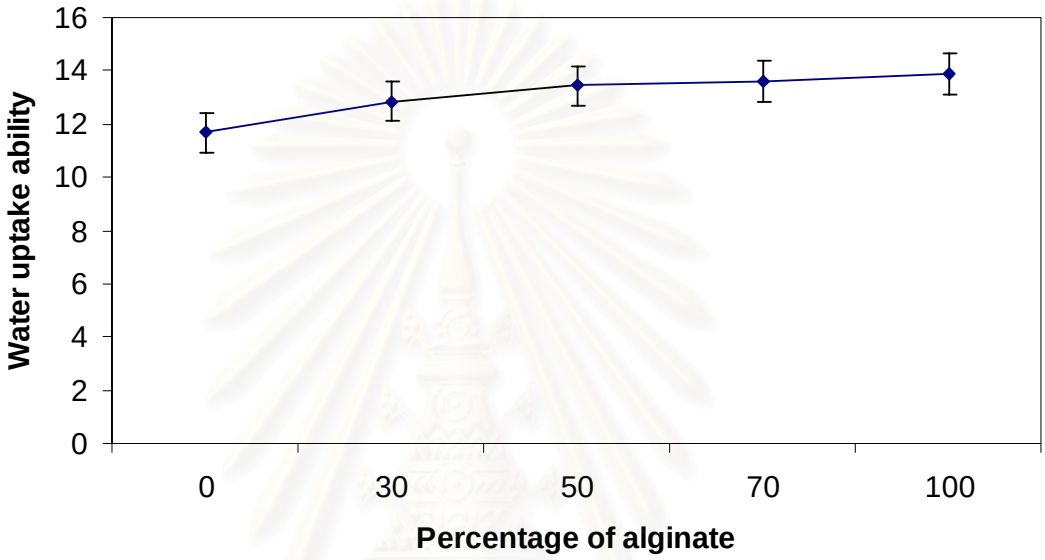


Figure 5.4 Swelling ability of bacterial cellulose/alginate sponges at various blending composition

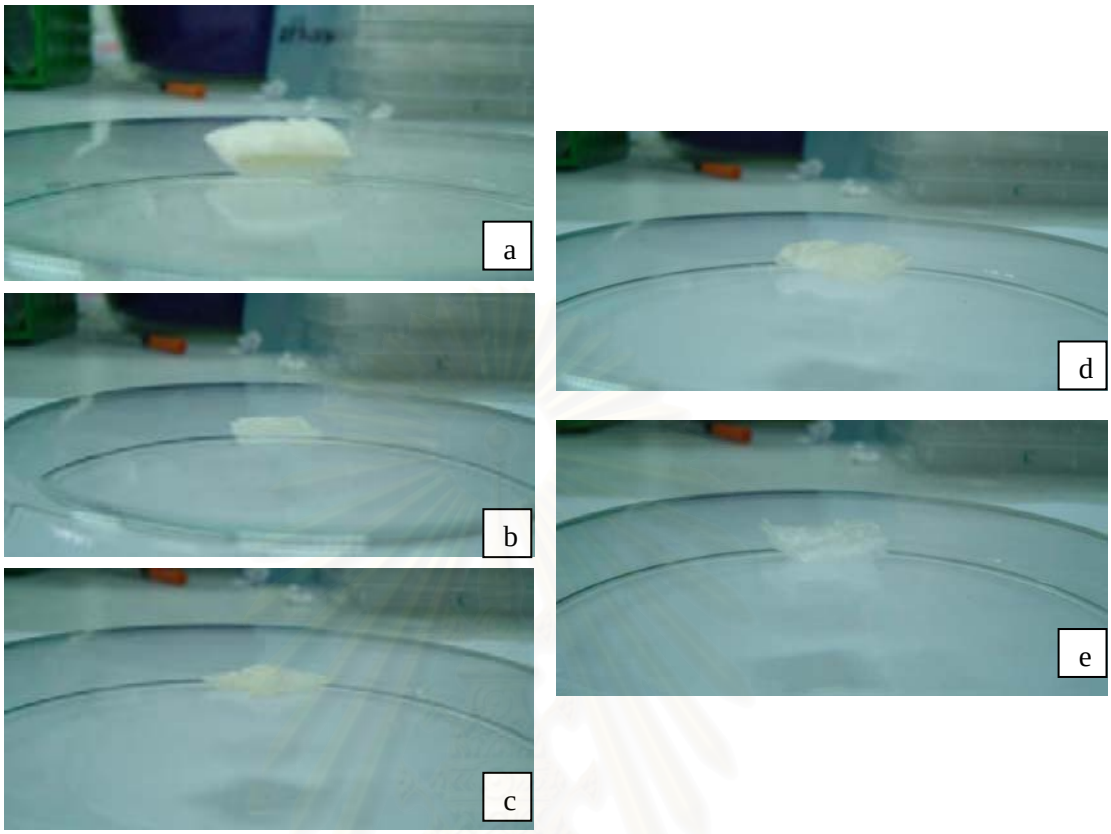


Figure 5.5 Freeze-dried sponges with variation blending composition between bacterial cellulose and alginate. (a) 100/0, (b) 70/30, (c) 50/50,(d) 30/70, (e) 0/100. (all samples were approximately 1x1x0.1 cm)

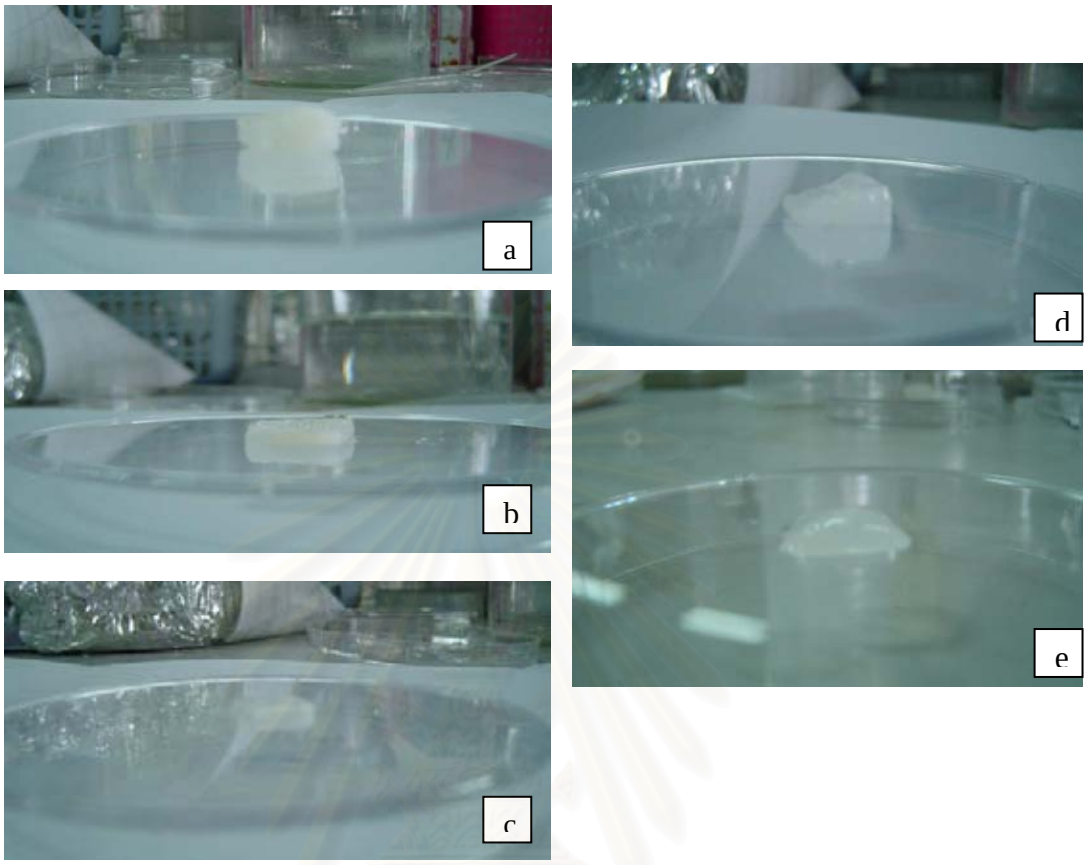


Figure 5.6 Hydrated sponges with variation blends between bacterial cellulose and alginate. (a) 100/0, (b) 70/30, (c) 50/50,(d) 30/70, (e) 0/100.

The water uptake ability is an important aspect to evaluate the material property for biomedical applications. As shown in Figure 5.5, bacterial cellulose showed good water uptake ability. Bacterial cellulose itself in wet state is extremely hydrophilic, absorbing 60-700 times of its weight in water. Being blended with alginate could reduce hydrogen bonding of cellulose chain and could result in increasing of water uptake ability of bacterial cellulose. In this study, the increasing of alginate content in sponges resulted in increasing of water uptake capacity. The water

molecules were easily adsorbed into the alginate due to its hydrophilic property (Lee, 1997).

As shown in Figure 5.6 and 5.7, the swelling behavior and structural stability of the sponges in a phosphate buffer salin (PBS), buffer solution, were also observed. The blends having alginate composition more than 30% showed good swelling properties but the sponges gradually disrupted and dissolved in the medium. This was because they lost their gel-like structure after swollen in PBS. Whereas, the bacterial cellulose/alginate sponges were stable. They retained their overall size through the period of study.

Jeanie *et* al. (2003) proposed that the dissolution of alginate hydrogel leads to weakening of the sponges. This might be the effect of the reduction in the degree of crosslinks between alginate and calcium ions. The reduction in crosslinking is induced by phosphate and sodium ions contained in the buffer solution. Since the crosslinked calcium ions can be exchanged with other non-gel-inducing cations such as sodium ions. In addition, phosphate sequesters the calcium ions cross-linked with alginate, consequently destabilizing the calcium-alginate gel.

The bacterial cellulose/alginate sponges with the blending composition of 100/0 and 70/30 were selected to test the morphology and further biological characterization because of their excellent swelling ability and mechanical strength. The good swelling ratios could facilitate the cells to grow during *in vitro* cell culture studies.

5.2.3 Moisture content

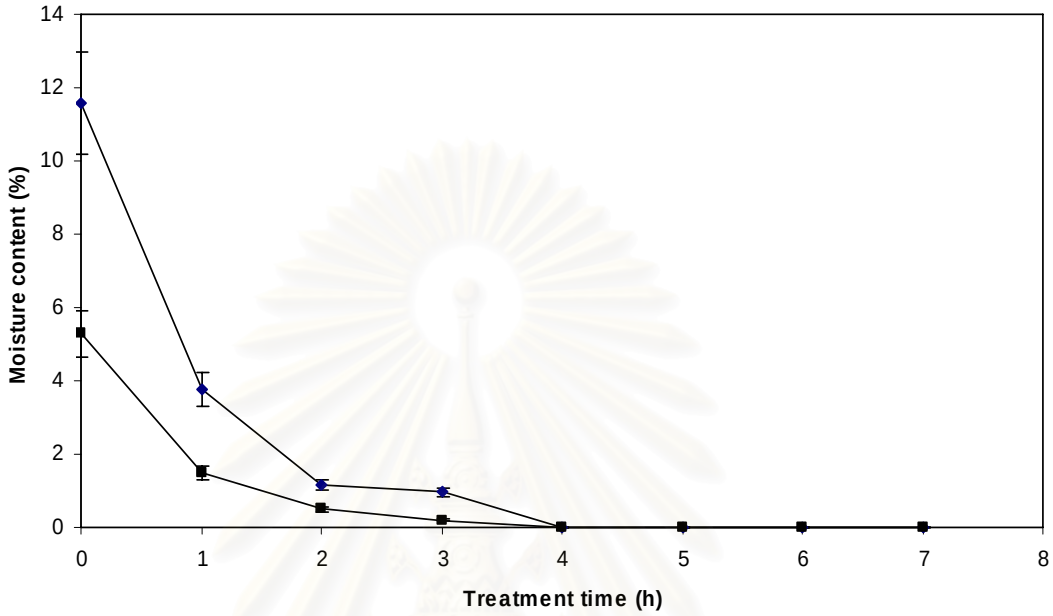


Figure 5.7 Moisture content of various blending compositions of sponges: (♦) 100/0 and (■) 70/30 (ratios of bacterial cellulose to alginate)

The moisture content influences the physical properties of a substance such as weight, density and many more. Oven-drying method was used to determine the moisture content (%) for each of the materials.

Samples were heated in oven at the temperature of 50°C. They were dried until they reached constant weight. Figure 5.7 represented the percentage of moisture content. The results showed that the moisture content of sponges was 5.29% and 11.57% for the sponges with 0% alginate and 30% alginate, respectively. The increasing of moisture content was related to by the increase of alginate proportion.

5.2.4 Shape retention test

The swelling behavior and structural stability of sponges plays an important role for their practical use in biomedical applications. The shape retention tests were determined by using both of distilled water and phosphate buffer saline (PBS). Figure 5.8, 5.9, 5.10 showed optical images of sponges

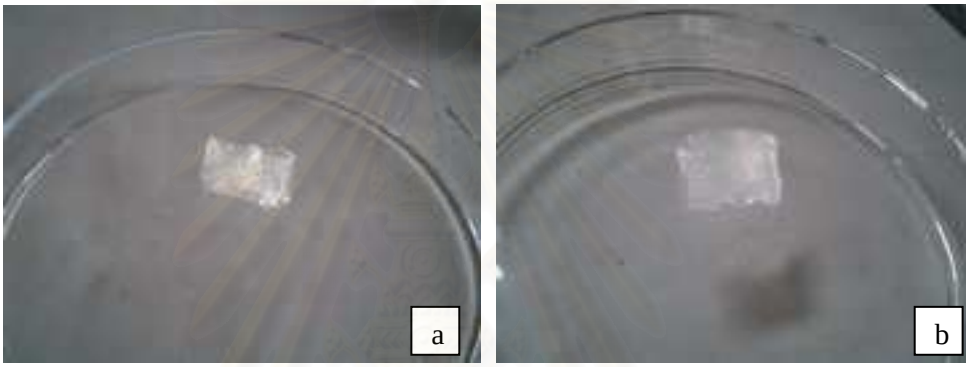


Figure 5.8A Changes of alginate sponges as a function of sample immersion time in distilled water (a) before immersion (b) after 60 mins

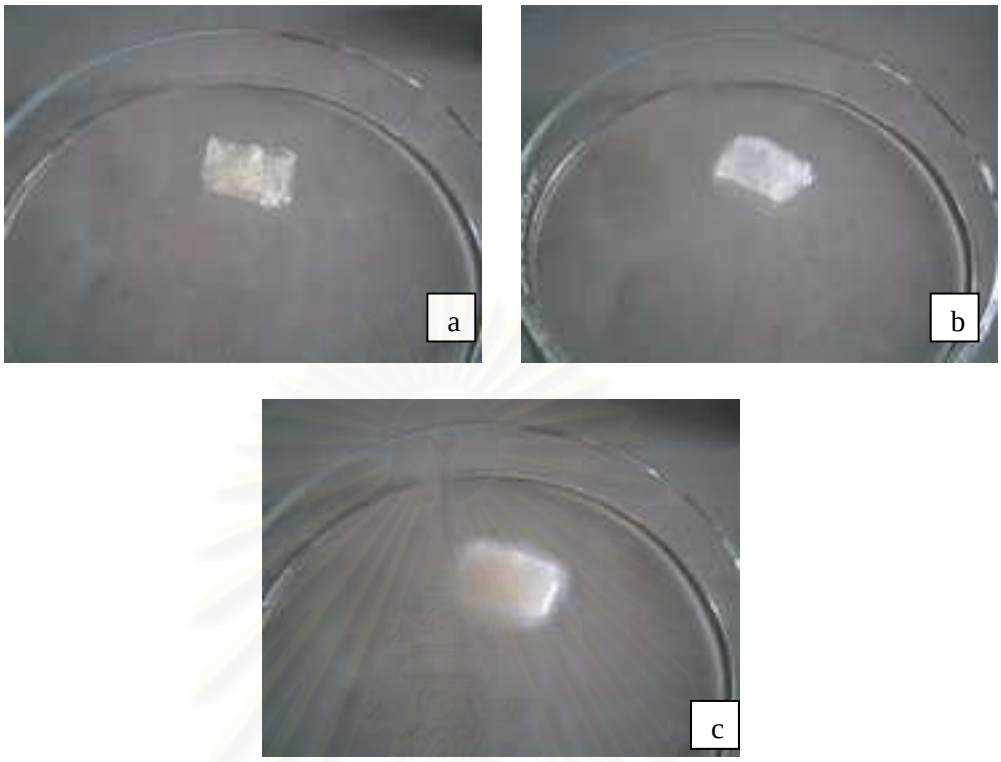


Figure 5.8B . Changes of alginate sponges as a function of sample immersion time in PBS (a) before immersion (b) after 15 mins (c) after 30 mins

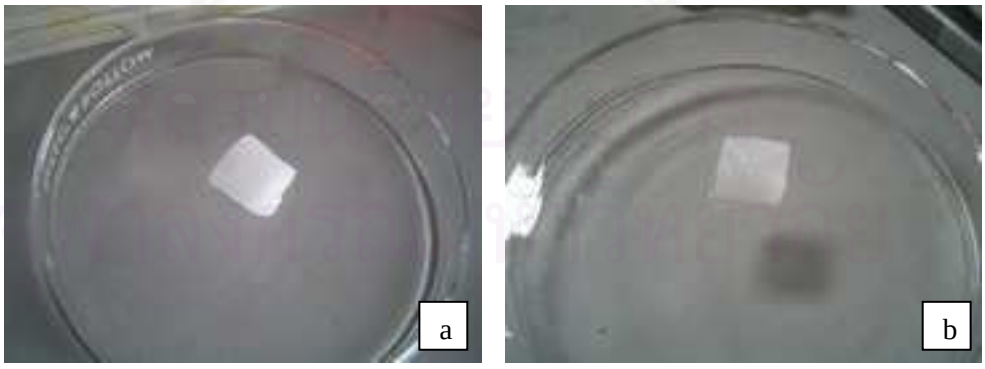


Figure 5.9A Changes of 30% alginate sponges as a function of sample immersion in distilled water (a) after 2 hours (b) after 4 hours

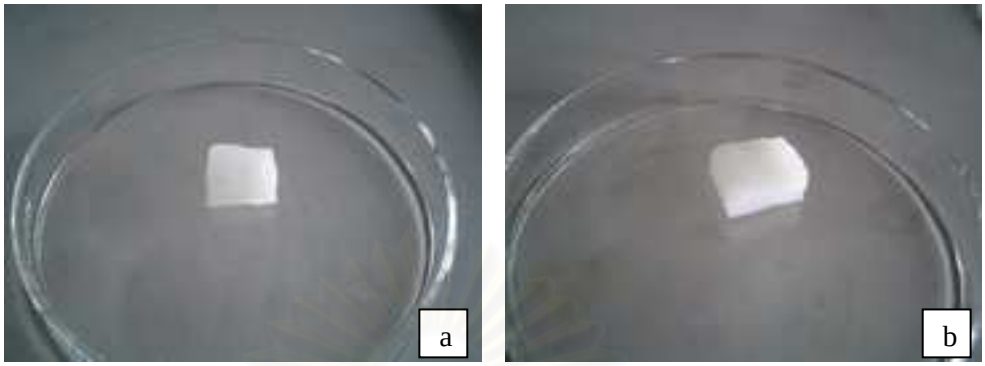


Figure 5.9B Changes of 30% alginate sponges as a function of sample immersion time in PBS (a) after 2 hours (b) after 4 hours

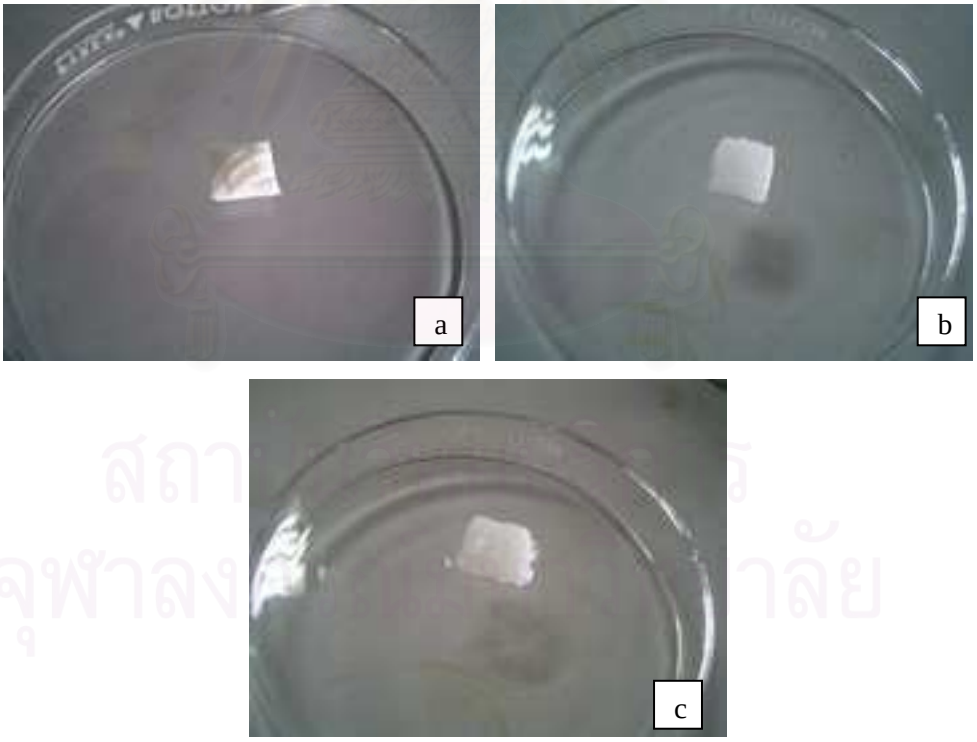


Figure 5.10A Changes of pure bacterial cellulose sponges as a function of sample immersion time in distilled water (a) before immersion (b) after 2 hours (c) after 3 hours

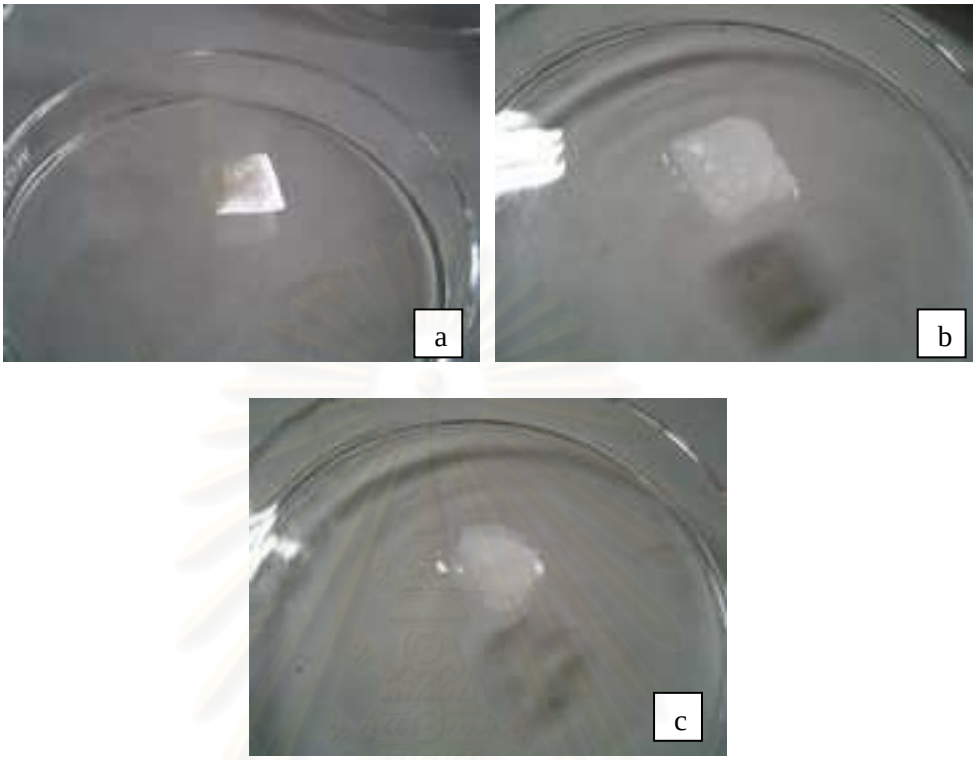


Figure 5.10B Changes of pure bacterial cellulose sponges as a function of sample immersion time in PBS (a) before immersion (b) after 1 hour (c) after 2 hours

The stability of the sponges differed in both solution. The sponges with 30% alginate appeared to retain their overall size through the study period. The alginate sponges in PBS solution were gradually dissolved in 30 mins. Pure bacterial cellulose sponges could swell rapidly and gradually collapsed within 2 hours in PBS solution and steadily dissolved after 3 hours immersion in distilled water. This suggested that the 30% alginate sponge is more stable than the others. This makes the bacterial cellulose/alginate sponge potentially applicable in a wider range of clinical use.

5.2.5 Morphology

Morphology of pure bacterial cellulose sponges by SEM photographs illustrated in Figure 5.12 indicated the porous structure with three-dimensional interconnection throughout the sponges.

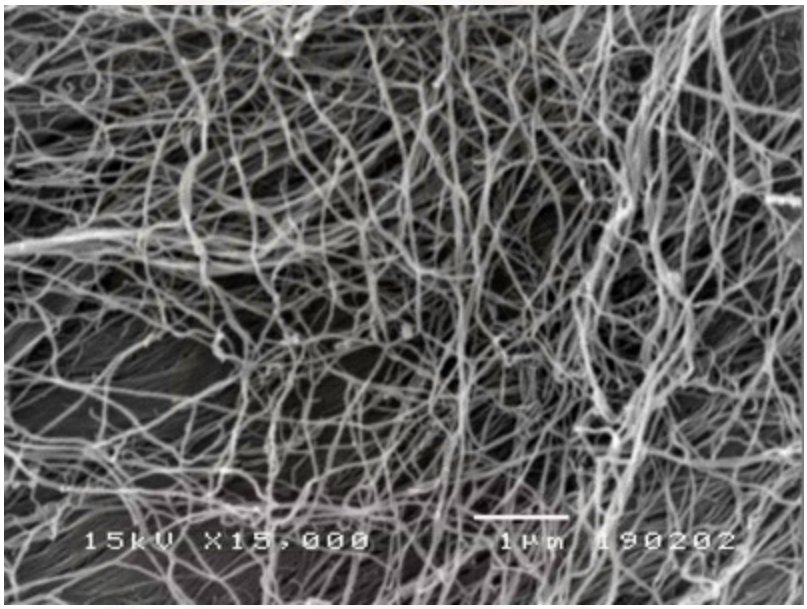


Figure 5.11 SEM of the surface of pure bacterial cellulose after using super critical drying method (×15000)

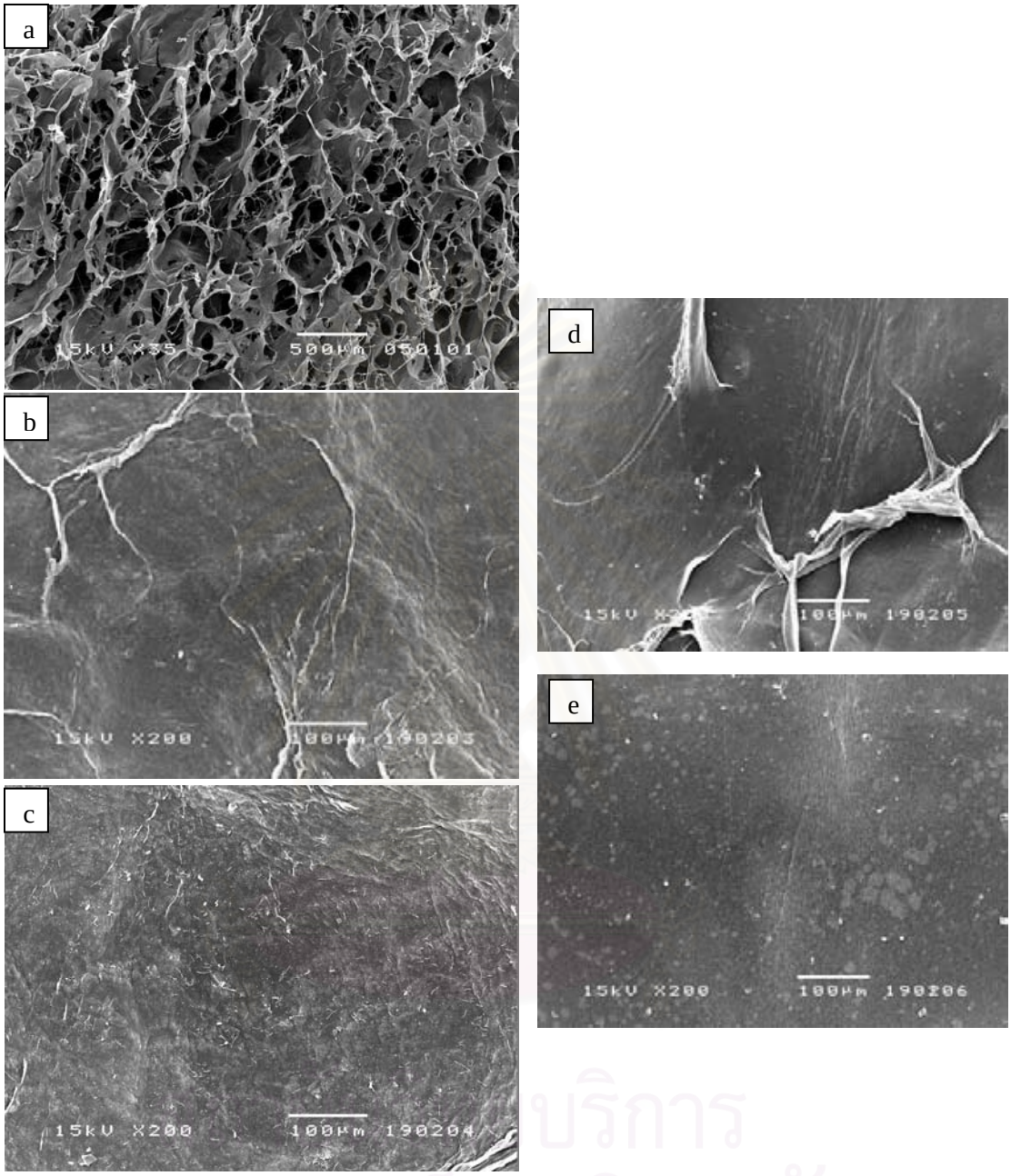


Figure 5.12 SEM micrographs of horizontal plane of bacterial cellulose/alginate sponges: (a) 100/0, (b) 70/30, (c) 50/50, (d) 30/70, (e) 0/100.

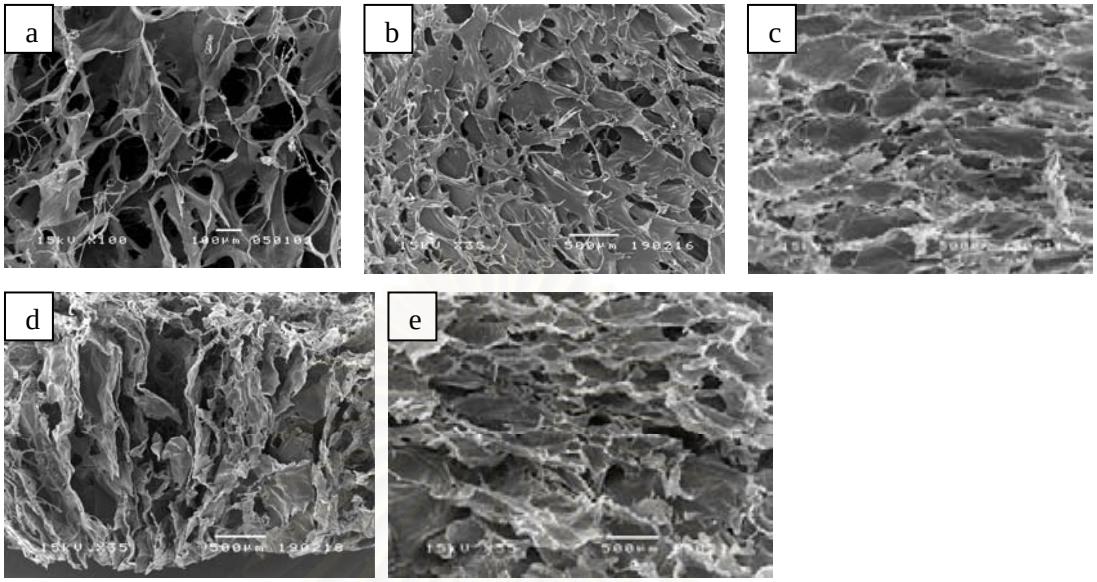


Figure 5.13 SEM micrographs of cross-sectional plane of bacterial cellulose/alginate sponges: (a) 100/0, (b)70/30, (c) 50/50, (d) 30/70, (e), 0/100.

The morphology of bacterial cellulose/alginate showed the asymmetric structure. This was similar to the work of Mi and coworkers (2002). They proposed a new type of wound dressing which is an asymmetric chitosan membrane. The asymmetric membrane consists of a top skin layer and sponge-like porous layer. The outer layer helps prevent bacterial invasion and avoid wound dehydration, whereas the porous support layer provide the drainage of wound exudates and mechanical strength. The design of asymmetric sponge as a functional wound dressing is shown in Figure 5.14.

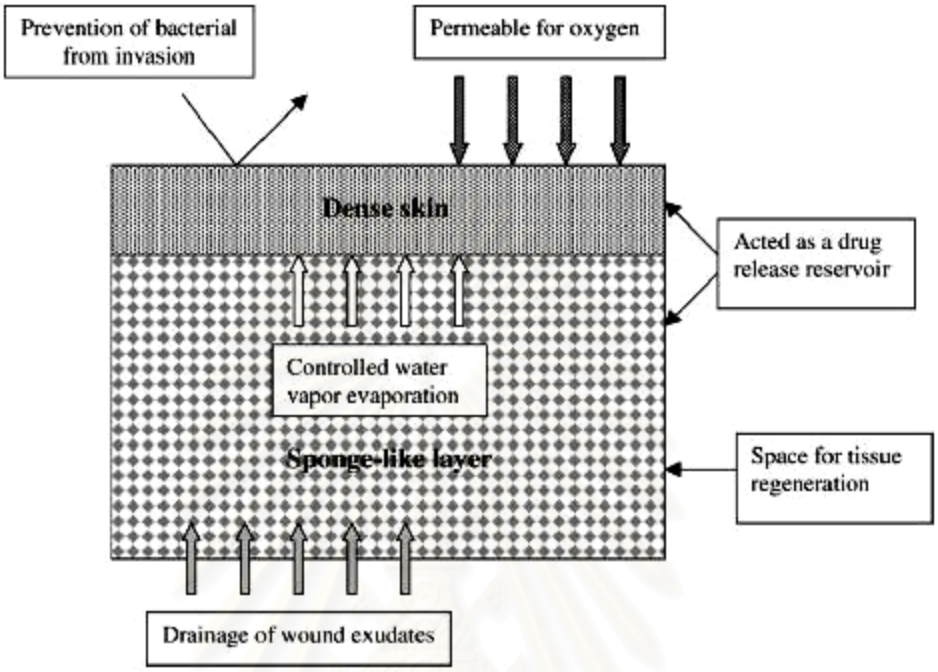


Figure 5.14 Design of asymmetric membrane for used as a wound dressing (Mi *et al.*, 2002)

Pore size of sponges fell in the range of 100-300 μm which was suitable for usage in tissue engineering (Yang *et al.*, 2001). For sponges at blending composition of 70/30 and 100/0 bacterial cellulose/alginate as shown in Figure 5.12 and 5.13, the morphology appeared to have more ordered structure and less dense than the other blending compositions. This was similar to the study of Lai *et al.* (2002). They reported that the mixing of alginate and chitosan leading to a more randomly ordered network. This could be caused by the interaction between the two polymers in pre-freezing step.

5.3 Biological characterization of sponges

5.3.1 Biodegradation

Figure 5.15 Samples of biodegradation of sponges (day 5)

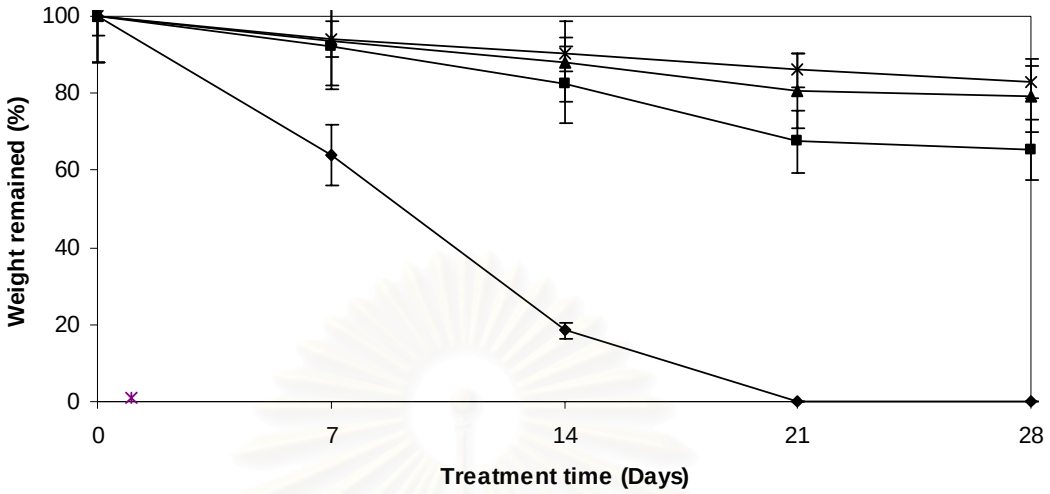


Figure 5.16 Biodegradation of sponges made of bacterial cellulose (BC) blended with alginate: (♦) 100/0, (■) 70/30, (▲) 50/50, (×) 30/70 (ratio of BC/alginate)

As describe in section 2.14, a prerequisite for hydrolysis to occur is direct physical contact between the enzyme molecules and the surface of cellulose (Lee and Fan, 1982). Cellulase enzymes have a specific three-dimensional shape and their catalytic power depends on adsorption onto the surface of a substrate in lock-and-key fashion (Etters, 1998). Since cellulases are highly substrate specific in their action, any changes in the structure and accessibility of the substrate has a profound influence on the hydrolysis reaction.

The biodegradation results were depicted in Figure 5.16. Pure bacterial cellulose sponges had the highest weight reduction and completely degraded within 21 days. The addition of alginate reduced degradation of sponges. This suggested that the physical interaction between bacterial cellulose and alginate possessed more hindrance effect to specific cleavage site of cellulases than that of the pure bacterial cellulose. The addition of alginate decreased the enzymatic accessibility.

The results revealed that the presence of alginate could prolong the degradability of sponges.

5.3.2 Cell Study

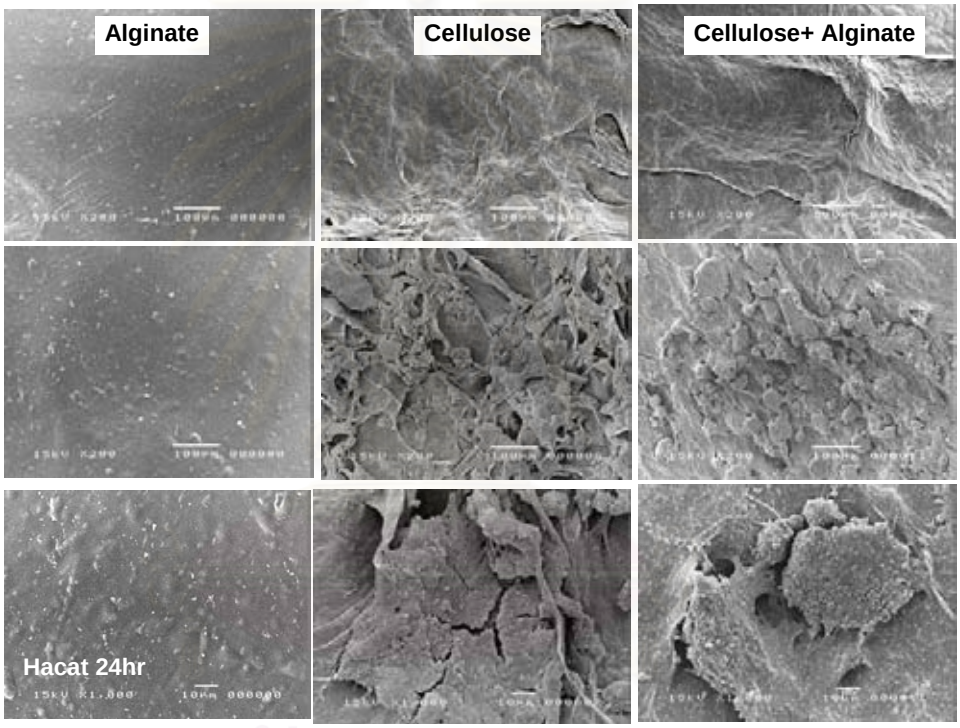


Figure 5.17 Morphology and distribution of human skin keratinocytes (A) on the alginate (Al), bacterial cellulose (BC) and alginate modified bacterial cellulose (Al-BC) scaffolds demonstrated at 24 h by using phase-contrast microscopy.

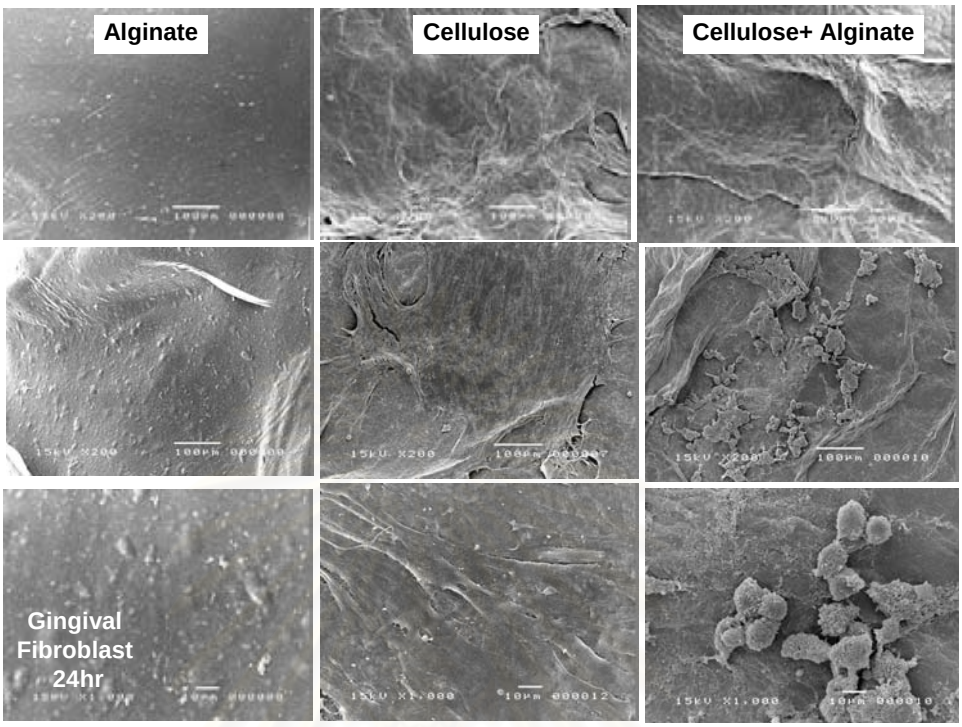


Figure 5.18 Morphology and distribution of Gingival fibroblasts on alginate (Al), bacterial cellulose (BC) and alginate modified bacterial cellulose (Al-BC) scaffolds demonstrated at 24 h by using phase-contrast microscopy.

Cell adhesion and proliferation are crucial for sponge to support and guide tissue regeneration. Experiments of cell culture were carried out to test the biocompatibility of sponges. Keratinocytes and gingival fibroblasts were used in this work. The appropriate sponges were selected to test their biological properties.

The SEM images of cell attachment and proliferation behavior on bacterial cellulose, bacterial cellulose/alginate and alginate sponges are shown in Figure 5.17 and 5.18. Figure 5.19 and 5.20 represented the results of the mitochondrial activity (MTT) assay for cell proliferation at 6, 24 and 48 h after seeding. Cells were seeded on the tissue culture plastic (control), pure bacterial cellulose, bacterial cellulose/alginate and alginate sponges to compare their cell behavior.

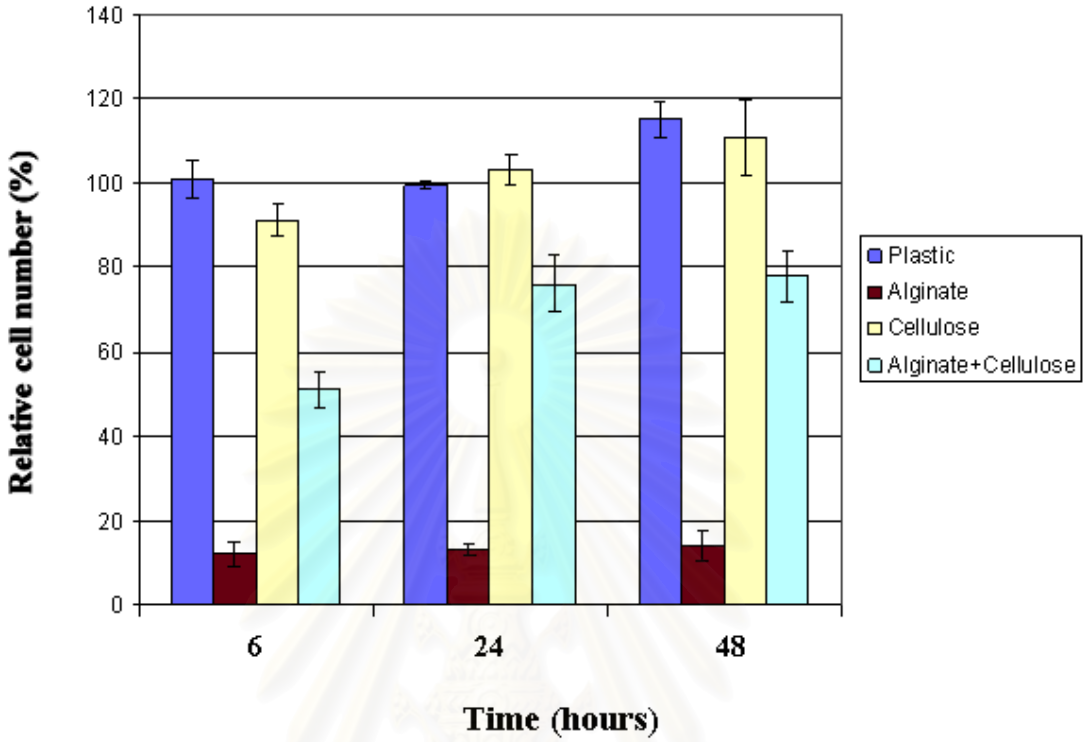


Figure 5.19 Proliferations of Gingival fibroblasts on the alginate (Al), bacterial cellulose (BC) and alginate modified bacterial cellulose (Al-BC) scaffolds. Percentage of living cells was assessed at 6, 24, and 48 h of culture by MTT assay.

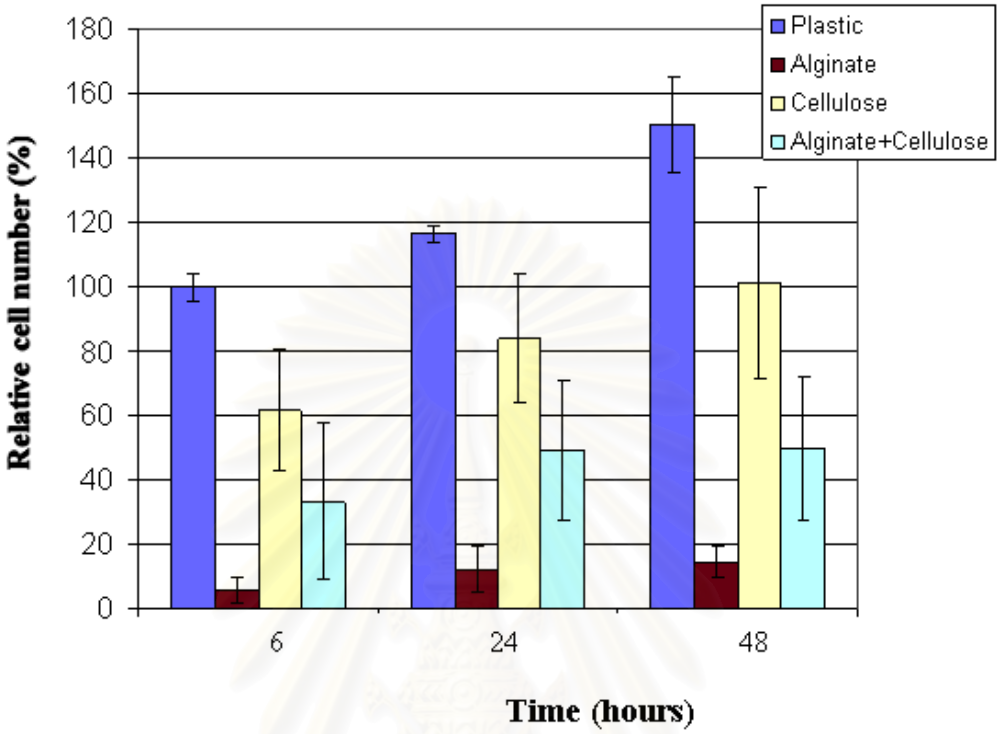


Figure 5.20 Proliferations of human skin keratinocytes (A) on the alginate (Al), bacterial cellulose (BC) and alginate modified bacterial cellulose (Al-BC) sponges. Percentage of living cells was assessed at 6, 24, and 48 h of culture by MTT assay.

From the cell study, pure bacterial cellulose supported growth and spreading of keratinocytes and gingival fibroblasts. Few of both cell types were detected on alginate. Phase contrast microscopy demonstrated that keratinocytes spread over the surface of bacterial cellulose/alginate sponges at 24h and became confluent monolayer at 48 h whereas fibroblasts partially spread and formed some clumps in various sizes. Both cell types appeared to proliferate more rapidly on tissue culture plastic and pure bacterial cellulose than on the other materials.

Our results indicated that the bacterial cellulose/alginate sponges had no toxicity and supported cell proliferation in both cell types. However, the pattern of cell distribution and stability on bacterial cellulose/alginate sponges were poorer in fibroblast culture. These results demonstrated that keratinocytes responded to the alginate modified bacterial cellulose scaffolds better by exhibiting normal cell proliferation and spreading.

Our cell proliferation results were similar to the study on the chondrocyte proliferation reported by Svensson and coworkers (2004). The proliferation tests were observed in chondrocyte on native and chemically modified bacterial cellulose material. They found that the percentage of relative cell viability of native bacterial cellulose was greater than others chemically modified ones.

Bacterial cellulose sponges showed high levels of cells growth, however the disadvantage is they could not retain their shapes throughout the cell culture study. They lost the mechanical integrity and could lead to sponge disruption. The ability of

bacterial cellulose/alginate to support cell survival in combination with good tear resistance property suggested that the modified bacterial cellulose sponge has a good potential to be used as a biocompatible material for tissue engineering.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study described the development of sponges of biopolymer blends between bacterial cellulose and alginate by freeze drying technique. Physical interaction between bacterial cellulose and alginate could affect both physical and biological properties of sponges. The biodegradation test demonstrated that the addition of alginate prolonged the biodegradation of bacterial cellulose/alginate sponges. The cell-material interaction study using bacterial cellulose-based sponges containing 30% alginate indicated that both human keratinocytes and gingival fibroblasts seeded on the sponges appeared to attach and proliferate well. Although the pure bacterial cellulose sponges could support the cell growth greater than that of the bacterial/cellulose sponges, but the bacterial/alginate sponges could retain their shape throughout the cell studies. The sponges could also resist the tear from sewing procedure. The results from cell culture expressed the good biocompatibility and could successfully induce cell growth. This study accomplished the utilization of bacterial cellulose (in wet state) and alginate. The sponges had promising properties of mechanical strength, water uptake capability and cell proliferation ability. The fabrication method of this biomaterial is environmentally friendly, non-toxic and easy to be scaled up and does not require complicated procedures. These encouraging results support the potential applications of the bacterial cellulose/alginate sponges as an improved alternative to other natural polymer-based material for biomedical applications.

6.2 Recommendations

Although the chemical, physical and biological properties of bacterial cellulose/alginate sponges have been studied in this work, there are still some interesting points which can be further investigated. These are some recommendations.

1. Although pure bacterial cellulose sponges showed a high degree of cell compatibility but they can not retain their overall size throughout the period of cell culture study. Therefore, the stability improvement should be ongoing.

2. The improvement of bacterial cellulose properties by modifying with other natural polymer during its biosynthesis could be further studied.

3. Further study on and biocompatibility of bacterial cellulose/alginate sponges with other cell type such as osteoblast should be explored in order to use in bone tissue engineering

REFERENCES

Alsberg V., Anderson K., Albeiruti A., Franceshi RT., Mooney DJ. “Cell interactive alginate hydrogels for bone tissue engineering”,*J Dent Res*, 2001, **80**, 2025-2029.

Brown, Jr. R. M., “Bacterial cellulose”, *Cellulose: Structural and Functional Aspects*, Ed. By Kennedy, Phillips, & Williams, Ellis Horwood Ltd, 1989, pp 145-151.

Brown, Jr. R.M., “Advances in Cellulose Biosynthesis”, *Polymers from biobased Materials,* Ed. By Chum, H.L., Doyes Data Corp., New Jersey, 1991.

Buckley, C. and O’Kelly, K. “Regular scaffold fabrication techniques for investigations in tissue engineering” Dublin: Centre for bioengineering, Department of mechanical and manufacturing engineering, Trinity College, Ireland, 2004.

Chapin III, F.S., P.A. Matson, H.A. Mooney. *Principles of Terrestrial Ecosystem Ecology*. Springer-Verlag NewYork, NY. 2002.

Danuta C.. “Multifunctional bacterial cellulose/chitosan composite materials for medical applications”, *Fibre & Textiles in Eastern Europe*, 2004, **12**, 69-72.

Davey, J. and Lord, M. “Essential cell biology Volume 1: Cell structure A practical approach”, *Oxford University Press*, 2003.

Dorothy M. and Boyce, S.T “Engineered skin substitutes: practices and potentials”, *Clinics in Dermatology*, 2005, **23**, 403–412.

Entcheva, E., Bien H., Yin L., Chung C., Farrell M., Kostov Y., “Functional cardiac cell constructs on cellulose-based scaffolding”, *Biomaterials*, **25**, 5753-5762.

Etter, J.N. “Emerging opportunities for enzymes in textiles”, *Colourage Annu*, 1998, 87-92.

Evans, B., O'Neill, H., Malyvanh, V., Lee, I. and Woodward, J., “Palladium-bacterial cellulose membranes for fuel cells”, *Biosensors and Bioelectronics*, 2003, **18**, 917-923.

Fontana, J.D., de Souza, A.M., Fontana, C.K., Torriani, I.L., Moreschi, J.C., Gallotti,

B.J., de Souza, S.J., Narcisco, G.P., Bichara, J.A., and Farah,, L.F.X., "Acetobacter cellulose pellicle as a temporary skin substitute", *Applied Biochemistry and Biotechnology*,1990, **24/25**, 253-263.

Gannadios, A., Hanna, M.A. and Kurth, L.B., "Application of edible coatings on meat, poultry and seafoods: A review", *Lebens. Wiss. Und Technol.*, 1997, **30**, 337-350.

Gimmestad, M., Sletta, H., Ertesvag., H., Bakkevig, K., Jain, S., Suh, S.J, Skjak-Braek, G., Ellingsen, T.E., Ohman, D.E. and Valla, S., "The Pseudomonas fluorescens AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation", *Journal of Bacteriology*, 2003, **185**, 3515-3523.

Glicksman, M., "Hydrocolloids in fabricated foods", *Food Technol*., 1985, **20**, 79-82.

Hart, J., Silcock, D., Gunnigle, S., Cullen, B., Light, N., Watt, P., "The role of oxidized regenerated cellulose/collagen in wound repair: effects *in vitro* on fibroblasts biology and *in vivo* in a model of compromised healing", *The international Journal of Biochemistry & Cell Biology*, 2002, **34**, 1557-1570.

Jarvis, P.M.,Galvin, D.A.J., Blair, S.D., Mccollum, C.N. "How does calcium alginate archieve hemostatis in surgery", *Thermobosis and Haemostasis*, 1987, **58(1)**, 80.

Jeanie L.,David J. "Hydrogels for tissue engineering: scaffold design variables and applications", *Biomaterials*, 2003, **24**, 4334-4351.

Jeanie L. Drury, Robert G. Dennis, David J. Mooney. "The tensile properties of alginate hydrogels, *Biomaterials*, 2004, **25,** 3187-3199.

Jonas, R., Farah, L. F., "Production and application of microbial cellulose", *Polymer Degradation and Stability*, 1998, **59**, 101-106.

Jones, I., Currie, L. and Martin, R., "A guide to biological skin substitutes", *British Journal of Plastic Surgery*, 2002, **55**, 185-193.

Kang, H., Tabata, Y. and Ikada, Y. "Fabrication of porous gelatin scaffolds for tissue engineering", *Biomaterials*, 1999, **20**, 1339-1344.

Karmakar, S.R., "Application of biotechnology in the pretreatment processes of textiles", *Colourage Annu*, 1998, 75-76.

Klemm, D., Heublein, B., Fink, H.P. and Bohn, A., "Cellulose:Fascinating Biopolymer and Sustainable Raw material", *Angew. Chem. Int. Ed.*, 2005, **44,** 2-37.

Klemm, D., Schumann, D., Udhardt, U. and Marsch, S., "Bacterial synthesized cellulose—artificial blood vessels for microsurgery", *Progress in Polymer Science*, 2001, **26**, 101-106.

Kliventov, A.S. and Mjagkostupova, V.P., "Studies of wood irradiative destroyed: Research of chemical content of larch wood exposed on gamma radiation", *Khimia Drev.*, 1991, **4**, 95-97 [in Russian].

Koenig, J.L. *Spectroscopy of Polymer*. Amsterdam:Elsevier, 1999.

Kolpak, F. J., Blackwell, J., "Determination of the structure of cellulose II", *Macromolecules, 1976,* **9**, 273-278.

Lai, H., Abu'Khalil, A., Craig, D. "The preparation and characterisation of drug-loaded alginate and chitosan sponges", *International Journal of Pharmaceutics*, 2004, **251**, 175-181.

Lee, Y., and L.T. Fan, "Kinetic studies of enzymatic hydrolysis of insoluble cellulose (II): Analysis of extended hydrolysis times", *Biotechnol.Bioeng*, 1983, **25**, 939-966.

Li, S., Ramay, H., Hauch, K. Xiao, D., Zhang, M.. "Chitosan-alginate hybrid scaffolds for bone tissue engineering", *Biomaterials*, 2005, **26**, 3919-3928.

Mi, F., Wu, Y., Shyu, S., Chao, A., Lai, J., Su, C., "Asymmetric chitosan membranes prepared by dry/wet phase separation: a new type of wound dressing for controlled antibacterial release", *Journal of Membrane Science*., 2003, **212**, 237-254.

Mosmann, T, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays", *Journal of Immunological Methods*, 1983, **65**, 55-63.

Okiyama, A., Motoki, M. and Yamanaka, S., "Bacterial Cellulose II. Processing of the Gelatinous Cellulose for Food Materials", *Food Hydrocolloids*, 1992, **6**, 479-487.

Okiyama, A., Motoki, M. and Yamanaka, S., "Bacterial Cellulose IV. Application to Processed Foods", *Food Hydrocolloids*, 1993, **6**, 503-511.

Ross, P., Mayer, R., and Benziman, M., "Cellulose Biosynthesis and Function in Bacteria", *Microbiol. Rev.*, 1991, **55**, 35-58.

Phisalaphong M., Suwanmajo T., Tammarate P. "Synthesis and Characterization of bacterial cellulose/alginate blend membranes", *Journal of applied Polymer Science*, 2007, **107**, 3419-3424.

Salgado A.J., Gomes M.E., Chou A., Coutinho O.P. , Reis R.L. , Hutmacher D.W.. "Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds", *Materials Science and Engineering*, 2002, **20**, 27–33.

Sherratta, J.A.; and Dallon, J.C. "Theoretical models of wound healing: past successes and future challenges", *C R Biologies*, 2002, **325**, 557-564.

Shibazaki, H., Kuga, S.,, Onabe, F., and Usada, M., "Bacterial cellulose membrane as Separation Medium", *J. Applied Polymer Sci.*, 1993, **50**, 965-969.

Smidsrod O., Draget KI. "Chemistry and physical properties of alginates" *Carbohydr Eur*, 1996, **14**, 6-13.

Snowman, J.W. *Downstream Processes: Equipment and Techniques.* London: Alan R. Liss, 1998.

Stuart, B.H, *Polymer Analysis.* New York: John Wiley & Sons, 2002.

Svesson, A., Nicklasson, E., Harrah, T., Panilaiis, B., Kaplan, D.L., Brittberg, M. and Gatenholm, P., "Bacterial cellulose as a potential scaffold for tissue engineering of cartilage", *Biomaterials*, 2005, **26**, 419-431.

Thomas S. "Wound management and dressings", London: *Pharmaceutical Press*, 1990.

Timothy, P.E "Lyophilization processing: Freeze-dried microspheres offer new IVD packaging and product opportunities", IVD *technology magazine*, 2002.

Wan Y.Z., Huang Y., Yuan C.D., Raman S., Zhu Y., Jiang H.J., He F., Gao C., "Biomimetric synthesis of hydroxyapatite/bacterial cellulose nanocomposites for biomedical applications", *Materials science & Engineering*, 2007, **27**, 855-864.

Watanabe, K., Eto, T., Takano, S., Nakamori, S., Shibai, H. and Yamanaka, S.,, "A new bacterial cellulose substrate for mammalian cell culture", *Cytotechnology*, 1993, **13**, 107-114.

Winter G.D. “Formation of the scab and the rate of epithelialization of superficial wounds in the skin of the young domestic pig”, *Nature*, 1962, **193**, 293-294.

Wu, Y., Yu, S. Mi, F., Wu, C., Shyu, Peng, S. Chao, A. “Preparation and characterization on mechanical and antibacterial properties of chitosan/cellulose blends”, *Carbohydrate Polymers*, 2004, **57**, 435–440.

Yamanaka, S., Ishihara M. and Sugiyama J., “Structural modification of bacterial cellulose”. *Cellulose*, 2000, **7**, 213-255.

Yamanaka, S., Watanabe, K., “Applications of Bacterial Cellulose”, *Cellulose Polymers, Blends and Composites*, Ed. By Richard D. Gilbert, Hanser/Gardner Publications, Cincinnati, 1994, pp207-215.

Yang, G., Zhang, L., Peng, T., Zhong, W.. “Effect of Ca^{2+} bridge cross-linking on structure and pervaporation of cellulose/alginate blend membranes”, *Journal of Membrane Science*, 2002, **175**, 53-60.

Yang S, Leong KF, Du Z, Chua CK, “The design of scaffold for use in tissue engineering. Part I. Traditional Factors, *Tissue Eng*, 2001, **7**, 679-689.

Yannas, I.V. “Tissue and organ regeneration in adults” Massachusetts: Springer-Verlag, 2001.

Yoshino, K., Matsuoka, R., Nogami, K., Yamanaka, K., Watanabe, K., Takahashi, M., and Honma, M., “Graphite film prepared by pyrolysis of bacterial cellulose”, *Journal of Applied Physics*, 1990, **68,** 1720-1725.

APPENDICES

Appendix A

Data of Experiments

Table A-1 Tensile strength (MPa) of the sponges

Replication no.	Blending composition (BC/alginate)				
	100/0	70/30	50/50	30/70	0/100
1	3.40	2.31	1.41	1.16	0.77
2	2.58	1.86	1.43	1.35	0.79
3	3.46	1.87	1.23	1.13	0.75
4	2.52	2.36	1.40	1.34	0.86
Average	2.99	2.10	1.37	1.25	0.79
SD	0.51	0.27	0.09	0.12	0.05

Table A-2 Elongation at break (%) of sponges

Replication no.	Blending composition (BC/alginate)				
	100/0	70/30	50/50	30/70	0/100
1	23	20	12	10	8
2	20	17	13	13	10
3	24	14	10	11	9
4	18	18	14	11	10
Average	21.25	17.25	12.25	11.25	9.25
SD	2.75	2.5	1.71	1.26	0.96

Table B-1 Raw data of water uptake ability ratio of sponges

Blending composition (BC/alginate)	Replication no.	Weight (g)		Swelling ratio
		Dry	Swell	
100/0	1	0.1	1.21	11.10
	2	0.1	1.23	11.30
	3	0.1	1.36	12.60
	Average			11.67
	SD			3.59
70/30	1	0.4	5.51	12.78
	2	0.4	5.54	12.85
	3	0.4	5.57	12.93
	Average			12.85
	SD			0.076
50/50	1	0.2	2.89	13.45
	2	0.2	2.91	13.55
	3	0.2	2.87	13.35
	Average			13.45
	SD			0.1
30/70	1	0.1	1.44	13.40
	2	0.1	1.43	13.30
	3	0.1	1.51	14.10
	Average			13.6
	SD			0.44
0/100	1	0.2	3.04	14.20
	2	0.2	2.94	13.70
	3	0.2	2.95	13.75
	Average			13.88
	SD			0.28

Table C-1 Raw data of biodegradation of bacterial cellulose sponges

Time (Days)	Replication no.	Weight (g)		Weight remained (%)
		Initial	Final	
7	1	0.48	0.34	70.83
	2	0.48	0.30	62.5
	3	0.48	0.28	58.33
	Average			63.87
	SD			6.36
14	1	0.51	0.13	25.49
	2	0.51	0.06	11.76
	3	0.51	0.09	17.65
	Average			18.30
	SD			6.89
21	1	0.51	0	0
	2	0.51	0	0
	3	0.51	0	0
	Average			0
	SD			0

Table C-2 Raw data of biodegradation of bacterial cellulose/alginate sponges at 70/30 blending composition

Time (Days)	Replication no.	Weight (g)		Weight remained (%)
		Initial	Final	
7	1	0.46	0.42	91.30
	2	0.46	0.41	89.13
	3	0.46	0.44	95.65
	Average			92.03
	SD			3.32
14	1	0.46	0.36	78.26
	2	0.46	0.41	89.13
	3	0.46	0.38	82.61
	Average			83.33
	SD			5.47
21	1	0.45	0.31	68.89
	2	0.45	0.26	57.78
	3	0.45	0.34	75.56
	Average			67.41
	SD			8.98
28	1	0.45	0.31	68.89
	2	0.45	0.28	62.22
	3	0.45	0.29	64.44
	Average			65.18
	SD			3.40

Table C-3 Raw data of biodegradation of bacterial cellulose/alginate sponges at 50/50 blending composition

Time (Days)	Replication no.	Weight (g)		Weight remained (%)
		Initial	Final	
7	1	0.45	0.43	95.56
	2	0.45	0.42	93.33
	3	0.45	0.41	91.11
	Average			93.33
	SD			2.23
14	1	0.45	0.38	84.44
	2	0.45	0.41	91.11
	3	0.45	0.40	88.89
	Average			88.15
	SD			3.4
21	1	0.45	0.35	77.78
	2	0.45	0.36	80.00
	3	0.45	0.38	84.44
	Average			80.74
	SD			3.39
28	1	0.47	0.35	77.78
	2	0.47	0.36	80.00
	3	0.47	0.36	80.00
	Average			79.26
	SD			1.28

Table C-4 Raw data of biodegradation of bacterial cellulose/alginate sponges at 30/70 blending composition

Time (Days)	Replication no.	Weight (g)		Weight remained (%)
		Initial	Final	
7	1	0.45	0.42	93.33
	2	0.45	0.42	93.33
	3	0.45	0.43	95.56
	Average			94.07
	SD			1.29
14	1	0.47	0.42	89.36
	2	0.47	0.45	95.74
	3	0.47	0.40	85.12
	Average			90.07
	SD			5.35
21	1	0.45	0.39	86.67
	2	0.45	0.39	86.67
	3	0.45	0.38	84.44
	Average			85.93
	SD			1.29
28	1	0.45	0.37	82.22
	2	0.45	0.38	84.44
	3	0.45	0.37	82.22
	Average			82.96
	SD			1.28

Table C-5 Raw data of biodegradation of bacterial cellulose/alginate sponges at 0/100 blending composition

Time (Days)	Replication no.	Weight (g)		Weight remained (%)
		Initial	Final	
7	1	0.47	0.46	97.87
	2	0.47	0.47	100.00
	3	0.47	0.43	91.49
	Average			96.45
	SD			4.43
14	1	0.4	0.38	95.0
	2	0.4	0.37	92.5
	3	0.4	0.37	92.5
	Average			93.33
	SD			1.44
21	1	0.42	0.36	85.71
	2	0.42	0.37	88.10
	3	0.42	0.36	85.71
	Average			86.51
	SD			1.38
28	1	0.4	0.34	85.0
	2	0.4	0.34	85.0
	3	0.4	0.33	82.5
	Average			84.17
	SD			1.44

Table D-1 Raw data of gingival fibroblasts cell proliferation test (Absorbance at 570 nm at 6 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	99.39	23.74	19.87	7.8
	2	100.6	65.98	15.67	1.9
	3	94.89	73.09	17.32	3
	4	105.1	68.22	17.61	2.4
	5	96.2	74.45	61.87	7.18
	6	103.79	63.07	67.06	11.97
	Average	99.99	61.425	32.23	5.71
	SD	4.04	18.94998	24.28	3.96

Table D-2 Raw data of gingival fibroblasts cell proliferation test (Absorbance at 570 nm at 24 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	119.6	46.38	47.7	15.79
	2	113.41	101.99	36	4.28
	3	120.09	96.15	28.22	5.15
	4	115.71	94.89	30.85	8.85
	5	116.16	77.44	69.06	19.56
	6	114.57	85.82	81.03	19.6
	Average	116.59	83.78	48.81	12.21
	SD	2.70	20.25	21.73	7.01

Table D-3 Raw data of gingival fibroblasts cell proliferation test (Absorbance at 570 nm at 48 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	145.22	52.34	47.7	10.49
	2	165.54	127.49	34.84	12.26
	3	130.99	126.52	29.19	12.27
	4	137.32	122.04	32.4	10.5
	5	165.6	96.2	78.6	19.16
	6	158.8	82.2	75.04	21.95
	Average	150.58	101.13	49.63	14.44
	SD	14.87	30.13	22.01	4.88

Table E-1 Raw data of human keratinocyte cell proliferation test (Absorbance at 570 nm at 6 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	106.19	94.21	55.86	8.8
	2	98.59	87.02	49.83	12.3
	3	98.2	92.64	47.35	14.46
	Average	100.99	91.29	51.01	11.85
	SD	4.50	3.78	4.38	2.86

Table E-2 Raw data of human keratinocyte cell proliferation test (Absorbance at 570 nm at 24 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	98.76	100.16	81.65	14.6
	2	100.4	102.14	78.09	11.94
	3	99.25	107.43	68.84	12.8
	Average	99.47	103.24	76.19	13.11
	SD	0.84	3.76	6.61	1.36

Table E-1 Raw data of human keratinocyte cell proliferation test (Absorbance at 570 nm at 48 h after seeding)

Sample	Replication	Control	100/0	70/30	0/100
BC/alginate	1	111.1	115.6	71.2	15.6
	2	114.5	116.5	80.16	9.58
	3	119.66	100.49	82.56	16.4
	Average	115.09	110.86	77.97	13.86
	SD	4.31	8.99	5.99	3.73

Table F-1 Raw data of moisture content determination of sponges

Blending composition (BC/alginate)	Replication no.	Weight (g)		Moisture content (%)
		wet	dry	
100/0	1	0.0236	0.0221	6.36
	2	0.0160	0.0152	5
	3	0.0222	0.0212	4.5
	Average			5.29
	SD			0.96
70/30	1	0.0372	0.0334	10.22
	2	0.0355	0.0320	9.86
	3	0.0287	0.0245	14.63
	Average			11.57
	SD			2.66

VITAE

Miss Nadda Chiaoprakobkij was born in Bangkok, Thailand on August 17, 1982. She finished her high school education in 1999 from Propittayapayat school. In 2003, she received her Bachelor Degree of Engineering with a major of Chemical Engineering from Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang. After graduation, she pursued her graduate study to a Master of Engineering (chemical engineering), the Faculty of Engineering, Chulalongkorn University.

VITAE

Miss Nadda Chiaoprakobkij was born in Bangkok, Thailand on August 17, 1982. She finished her high school education in 1999 from Propittayapayat school. In 2003, she received her Bachelor Degree of Engineering with a major of Chemical Engineering from Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang. After graduation, she pursued her graduate study to a Master of Engineering (chemical engineering), the Faculty of Engineering, Chulalongkorn University.

NEERACHA S.

Program & Abstracts

International Association for Dental Research
South-East Asian Division
21st Annual Scientific Meeting

South-East Asian Association for Dental Education
18th Annual Scientific Meeting

SEAADE ORAL COMMUNICATION
Chairperson - Assoc Prof Dr Leung Wai Keung
Venue: Kharisma Ballroom

Time	Title and Author(s)	Code
12:00 – 12:10	**The Effectiveness Of An Intensified Dental Support Programme In Reducing Orofacial Pain *And Promoting Oral Hygiene Among Navy Personnel*** N Ab Murat, Z Mohd Taha, RL Raja Jallaludin	SO-1
12:10 – 12:20	***Tobacco Use Cessation Initiatives In The Dental Curriculum*** NA Yahya, TI Rosli, TN Mohd Dom	SO-2
12:20 – 12:30	***Enhancing Dental Education Using Et And Ict*** Keng SB, Foong KWC, Tan KBC, Tok WW.	SO-3
12:30 – 12:40	***Evidence-Based Practice (Ebp) Among General Dental Practitioners (Gdps) In Selangor Malaysia*** ZYM Yusof, Lee JH, Poon PS	SO-4
12:40 – 12:50	***Teaching Reform And Graduates' Knowledge Attitude Practice Of Preventive Dentistry*** Chin-Ying S Hsu, Teresa Loh, Xiao-Li Gao, Grace Ong	SO-5
12:50 – 13:00	***Incorporating Periodontal Disease Risk Assessment In Treatment Planning*** S Mohd Said, TN Mohd Dom	SO-6

Time	Event	Venue
13:00 – 14:00	IADR – SEAADE Council Join Lunch	The Pond Cafe
13:00 – 15:00	Open to public	Arjuna Room

SEAADE POSTER SESSION
Time: 14:00 – 16:00 *Venue: Kharisma Ballroom*

Title and Author(s)	Code
Cross-Cultural Comparison Of Stress Between Malaysian And Indonesian Clinical Dental Students Fara Azwin A, Norhayati R, R Abdul Kadir, Sri S, Eky SSS	SP-1
Self-Reported Factors Anticipated By Dentalstudents To Be Associated With Successful Working Impression Registration Abd Shukor SS, Musa KA, Wan Daud WJA, Yahaya N	SP-2
Personality Profiles Amongst First Year Universiti Kebangsaan Malaysia And Relation To Their Semester 1 Academic Performance. AM Mohamed, Z Zanul Abidin, SH Jamhari, N Daud, M Ahamd, SR Abd Rahman	SP-3
Undergraduate Paediatric Dentistry Module: Dentistry For Children With Special Needs A Mahyuddin	SP-4
Survey On Problems In Removable Partial Denture Constructions In Faculty Of Dentistry, University Of Malaya SF Ahmad, S Slim, AA Aziz	SP-5
Teaching Of Geriatric Dentistry In The Undergraduate Curriculum TI Rosli, TN Mohd Dom	SP-6
Introducing Basic Clinical Dentistry To The Undergraduates In Universiti Kebangsaan Malaysia S Mohd Said, TN Mohd Dom, D Abdullah	SP-7
Multimedia Cases Designs For Pbl P Jessica Goset	SP-8
Effects Of Knowledge-Attitude-Behavior On Student Learning In Oral Epidemiology Chin-Ying SH, Tuo Sheng JK	SP-9

IADR/SEA 21th Annual Meeting Programme

POSTER SESSION I — 15:30-17:00 pm
Friday 7th September 2007 — Venue: Corridor

Abstract	Tittle
IP-017	A retrospective multi center of the clinical performance of Implant S.S. SHAYEGH
IP-018	Biocompatibility of Resin Acrylic after being soaked in Sodium Hypochlorite N. HENDRIJANTINI
IP-019	The Influence of Oral Appliances for OSA on Stomatognathic System K. SAITO
IP-020	Difference in Resonance Frequency of Post Varying Levels of Cement K.-Z. CHANG
IP-021	Effect of Oral Application of *Piper betle* Linn on Caries M.O. ROESLAN
IP-022	Salivary cholesterol level in atherosclerotic and healthy subjects SUHARNO
IP-023	Effectiveness Of Topical Fluoride Gels To Inhibit Artificial Caries Formation C. SUKSAROJ
IP-024	Effects of Dentifrices on Artificial Caries-like Lesions: a pH-cycling Study A. ITTHAGARUN
IP-025	Micro-CT evaluation of laser-fluoride effect on enamel demineralization Y.Y. LIU
IP-026	Microanalysis of Tooth Decay Caused By Clove Cigarette S. FARIDA
IP-027	Precision of clinical diagnosis of fissure caries by different methods D.S.H. YOU
IP-028	Accuracy of Demirjian"s age estimation method for Kuwaiti Children M.A. QUDEIMAT
IP-029	Contraction Characteristics of Lateral Pterygoid Muscle Fibers in Rats M. MIZUTANI
IP-030	Disproportional Growth Of Dentofacial Components In Hemoglobin-E Beta Thalassemia Patients L.D. SJAHRUDDIN
IP-031	The Effect of S. spontaneum L. on Rat's Heart M.D. SADONO
IP-032	Biocompatibility of Alginate Modified Bacterial Cellulose on Human Keratinocytes N. SANCHAVANAKIT
IP-033	Development of a mesoporous biomaterial for treatment of dentin hypersensitivity H.-J. CHEN
IP-034	Factors related to salivary flow rate and xerostomia in elderly L. MEUTIA SARI
IP-035	Oral Health Status of Elderly in West and South Jakarta A. ZAIRINA
IP-036	Apical Size Effect on Electronic Root Canal Length Determination K.-H. HUANG
IP-037	Correlation between the developmental stages of MP3 and cervical vertebrae J.W. SUDHANA

The Effect of *S. spontaneum* L. on Rat's Heart

M.D. SADONO[1], K. YULIYANTI[1], E.H. SUNDORO[2], S. MAAT[3], and S. KOSELA[2], [1]Trisakti University-INDONESIA, Jakarta Barat, Indonesia, [2]University of Indonesia, Jakarta Pusat, Indonesia, [3]University of Airlangga, Surabaya, Indonesia

Previous research to *S. spontaneum* L. revealed that a bleaching agent, who traditionally used for tooth discoloration. Therefore application should be under professional supervision. Since the safety level properties have not yet been established, especially to the vital organ. Objectives: The acute toxicity test of white Wistar rat's heart had been done to prove the safety level of *S. spontaneum* L. to one of the vital organ. Methods: Twenty four Wistar rats were used and devided into eight groups. The treated groups were given extracts with dosages of 0.5g/kgBW, 5g/kgBW and 15g/kgBW of the S. spontaneum L. orally, and were killed after 2 hours and 24 hours. The control group was also killed after two hours and 24 hours after applications with aquadest. Results: The Chi-square statistical test shows that χ^2 calculation of nuclear and myocardium fiber hypertrophy (χ^2=11.5000) less than χ^2 table (χ^2=12.017). Meanwhile the χ^2 of inflamed cells (χ^2=13.723) less than χ^2 table (χ^2=14.067). Conclusions: *S. spontaneum* L extract did not show any toxic effect to white Wistar rat's heart after 2 and 24 hours intake.

Biocompatibility of Alginate Modified Bacterial Cellulose on Human Keratinocytes

N. SANCHAVANAKIT, M. PHISALAPHONG, N. CHIEWPRAKOPKIT, and P. PAVASANT, Chulalongkorn University, Bangkok, Thailand

Bacterial cellulose is a potential biomaterial that can be used in several medical aspects. We have modified bacterial cellulose with alginate to enhance the tear resistance to sewing procedure when used as mucosal flap in oral tissue regeneration. **Objectives:** To investigate the growth of human keratinocytes (HaCat) and gingival fibroblasts (GF) on alginate modified bacterial cellulose. **Methods:** Cytotoxicity test was performed in L-929 mouse fibroblasts. Growth and spreading of HaCat and GF on each material were compared and analyzed by MTT and scanning electron microscopy. **Results:** No toxicity of the material was observed. Unmodified cellulose, but not alginate, supported growth and spreading of HaCat and GF. However modified bacterial cellulose could support only HaCat. **Conclusions:** Biocompatibility of modified cellulose to keratinocytes in combination with good tear resistance property suggests that modified bacterial cellulose has a good potential to be used in oral cavity to cover the surgical wound. This study was supported by Ratchadapisek Somphot Endowment, Chulalongkorn University 2006.