ผลของฟิล์มแบคทีเรียเซลลูโลสที่บรรจุเคอร์คูมินต่อเซลล์มะเร็ง

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EFFECTS OF CURCUMIN-LOADED BACTERIAL CELLULOSE FILMS ON CANCER CELLS

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ชยุตม์ ทรัพย์ทวีสิน : ผลของฟิล์มแบคทีเรียเซลลูโลสที่บรรจุเคอร์คูมินต่อเซลล์มะเร็ง (EFFECTS OF CURCUMIN-LOADED BACTERIAL CELLULOSE FILMS ON CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. เหมือนเดือน พิศาลพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. นพ. อมรพันธุ์ เสรีมาศพันธุ์, 91 หน้า.

งานวิจัยนี้ได้ทำการพัฒนาแผ่นฟิล์มแบคทีเรียเซลลูโลสที่บรรจุเคอร์คูมิน โดยแผ่นฟิล์มแบคทีเรียเซลลูโลสได้ถูกสังเคราะห์ด้วยการเลี้ยงแบคทีเรีย *Gluconacetobacter xylinus* ในอาหารเลี้ยงเชื้อที่มีน้ำมะพร้าวเป็นตัวทำละลาย จากนั้นแผ่นฟิล์มได้ถูกนำไปแช่ในสารละลายเคอร์คูมิน สารออกฤทธิ์หลักที่พบในขมิ้นชัน (*Curcuma longa* Linn) ที่ความเข้มข้น 0.5 และ 1.0 มิลลิกรัมต่อมิลลิลิตร โดยมีเอทานอลบริสุทธิ์เป็นตัวทำละลาย เป็นเวลา 24 ชั่วโมง แล้วตากให้แห้ง จากการทดสอบการปลดปล่อยของเคอร์คูมินในสารละลายบัฟเฟอร์ที่มีการเติมแต่งด้วย Tween 80 และ Methanol พบว่าอัตราการปลดปล่อยของเคอร์คูมิน เป็นไปในอัตราที่มีการควบคุม (Controlled release) และจากการทดสอบกับเซลล์มะเร็งของมนุษย์ ได้แก่ เซลล์มะเร็งผิวหนัง A375 malignant melanoma เซลล์มะเร็งลำไส้ใหญ่ HT29 และเซลล์มะเร็งเต้านม MCF7 พบว่าแผ่นฟิล์มแบคทีเรียเซลลูโลสที่บรรจุเคอร์คูมินมีความเป็นพิษต่อเซลล์มะเร็ง แต่ไม่เป็นพิษต่อเซลล์ปกติที่ทำการทดสอบ ได้แก่ เซลล์ผิวหนัง Keratinocytes และ Dermal fibroblasts รวมไปถึงเซลล์ kidney epithelium จากไตลิง (Vero) ดังนั้นคุณสมบัติของแผ่นฟิล์มนี้จึงเป็นประโยชน์ในการประยุกต์ใช้ในเชิงชีวเวช

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CHAYUT SUBTAWEESIN: EFFECTS OF CURCUMIN-LOADED BACTERIAL CELLULOSE FILMS ON CANCER CELLS. ADVISOR: PROF. MUENDUEN PHISALAPHONG, Ph.D., CO-ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., 91 pp.

In this study, a curcumin-loaded bacterial cellulose films were developed. Bacterial cellulose films were prepared by culturing *Gluconacetobacter xylinus* in coconut water-based medium. Curcumin, an active substance found in turmeric (*Curcuma longa* Linn), was then absorbed into never-dried bacterial cellulose pellicles by immersion in 0.5 and 1.0 mg/ml curcumin solutions, with absolute ethanol as solvent, for 24 hours. The curcumin-loaded bacterial cellulose pellicles were then air-dried. Controlled release of curcumin was achieved in buffer solutions containing Tween 80 and methanol additives, at pH 5.5 and 7.4. Curcumin-loaded bacterial cellulose films prepared with curcumin solutions at concentrations of 0.5 and 1.0 mg/ml demonstrated anticancer activity against A375 human malignant melanoma cells, HT29 human colon cancer cells, and MCF7 human breast cancer cells. On normal cells, specifically human keratinocytes and human dermal fibroblasts, along with Vero kidney epithelial cells, no significant cytotoxic effect was observed. These developed films have properties that would be beneficial in biomedical applications.

Field of Study: Biomedical Engineering Student's Signature .....

Academic Year: 2017 Advisor's Signature .....

Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

1.1 Background and Rationale

Skin cancer is currently one of the most common types of cancers, which is found in almost every types of society worldwide[1], particularly in western nations that majority of citizens have light-colored skin, such as United Kingdom[2, 3], United States of America[4], and Australia[5]. The incidence of malignant melanoma had been quadrupled between 1982-2012, with the riskiest group is in patients at the age of equal to or above 65 years old[2, 3]. Despite representing only 4% of all skin cancer cases, malignant melanoma is the cause of 65% of all deaths related to skin cancer[1]. Chronic and repeated direct exposure to ultraviolet radiation, mostly from direct sunlight, is the most important risk factor for malignant melanoma. There are also many risk factors that lead to malignant melanoma. The notable examples are prolonged sun exposure history during childhood, family history, immunosuppression, organ transplantation, aging, etc.[1, 2]. Occupations that involve extended period of sun exposure without protection is also a risk factor, such as soldiers[4]. The current practical treatment for skin cancer in the present is surgery, which involves incisions to remove the malignant melanoma tissue. Then the wound is either closed directly to allow natural healing, or re-constructed with skin graft, in which tissue engineered scaffold plays major role[2].

Colorectal cancer, or bowel cancer, is currently one of the leading causes of cancer-related death worldwide, especially in western-influenced lifestyle [6-12], with about 1-2 million new cases diagnosed, and more than 600,000 people were killed each year[13-15]. The major risk factors of colorectal cancer are family history, marital status, age (65 year old or older individuals have higher risk), inflammatory bowel disease, cigarette smoking, excessive drinking, high consumption of red and processed meat, obesity, and diabetes[14, 16, 17]. Currently, the treatment for this type of cancer is surgery[16], in which about 75% of patients accept treatment, and about 50% of patients can be treated without further treatments[18, 19].

Breast cancer is one of the most common cancers in existence in females, which is also currently one of the leading causes of female deaths[20]. In 2012, there were about 1.7 million new cases and 521,900 fatalities [21, 22]. It represents about 25% of all cancers and 15% of all cancer fatalities[23]. Globally, the annual trend of breast cancer is increasing [24]. The most significant risk factor is family history[20], because 5-10% of all breast cancer cases are caused by autosomal-dominant germline genetic mutations. In breast cancer patients with age below 45, they have high likelihood of mutation due to strong family history of breast and/or ovarian cancer[20]. Other risk factors are age, obesity, excessive drinking, lack of exercise, history of reproduction activities and menstruation in patient, benign breast lumps, hormonal conditions, marital status, high-risk occupations (e.g. farmers and workers in industries that patients expose themselves excessively to chemicals that are associated with breast carcinogenesis) and excessive radiation exposure[17, 23]. Currently, the majority of breast cancer treatment is surgical removal of cancer tissue from the breast, with some cases require supplementary treatments, such as chemotherapy, radiation therapy, or hormone therapy[20]. This is a highly risky approach that also requires the approval of the relatives of the patient before committing, and also impact the lifestyle of the patient significantly.

Curcumin is a natural, yellow hydrophobic polyphenolic compound, commonly found in turmeric[25-32], which is called as “Khamin Chan” (ขมิ้นชัน) in Thai[26]. It is one of the most common spices used in Thai cuisine, such as yellow curry, called as “Kang Kari” (แกงกะหรี่) in Thai, or northern Thai noodles, called as “Khao Soi” (ข้าวซอย) in Thai. There was an evidence that it has potential as a therapeutic agent in wound healing[33], diabetes[26], cardiovascular diseases[34], gastrointestinal diseases[25], and pulmonary diseases[35]. It also has antianglogenic, antiproliferative, anti-invasive, and chemopreventive properties[26]. These properties have made curcumin to be proved in some clinical trials that curcumin can treat colon cancer, inflammatory bowel disease, pancreatic cancer, etc[26]. US Food and Drug Administration (FDA) declared that curcumin is safe for humans[36, 37]. The joint FAO/WHO Expert Committee on Food Additives recommended daily intake of 0.1-3mg

of curcumin per 1kg of human body weight. However, curcumin has several limitations, particularly pharmacological properties, such as short half-life, poor absorption, poor bioavailability, and rapid metabolic rate when administered through gastrointestinal (GI) tract[36]. In order to solve this problem, development of proper drug delivery system is required.

Bacterial Cellulose (BC), a nanomaterial produced by *Gluconacetobacter xylinus[38, 39]* is a promising material to be used in biomedical applications and drug delivery system due to its unique properties, including outstanding biocompatibility, low toxicity, high porosity, and biodegradability, along with its remarkable physical properties[40-43]. BC has been used as tissue engineering scaffolds, because it facilitates cell attachment and proliferation on the BC scaffold itself[40, 44]. Due to its compaction property, it is one of the materials with long history in pharmaceutical industry. In pharmaceutical applications, it extends the period of model drug's release, making the release occurs in more controlled manner, improving pharmaceutical properties[40, 45].

Therefore, BC is one of the most promising candidates to be used in drug delivery, due to the properties previously discussed. When combined with curcumin, a natural substance declared with anti-cancer effects that is safe for humans, makes curcumin-loaded BC an ideal candidate for cancer treatment.

1.2 Objectives

1. To develop BC films containing curcumin
2. To investigate the cytotoxic effects of BC films containing curcumin on human colon, breast, and skin cancer cells.

1.3 Research Scopes

1. Preparation of BC film by using *Gluconacetobacter xylinus* bacterial strain.
2. Loading of curcumin into BC film.
3. Determination of actual curcumin content in curcumin-loaded BC film.
4. Investigate cytotoxic effects of curcumin-loaded BC film on colon, breast and malignant melanoma skin cancer cells, and compare with normal cells, including monkey's epithelial kidney cells and human skin cells (dermal fibroblasts, keratinocytes).

1.4 Benefits

This study will demonstrate effectiveness of cancer treatment by using BC films which encapsulate curcumin.

CHAPTER II

THEORY

2.1 Cellulose

Cellulose is the most abundant natural polymer in the world[39], and also important for the global economy[46]. It is estimated that 10^{14} tons of cellulose pulp is produced annually[46-48]. It is a homopolysaccharide that is well-known as the main structural molecule in cell walls of plant cells[49]. It was first discovered in 1838 by Payen[50]. Its molecular structure is a chain made of about 10,000 to 15,000 D-glucose blocks, linked by β1-4 glycosidic bonds[51], with chemical formula $(C_6H_{10}O_5)_n$[39]. Its abundance of hydroxyl (-OH) groups is from hydrogen bonds between different polymer chains and within the same polymer chain, contributing to its remarkable hydrophilicity, chemical modifiability, and biocompatibility[44]. The most stable conformation of glucose is when each of the "chair" of glucose building blocks is turned 180° relative to its neighbors that give out straight chains[40]. When several chains are placed side-by-side, the hydrogen bonds between chains' hydroxyl groups and within each of specific cellulose chains themselves, are formed, leading to its stability[52]. This results in its distinctive material properties, including its tensile strength, and its insolubility in common solvents that makes it a natural reinforcing agent. Furthermore, it has thermal stability of 250-300°C[44]. Many useful materials, such as paper, cardboard, etc., are manufactured from cellulose[39].

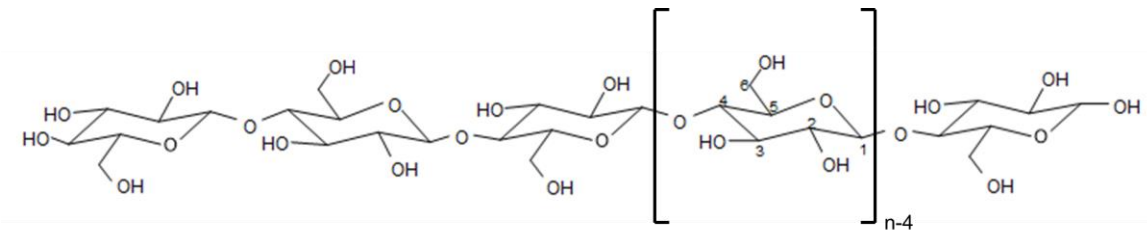


Figure 1: Molecular Structure of Cellulose[44, 52]. n=degree of polymerization

However, most of raw materials for cellulose products are plant-derived, which contain other components such as lignin and hemicellulose that are present in natural wood[41, 47, 53]. This requires multiple chemical treatments in order to obtain pure cellulose and contaminants of residues from chemicals used in treatment are mostly toxic. If cellulose purification was not properly done, toxins may present. This might react with any reagents during preparation of cellulose-based products, and eventually cause effects to health if contaminants are unintentionally left over to the biomedical applications[53]. Furthermore, plant-derived cellulose has long production time, due to the time required for plant growth, and also require more energy compared to BC production[53].

2.2 Bacterial Cellulose

Bacterial Cellulose (BC) is a type of nanocellulose[40], in which the terminology can be described as the nanostructured derivatives of native cellulose. The molecular structure of BC is identical to plant cellulose[39, 45, 50].

Acetic acid bacteria of the genus *Gluconacetobacter* are microorganisms that are the most efficient cellulose producers[39, 48, 54]. The cellulose-producing species of *Gluconacetobacter* was first isolated in 1886 and initially named as *Acetobacter xylinum (A. xylinum),* which was then later changed to *Acetobacter xylinus (A. xylinus),* and eventually *Gluconacetobacter xylinus (G. xylinus)*[38]. This genus is further divided into two groups, which are motile *Gluconacetobacter liquefaciens,* with presence of flagella, and non-motile *Gluconacetobacter xylinus,* that do not have flagella[54]. Naturally, *G.xylinus* is found on the fruit and leaf surfaces, and the role of bacterial cellulose synthesized in the nature is to protect a bacterial colony on the surface of the fruit and leaf from unfavorable environmental factors [55]. In cellulose-producing bacteria, the key enzyme of cellulose biosynthesis is cellulose synthase, which synthesis of this enzyme is from bcs “bacterial cellulose synthesis” operon located in genes of bacterial cells. These operons usually consist of three (bcsAB, bcsC, bcsD) or four genes (bcsA, bcsB, bcsC, bcsD), which, in *G.xylinus*, is expressed constitutively [54, 56].

According to M. Iguchi et al (2000), Cellulose molecules are synthesized in the interior of bacterial cell, which are then spun out through cellulose export components (Figure 2). During cell division, three-way branch of fibers are inevitably formed when fibril extrusion from cells is continued from mother to daughter generation of cells. The fibrils are narrower at the branching point if number of nozzles at the cell division state is not normal, but normal fibril diameter may be recovered when time progresses, causing non-linear appearance of fibers. The average segmental length of fibers between branching points is around 580-960μm due to bacterial lifetime and fibril growth rate. The existence of branching fibers is related to toughness of the pellicle and resistance against stretching, which contribute to its excellent mechanical properties[57].

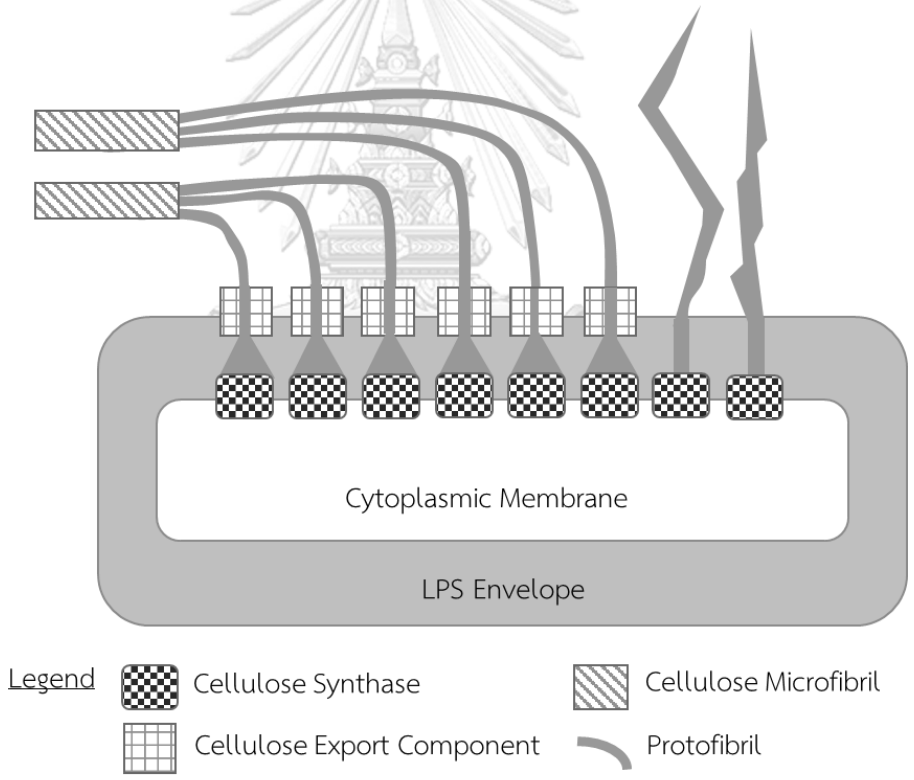


Figure 2: schematic diagram of BC synthesis from bacterial cell[48, 57].

The synthesis process of BC is commenced when the cellulose-producing bacteria species is cultured in an optimal culture medium[57]. There are two main methodologies for producing BC by using microorganisms. This includes static culture

and stirred culture [47, 48]. For static culture, it is carried out in static (stationary) condition at 28-30ºC. When the culture is progressed, the system becomes more turbid, and then thick, white, and translucent pellicle appears, which the thickness of the pellicle will be increased steadily with time [47, 57]. (Figure 3) It is important to note that the cellulose synthesis is an aerobic process, so the production of BC is done only in the vicinity of surface. Therefore, surface area is an important factor on production of BC. Larger the surface area, higher the production rate of BC. As long as the system is stationary, the disc-shaped gel is suspended in the culture by cohesion to the culture to the interior wall of a culture vessel, and slides downwards steadily when the gel thickens further. However, if the culture is done in conical flask, the growth of continuous gel is tended to fail[57]. BC pellicles produced by this methodology have good mechanical properties and yield rate. In stirred culture, BC is formed in dispersed manner, which results in irregular pellets or suspended fibers that use less culture time than static culture. However, the pellets produced by this methodology have inferior mechanical properties and lower yields than static culture. There are also higher risks of microorganism mutation that may affect BC production process [47]. Therefore, the choices of methodologies for BC culture depend on applications, because when designing the products, the material properties (i.e. physical, morphological, mechanical properties) always play major role in production of components, and most importantly, cost of production, especially when scaling up to industrial production is concerned.

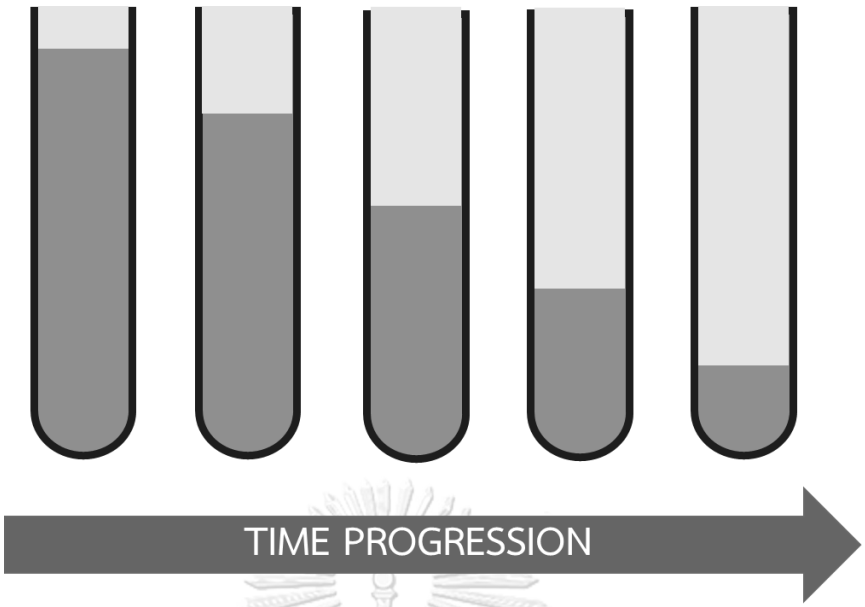


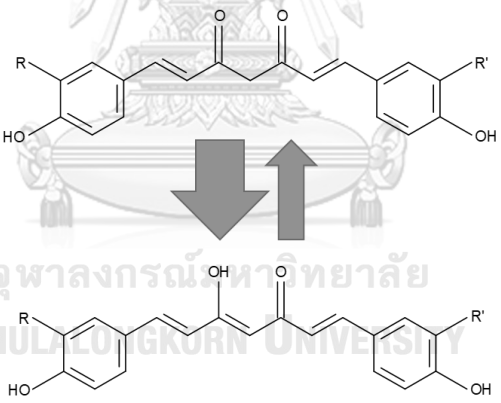
Figure 3: BC growth progression[57]. Darker shade (lower portion) represents BC, and lighter shade (upper portion) represents culture medium containing cellulose-producing cells.

In biomedical applications, where purity is concerned, residual bacteria contained in BC pellicles can be removed after harvesting from culture by immersing the pellicles in dilute alkaline solution and wash with water. Treatment by oxidant may be applied after previous methodologies are done if purification stated previously is not sufficient [57].

BC has been increasing popular in current biomedical research because of its desirable properties, including its environmentally friendliness, high water holding capacity, insolubility, biodegradability, biocompatibility, biofunctionality, elasticity, transparency, non-toxic, large surface area to volume ratio, and ease of sterilization, especially ability to be autoclaved at standard conditions (121°C, 15min) [45, 47, 50, 58]. Examples of significant biomedical applications of BC, are artificial skins, artificial blood vessels, tissue engineering scaffolds, drug delivery system, protein adsorbent, nanocomposite membranes, cardiovascular tissue replacements, and dental implants. It is also used as component of nanocomposites in other applications [53, 58].

2.3 Curcumin

Curcumin is a polyphenolic compound, with scientific name "diferuloylmethane", or fully identified as "1,6-hepta-diene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E)"[26, 59-61], or "1, 7-bis 4-hydroxy-3-methoxyphenyl-1,6-heptadien-3,5-dione"[62]. It is a bis-a,b-unsaturated b-diketone, with chemical formula $C_{21}H_{20}O_6$, molecular mass of 368.37g/mol[59], and melting point of 183°C[26]. It was first isolated in 1815, and its crystalline form was first obtained in 1870[26, 61]. Its appearance is a yellowish-orange powder that is relatively hydrophobic – insoluble in water but soluble in ethanol, dimethylsulfoxide (DMSO), and acetone[26, 36]. The maximum spectrophotometric absorption of curcumin occurs at 430nm in methanol, and 415-420nm in acetone, appearing as bright yellow solution at pH 2.5-7 and red when pH is more than 7[26].



Variant	R	R'
Curcumin	OCH_3	OCH_3
Demethoxycurcumin	H	OCH_3
Bisdemethoxycurcumin	H	H

Figure 4: Molecular structure of curcumin and curcuminoids (Curcumins II and III) found in turmeric[59]

It is an active compound found in turmeric, a spice produced by extracting the rhizomes of *Curcuma longa Linn*[25, 29, 32, 36, 62-64], the herb commonly cultivated in tropical areas of India, southeast Asia, and China[26, 29, 59]. It is a plant that belongs to the same family as ginger[36]. It is traditionally used as food ingredients, especially spice in food such as yellow curry, that curcumin contributes to its distinctive color and odor, and also used in cosmetics, and traditional medicine[26]. In traditional medicine, the yellow fraction contains curcuminoids, which are molecular derivatives of curcumin[59, 65]. The extract of Curcuma Longa consists of 60-70% carbohydrates, 6-8% protein, 3-7% essential oils, 5-10% fixed oils, 2-7% fiber, 3-7% minerals, and 2-6% curcuminoids[59]. It was also recently founded that the natural cellulose fibers extracted from the stems of this plant has antimicrobial activities against both gram positive and gram negative bacteria[64].

In the turmeric extract, the majority of curcuminoids found are demethoxycurcumin (Curcumin II) and bisdemethoxycurcumin (Curcumin III). However, in commercially available curcumin, the approximated composition is as follows: 77% Curcumin I; 17% Curcumin II; 3% Curcumin III[26, 30, 60].

There are two types of curcumin conformations, enolic, found above pH 8, and beta-diketonic form, found in acidic and neutral conditions[59], and in solid phase[36]. In most solutions, curcumin exists in enolic form[26]. This conformation plays a major role in radial-scavenging ability of curcumin[26], and makes curcumin an ideal chelator of metallic ions that are found in active sites of the protein [59].

Curcumin is relatively stable in an acidic solution, because the degradation process occurs extremely slowly at pH 1-6 .However, it is unstable in neutral or basic pH ranges, which makes rapid degradation into ferulic acid and feruloylmethane, as there was a study that 90% degraded in phosphate buffer at physiological pH (7.4) [26, 66]. Furthermore, it is relative stable in human cell culture medium containing 10% fetal bovine serum (FBS) and in human blood, as the half-life of curcumin is increased by the presence of protein content [26, 67].

When curcumin is ingested through digestive tract, the degradation of curcumin occurs by reduction and conjugation [67]. The process commences with O-conjugation of curcumin, forming curcumin glucuronide and curcumin sulphate, then reduced into tetrahydrocurcumin, hexahydrocurcumin, octahydrocurcumin, and hexahydrocurcuminol, and finally undergone glucuronidation into curcumin glucuronide, dihydrocurcumin glucuronide, tetrahydrocurcumin glucuronide, and curcumin sulphate[36]. Furthermore, the poor permeability of intestinal cells also contribute to poor bioavailability in this path[68].

Due to its relatively bad bioavailability, the delivery systems have been implemented to improve. The examples of curcumin delivery systems previously studied are curcumin nanodisks[69], liposome encapsulation[70, 71], gelatin/silk fibroin based delivery systems [29, 63], magnetic nanoparticles[72], polymeric nanoparticles[73, 74], peptide nanofibers[75], catanionic lipid nanocarriers[76], whey protein encapsulation[77], mesoporous silica particles[78], chitosan/cellulose microcrystals[79], poly(butylene adipate-co-terephthalate) electrospun nanofibers[80], and nanovesicles[81].

2.4 Controlled Release drug delivery systems

Drug delivery system (DDS) is an approach to improve the delivery of the active component (drug) to its intended target[82]. The development of DDS encompasses multidisciplinary approaches[83], which addresses the limitations of the conventional therapies[84]. Controlled release DDS is a DDS which the active component is loaded into developed carriers, designed to provide a predictable rate of *in vivo* release when administered into the body, either through invasive (injection) or non-invasive (non-injection) approaches [84, 85].

The objectives of DDS are not only providing a targeting towards the target of administration (such as tumors in anticancer drugs) with enhanced pharmacological properties, but also improving convenience of use and reduce side effects to the patients compared to conventional therapies[86]. In case of applications that require

extended release, or larger therapeutic window, the controlled release system is designed to extend the duration of release, in which the system is described as “sustained delivery DDS”[87]. Therefore, the theoretically ideal DDS must deliver the active component in specific, intended target in desirable properties, including release time, release rate, and release pattern[88]. The choices of the material used for the DDS are dependent on these following factors: target drug release profile, the properties of an active component itself, and the site of application[89].

According to Kunal J. Rambhia et al (2015), controlled release DDS is created by loading the active component into the material, which will be used as a controlled release matrix. This can be done in any methodologies, whether chemically or physically. When the controlled release DDS is in real use, the active component was released. This can be achieved by either diffusion of an active component out of the matrix, or degradation of the matrix material itself. The release rate can be controlled by adjustments of the absorption process when loading an active component into the matrix and/or the adjustments of the properties of the material used as the matrix[90]. The tissue engineering scaffolds can also be controlled release matrix, as active components that promote tissue regeneration may be loaded into the scaffold material, and controlled release facilitates tissue regeneration[91].

The examples of the previously developed controlled release systems are bacterial cellulose films for controlled release of ethanolic extract of mangosteen peel[41], bacterial cellulose for transdermal delivery of diclofenac[92], injectable hydrogels for controlled antibody delivery[93], nano-ethosomes for transdermal ketoprofen delivery[94], silk fibroin and gelatin multilayer film system[86], calcium carbonate microcapsules which use chondroitin sulfate that enable selective control of loading and release of drug[95], methotrexate in oleic acid based liposomes for transepidermal delivery targeting psoriasis[96], cellulose acetate loaded with thymol[97], and poly(lactic-co-gycolic acid) (PLGA) nanoparticles loaded with Tamoxifen to treat breast cancer[98].

2.5 Skin Cancer cells

Skin cancer (cutaneous carcinoma) is a cancer that is formed from malignancies in epidermal cells[99]. It can be classified into two major types, including malignant melanoma, and non-melanoma[5, 100], which can be further classified into two major subtypes, basal cell carcinoma, and squamous cell carcinoma[1, 2, 100].

Basal cell carcinoma is a basal cell-derived malignant neoplasm, which usually appears without lesions that are precursors[1]. It is the most common type of malignant tumor occurring on the skin parts that are exposed to sunlight, which its name is derived from its similarity in terms of histological characteristics to the normal epidermal basal cells [3]. It appears as a red nodule with telanglectasia that has slow growth rate but frequent ulceration occurs. In some cases, erythematous macules or patches or scar-like plaque may occur[100].

Squamous cell carcinoma is a dermis-invading malignant tumor originated from epidermal keratinocytes[1]. Lighter the skin color, more likely it occurs[99]. Due to its invasion, it causes extensive tissue destruction and hematogenous metastases by spreading throughout body through lymphatic system (which eventually enters blood circulation) in more advanced stages[1]. It appears as an indurated nodule with rapid growth, or non-healing ulcer in the sun-exposed skin[100].

Malignant melanoma is a malignant tumor that is derived from epidermal melanocytes, which is induced through suppression of skin's immune function, melanocyte cell division induction, and production of free radicals, which eventually lead to DNA damage in melanocytes[1]. It appears as irregular bordered pigmented lesions that also have eventual changes such as enlargement, itching, and bleeding that patients report associated painful feeling[100].

2.6 Colorectal Cancer cells

Colorectal cancer is a multi-factorial disease that occurs from multistep progression of events beginning from mutations in the epithelial and preneoplastic cells in colon and rectum [8, 101]. The cancer is mostly originated from large, benign

high-grade pedunculated adenomas (polyps), with more than 50% of large polyps (diameter $\geq$ 2cm) undergone mutations, which eventually developed into cancer, while small polyps (diameter $\leq$ 5mm) almost never developed into cancer [16]. According to D. Treanor et al (2007), It can be classified into three major subtypes: Adenocarcinoma, Mucinous adenocarcinoma, and medullary carcinoma [102].

Adenocarcinoma is a cancer tissue which is originated from the proliferation of mutated columnar surface epithelium[103]. It is the most common type of colon cancer, representing about 90-95% of all cases[8]. It usually has moderate differentiation, and despite not having distinctive histological features like other types of colorectal carcinoma, it occasionally exhibits the cribriform patterns that have central necrosis[102].

Mucinous adenocarcinoma is a special case of adenocarcinoma that exhibits mucinous component, accumulated in glandular lumen or within the tumor cell itself[103]. Its distinctive feature is its ability to secrete extracellular mucin[102].

Medullary carcinoma is a solid type of adenocarcinoma with glandular differentiation and slight cellular pleomorphism almost approaches zero[103]. Its distinctive phenotype is a right-sided tumor that has sheets of cells, which includes tumor-infiltrating lymphocytes[102].

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2.7 Breast Cancer cells

Breast cancer is a disease when there are malignancies occur in a heterogenous group of epithelial tissue in mammary glands and milk ducts[20, 104]. Its heterogeneity makes this type of cancer challenging to diagnose and eventually treat[105].

There are two major subtypes of breast cancer, according to Parveen Kumar et al (2013), namely ductal carcinoma and lobular carcinomas[20].

Ductal carcinoma is a cancer that is originated from a proliferation of abnormal, mutated epithelial cells within the lumen of the breast ductal system, which supplies milk to the nipple, representing 90% of breast cancer cases[20, 106].

Lobular carcinoma is a cancer that is originated from the terminal duct lobular unit in the milk-producing glands, located deeper into the breast. The cells, usually small in size, non-coherent, and monomorphic, may migrate between the basement membrane and normal epithelial cells along the breast ductal system [20, 107, 108].

For both types of cancer, the tissue remains confined (in situ) in the early stages, which can be treated by surgical removal by lumpectomy, which is a surgical removal of only a tumor. However, in later stages, it is invaded into other areas of the breast tissue, becoming invasive cancer, which would require mastectomy, a surgical removal of the breast [20, 106, 109].

CHAPTER III
LITERATURE REVIEW

3.1 Effects of curcumin on cancer cells

In breast cancer cells, B.Chen et al (2014)[110] found that curcumin inhibited the proliferation by up-regulating the expression of Nrf2, the principal regulator of antioxidant defense system in cells. Curcumin also involved in inhibition of Fen1, a DNA repair-specific nuclease, expression, through RNA interference process. Furthermore, it downregulated Fen1 gene expression and promotor activity in Nrf2-dependent manner, and induced Nrf2 recruitment on Fen1 promotor in MCF-7 cells.

In thyroid cancer cells, XiaoBo Xu et al (2014)[111] found that curcumin had cytotoxicity to the cells, by inhibiting cell growth, induced cell cycle arrest through inhibition of DNA replication and protein synthesis, and interrupting the cell cycle of thyroid carcinoma cells. Furthermore, not only curcumin was cytotoxic due to actions to the cells previously described, but also due to its ability to induce apoptosis (programmed cell death), and suppressed cell metastasis through inhibitions of cell adhesion, cell migration, and cell invasion, which are essential processes that cells maintain their life. Furthermore, it also reduced p-Akt protein expression that led to reduction of expressions of Matrix Metalloproteinases, which are prerequisites for tumor invasion and metastatis due to its abilities of tissue remodeling via ECM, basement membrane hydrolysis, and angiogenesis (blood capillary formation) induction, and COX-2, which participates in malignant tumor development, in which its overexpression is associated with tumor angiogenesis, which formed blood capillaries to supply tumor cells with oxygen and nutrients, and might spread cancer to other parts of the body through bloodstream.

In papillary thyroid cancer cells, C. Zhang et al (2013)[112] found that curcumin inhibited cell viability, cell invasion, cell attachment, cell spreading, cell migration, and suppressed MMP9 activities and expressions.

In human colon cancer cells, Jane L. Watson et al (2008)[113] found that curcumin was cytotoxic, and curcumin-mediated killing of colon cancer cells was independent of p21, the major transcriptional target of p53 tumor suppressor. Cells were died mainly due to apoptosis. However, normal dermal fibroblast cells, which they used as control, were intact in low curcumin concentrations. However, at higher curcumin concentrations, normal cells were killed, which this study demonstrated that in order to be cytotoxic to cancer cells, but not cytotoxic to normal, healthy cells, suitable range of curcumin concentrations must be used. Yue Guo et al (2015)[114] found that curcumin suppressed anchorage-independent growth of colon cancer cells. Curcumin also increased the transcription of DLEC1 tumor suppressor, which its overexpression is associated with colony formation repression of the cancer cell line. Furthermore, they also found that curcumin altered the expression of epigenetic-modifying enzymes in HT29 cells.

In leukemia cells, A. Shakor et al (2014)[115] found that curcumin induced ceramide accumulation, which was related with cell viability reduction. Curcumin was also found to induce nSMase activation, reduce the activities of SMS, an enzyme that catalyzed the process of conversion of ceramide into sphingomyelin, and reduce the activities of GCS, which is an enzyme involved in ceramide metabolism. They concluded that curcumin induced ceramide-regulated apoptotic cell death.

In cutaneous T-cell lymphoma cells, D. Yosifov et al (2014)[116] found that curcumin reduced cell viability by exerting cytotoxic and proapoptotic effects. Curcumin also shrunk the nucleus, and induced chromatin condensation in cells.

In prostate cancer cells, Jian Sha et al (2016)[117] found that curcumin inhibited cell proliferation and induced apoptosis in cells. Furthermore, they found that curcumin induced G0/G1 cell cycle arrest.

In non-small cell lung cancer cells, S. Lev-Ari et al (2016)[118] found that curcumin suppressed biological signals that lead to cancer, leading to apoptosis and inhibition of cell proliferation.

In ovarian cancer cells, J. Seo et al (2016)[119] found curcumin involvement on decreasing cell viability and increased apoptosis, stimulated by increasing of Ca^{2+} concentration in cytosol.

In pancreatic cancer cells, Jia ma et al (2014)[120] found that curcumin inhibited cell proliferation, migration, and invasion in pancreatic cancer cells, while induced their cell apoptosis, by increasing miR-7 expression.

In malignant melanoma cells, Y.P. Zhang et al (2015)[121] found that curcumin decreased cell number. It also caused cell contraction, shrinkage, and detachment from adherent state. Their observations indicated that there were more scratches in cell culture in groups treated with curcumin than in control groups. It lowered the cell proliferation, and increased apoptosis of A375 cells. Furthermore, it caused significant dose-dependent decrease in Bcl-2, which is an apoptotic protein.

From literature previously discussed in this section, it can be concluded that curcumin inhibited the proliferation of cancer cells by inducing apoptosis, or "Programmed Cell Death". Furthermore, they also inhibited the vital processes of the cell, including cell adhesion, cell migration, cell invasion, and cell spreading, which means that curcumin is cytotoxic to cancer cells, but not to normal cells if used in right concentrations. The process was done through inhibitions of mechanisms of cell maintenance and growth in cancer cells, while promoting mechanisms that lead to apoptosis. The effects can be observed in both morphologically and in cell culture assays. Therefore, development of drug delivery system is required to further lower the effects on normal cells, while also increase its pharmaceutical properties. However, these works are done with raw curcumin without any encapsulation. Effects of curcumin encapsulated in BC on cancer cells remains unstudied.

3.2 Limitations of conventional curcumin administration

According to the review paper from K. Mahmood et al (2015), curcumin has extremely low water solubility, low stability, rapid metabolic rate, and poor absorption, which lowers its bioavailability and reduces health benefits. In order to overcome these

limitations, drug delivery systems (DDS) are means to improve the lifespan of curcumin in blood circulation, its permeability, or making it lesser-prone to molecular degradation, which is very vital of designing cancer-treating drugs. The main criteria for designing DDS is to maximize the therapeutic efficiency of the drug in targeted disease treatment, which in this case is to kill cancer cells, while decreasing its side effects, especially to normal cells that surrounding cancer cells[59].

3.3 Applications of drug delivery system on curcumin

Vaishakhi Mohanta et al (2014)[122] fabricated nanocrystalline cellulose thin films by layer-by-layer approach, and studied its feasibilities for delivering curcumin as a hydrophobic drug model. They conjugated curcumin with nanocrystalline cellulose, before thin film assembly by using layer by layer methodology, because they found that curcumin could not be loaded into layer-by-layer assembly by using conventional methodologies. However, they found that nanocrystalline cellulose could interact with curcumin favorably, and achieved sustained release rate.

Leticia Mazzarino et al (2014)[123] developed chitosan films that contain curcumin-loaded polycaprolactone nanoparticles. They used chitosan films that were prepared by casting/solvent evaporation method, which used glycerol as plasticizer. Then they loaded with nanoparticles previously stated. *In vitro* release studies, performed by Franz cells and commercially available dialysis cellulose membranes revealed that films containing free curcumin release significantly slower than curcumin-loaded nanoparticles. This correlated with low solubility of hydrophobic drug when loaded into chitosan films. Furthermore, films with free curcumin also had lower actual curcumin content compared to films loaded with curcumin-loaded nanoparticles.

Kantarat Lerdchai et al (2016)[63] encapsulated curcumin in Thai silk fibroin/gelatin sponges. They found that curcumin was released controllably during six-hour period, beginning with rapid burst at first 15 minutes, followed by controlled-rate release. *In vitro* tests revealed that curcumin-loaded sponges have no cytotoxicity to L929 mouse fibroblast cells, but had growth inhibition effects on cervical cancer cells.

The release rate was dependent on crosslinking in the matrix structure. The main factors for this was the ratio between silk fibroin and gelatin during sponge preparation.

S.K. Bajpai et al (2015)[79] loaded curcumin into chitosan/cellulose microcrystal composite films. They demonstrated controlled release of curcumin over a period of 36 hours, which the amount released was inversely dependent on cellulose microcrystal concentration in chitosan matrix. This was due to cellulose microcrystal produces additional crosslinks, which reduced release rate. The film demonstrated fair antibacterial and antifungal activities.

Premika Govindaraj et al (2014)[124] loaded curcumin in polyacrylonitrile films. They found that the curcumin/polyacrylonitrile film were not cytotoxic to mouse embryonic fibroblast cell line. *In vitro* release assays revealed the initial curcumin burst release in first five hours then steady, controlled release occurred until the end of 24-hour test period.

S. Manju et al (2010)[125] fabricated hollow polyelectrolyte multilayer microcapsules by layer by layer methodology for encapsulation of curcumin in order to allow controlled release. The microcapsules were fabricated with six double layers of poly(sodium 4-styrene sulfonic acid) and poly(ethyleneimine), then curcumin was loaded. They found that the permeation of curcumin through the capsule layers was a determining factor for release rate of curcumin. This could be explained that the release was diffusion-controlled. The system was capable of releasing curcumin for prolonged period without initial burst, suggesting that multilayer assembly plays major role for continuous, controlled release. Furthermore, they also proved that the bioactivity of curcumin was retained despite of encapsulation.

From literature previously discussed in this section, curcumin was highly compatible with controlled release applications. The activities of curcumin were preserved despite being loaded into controlled release matrix. However, the release rate was controlled by the design of the controlled release material. The loading efficiencies of curcumin on film-based applications may require modifications, such as encapsulations of curcumin in micro- or nanomaterials, before loaded on film or

material, in order to improve loading efficiencies. The examples previously discussed suggests that materials used for controlled release in different applications were dictated by implementations, and improvements may be required to meet the criteria in each application. The relationship between BC, an ideal material for drug delivery due to its exceptional activities, and curcumin is remain unstudied.

3.4 BC applications in drug delivery

Astrid Muller et al (2013)[45] studied the feasibility of applying BC in drug delivery system, by using albumin as model. They found that the loading and release processes were controlled by diffusion and swelling controlled mechanisms similar to the behavior of hydrogels. In never dried samples, the drug loading was better than in lyophilized samples, which correlates to structural changes caused by lyophilization. Therefore, they concluded that BC is an innovative drug delivery material due to its remarkable biological and physical properties, biodegradability, ease of sterilization, and its capability to absorb and release active agents at a controlled rate.

S. Taokaew et al (2014)[41] loaded BC film with ethanolic extract of mangosteen peel, in which its bioactive compounds have antioxidant, antibacterial, and anti-inflammatory effects. BC film in this case was used as a controlled release matrix of the crude extract of mangosteen peel, and its effects against melanoma and breast cancer cells were investigated. They found that the bioactive components of mangosteen were absorbed into BC pellicles within 24-hour immersion period. The interactions between mangosteen extract with BC was weak. In non-transdermal conditions, the factors that dictated the release rate were concentration, immersion time, and pH of the medium. In transdermal conditions, about 59-62% of loaded phenolic compounds were released, and more than 95% of bioactive compounds were released. When treated with melanoma and breast cancer cells, the viabilities were significantly reduced, and morphologies revealed significant damages to the cells, such as cell shrinkage and membrane damage.

Maximiliano L. Cacicedo et al (2016)[126] loaded doxorubicin, which is a drug in anthracycline family extensively used in cancer treatments, in BC film. The film was modified by adding alginate during bacterial culture. They found that the cell viabilities of human colorectal adenocarcinoma cell after specified release periods were significantly reduced when doxorubicin was encapsulated in BC film compared to free doxorubicin, which reflected the controlled rate of the release.

Wei Shao et al (2016)[127] loaded tetracycline hydrochloride in BC film, and they found that tetracycline hydrochloride (TCH) was steadily released after initial burst release. However, when compared to free TCH, the release rate of BC-encapsulated film was slowed down. This was happened due to electrostatic interactions between BC and TCH that each of them were oppositely charged, with negatively charged BC and positively charged TCH. This suggests that BC can be used as controlled release of drugs for treatment of infection and inflammation.

Eliane Trovatti et al (2012)[128] applied BC for transdermal delivery system of lidocaine hydrochloride and ibuprofen, which they used as model hydrophilic and hydrophobic drugs respectively. They found that the release of the lidocaine hydrochloride release more slowly than the release of ibuprofen, because BC is a hydrophilic environment, and less polarity means less interactions with BC ultrastructure. However, there are other factors that also affected the release rate, such as drug loading formulation and target the hydration status of the skin.

Sebastian Moritz et al (2014)[129] loaded BC with octenidine. They found that octenidine-loaded BC was highly biocompatible with human keratinocytes, and stable for up to six months of storage without penalties in terms of its biological and physicochemical properties. Its release rate began with an initial burst then released steadily to maintain drug level, making it suitable for wound dressing applications. Furthermore, loading octenidine into BC did not alter the mechanical properties of BC.

Nuno H.C.S. Silva et al (2014)[92] loaded diclofenac sodium salt, a water-soluble drug, into a BC membrane. They found that diclofenac-loaded BC membrane was homogenous, and the permeation rate of diclofenac when loaded in BC

membrane was similar to commercially available patches, but lower than in gels. It was also flexible, allowing easy manipulation and skin adhesion. They confirmed that BC was capable to be used as controlled-release matrix for diclofenac. When combined with the excellent properties of BC, this makes BC an ideal candidate for drug delivery applications.

From literature previously discussed in this section, BC is an ideal candidate for drug delivery because of its capability to absorb and release active agents at a controlled rate. It has excellent storage stability, and it has electrostatic interactions when applied with molecules that are positively charged, due to its negative charged functional groups. When it is used with hydrophobic molecules, it interacts less with its ultrastructure, preserving functionalities. The examples of BC applications previously discussed confirmed that BC, when combined with its remarkable biological, mechanical and physical properties, biodegradability, and ease of sterilization, makes BC an ideal drug delivery material. However, applications of BC in curcumin delivery remains unstudied.

CHAPTER IV
EXPERIMENT

4.1 Materials and Equipment

4.1.1 Chemicals and Reagents:

All of chemicals and reagents listed below were supplied in analytical grade, except as noted:

- Coconut Water (Extracted from fresh coconut, purchased from beverage/juice shop (no.8) in Chulalongkorn University Student Dormitory cafeteria)
- Sucrose (Ajax Finechem Pty Ltd)
- Ammonium Sulfate (Ajax Finechem Pty Ltd)
- Glacial Acetic Acid (QReC)
- Absolute Ethanol (Merck)
- Curcumin (Sigma-Aldrich)
- Sodium Hydroxide (LOBA Chemie, Ajax Finechem Pty Ltd)
- Sodium Acetate (Ajax Finechem Pty Ltd)
- Phosphate Buffer Saline (PBS) (supplied as tablets, each for 100mL of PBS) (VWR Lifesciences)
- Dulbecco's Modified Eagle's medium (DMEM) cell culture medium (Gibco)
- Roswell Park Memorial Institute (RPMI) #1640 (RPMI-1640) cell culture medium (Gibco)
- Fetal bovine serum (FBS) (Gibco)
- Antibiotic solution, containing Penicillin and Streptomycin (Gibco)
- Dimethylsulfoxide (DMSO)
- Acetone (RCI Labscan)

- Dimethylacetamide (RCI Labscan)
- Methanol (Ajax Finechem Pty Ltd)
- Tween® 80 (Sigma-Aldrich)
- MTT solution
- PrestoBlue solution (Life Technologies)

4.1.2 Bacterial Strain

Gluconacetobacter xylinus AGR60 (isolated from *nata de coco),* provided as stock culture, kindly supplied by Pramote Thammarad, Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand[41].

4.1.3 Cells

Monkey kidney epithelial cell line (Vero), Human Colon cancer cell line (HT29), and Human breast cancer cell line (MCF7), were provided by Scientific Instrument center, Faculty of Science, King Mongkut's Institute of Technology Lat Krabang (KMITL), Bangkok, Thailand. Human Dermal Fibroblast (HDF), skin cancer cell line (A375) and human keratinocyte cell line (HaCat) were provided as -80°C frozen stock, under courtesy from Amornpun Sereemaspun, M.D., Ph.D., Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[130].

4.1.4 Equipment

- 14.5cm glass Petri Dish with cover
- Alcohol Lamp
- Biosafety cabinet (Class 2), separate ones for bacteria and human cell cultures
- Magnetic Stirrer

- Fume Hood
- Autoclave
- Autopipette
- 24-well cell culture plate (Corning)
- 96-well cell culture plate (Corning)
- 5mL syringe
- Syringe filter
- 15mL, 50mL centrifuge tubes
- Hemocytometer
- Cover glass (square and 15mm round)
- UV-visible spectrophotometer
- Multiplate reader (Thermo Scientific)
- Cell culture flasks (T-25, T-75)
- Phase-Contrast inverted Microscope
- 37C CO_2 Incubator (Thermo scientific)
- Franz diffusion cells
- 1mL syringe
- Needle
- Serological tubes

4.2 Preparation of BC Film

BC was prepared by methodologies described by Siriporn Taokaew et al (2014)[41]. The medium for *G.xylinus* culture was coconut water containing 5% glucose, 0.5% ammonium sulfate ($(NH_4)_2SO_4$), and 1.0% acetic acid (CH_3COOH). It was sanitized by autoclaving at 110°C temperature for 5 minutes. 75mL of medium was added to

each of the 14.5cm petri dishes, which were used as culture dishes. After that, 3.75mL of bacterial preculture was added into each of the culture dishes. Then the culture dishes were shaken gently. After that, took out of biosafety cabinet and left at ambient conditions for 7-9 days until BC fully occupied culture dishes.

After 7 days of culture, BC pellicles were harvested from the culture, washed with water, then immersed in sodium hydroxide solution for 24 hours, and washed with running tap water for about 2 hours, or until no appearance of yellow spots that represent dead bacterial cells. Then they were washed with deionized (DI) water several times, and boiled in deionized water at 100°C for 1 hour. Boiling was then repeated until the water that soaked BC pellicles turned colorless. BC pellicles were then stored in DI water. The morphology of the film was then observed under Field Emission Scanning Electron Microscope (FE-SEM) (JEOL JSM-7610F, Tokyo, Japan).

4.3 Curcumin absorption in BC films

Curcumin solutions at concentrations of 0.25, 0.5 and 1.0 mg/mL were prepared by dissolving curcumin in absolute ethanol. After dissolution, the solutions were then stirred with magnetic stirrer to be homogenized. For each of the specified concentrations, 150mL of solutions were prepared for each BC pellicle to be absorbed with curcumin. BC pellicles that were to be absorbed were placed in the petri dishes, which were the same size as used in BC film preparation (section 4.2). After that, 150mL of curcumin solution at specified concentration was added into each of the petri dishes, which made an immersion were immersed one to one into each of the curcumin solutions for 24 hours. Then air-dried them at room temperature. Note that BC pellicles treated with 0.5 and 1.0 mg/mL concentrations of curcumin solutions were identified as BCC0.5, and BCC1.0 respectively[131]. The morphology of the curcumin-loaded films was then observed under Scanning Electron Microscope (SEM) (JEOL JSM-IT500HR, Tokyo, Japan).

4.4. Buffer Solution Preparations

Each 1000mL of acetate buffer solution used in this research was prepared by adding 150g of sodium acetate into 250mL of deionized water, then 15mL of glacial acetic acid was slowly added into the sodium acetate solution. After that, add deionized water to fill the volume[131]. If pH is below 5.5, 12M NaOH solution was added to the solution dropwise to adjust pH to 5.5, and if pH is above 5.5, 5M NaOH solution was used to adjust pH to 5.5.

Each 1000mL of physiological PBS used in this research was prepared by adding 8g NaCl, 1.44g Na_2HPO_4, 0.2g KCl, and 0.24g KH_2PO_4 into 1000mL deionized water, then stirred thoroughly with magnetic stirrer. After that, while magnetic stirrer was in operation, pH meter was then immersed in solution to measure real-time pH level. If pH is below 7.4, 12M NaOH solution was added to the solution dropwise to adjust pH to 7.4, and if pH is above 7.4, 5M NaOH solution was used to adjust pH to 7.4.

4.5. Determination of actual curcumin content in BCC films

Each of the curcumin-loaded films was undergone the determination of actual curcumin amount loaded[131]. Each of the specimen was cut into a 2.5x2.5cm square. The specimen was then immersed in 4mL of 2:1 v/v acetone/DMAc solution. After that, 0.5mL of immersion solution is transferred to 8mL of acetate buffer solution, and actual amount of curcumin content was determined by obtaining values obtained from measurements by UV-visible (UV-vis) spectrophotometry (Shimadzu UV-2550, Tokyo, Japan) at 420nm wavelength.

4.6. Curcumin Release Assay

The release of curcumin was evaluated by using modified Franz Diffusion cells [41]. The samples were cut into 35mm diameter circular sheets, and placed on the receptor compartment of the Franz cell before covering with the donor cell, in which its opening was sealed off with a plastic film on both sides.

The receptor chamber of the cell was fully filled with buffer solution. Physiological PBS (pH 7.4) and acetate buffer (pH 5.5), along with buffers containing 0.5% v/v Tween 80 and 3% v/v methanol were used as release medium[131, 132].

The system was maintained at physiological temperature of 37°C by using circulating water bath throughout the experiment. The receptor fluid was stirred by magnetic stirrer. During the assay, 0.3mL of sample was withdrawn from the receptor by using 1mL syringe with needle and the equal amount of fresh buffer will be refilled back to the system immediately. Sample acquisitions were performed hourly at 1st to 6th hour, then every two hours from 6th to 8th hour. After that, the samples were then acquired every 12 hours until the assay finishes at 48th hour. Then the solutions were diluted to 1.0mL, in which the amount of curcumin was determined by UV-vis spectrophotometry at 420nm by using 1mL semi-micro cuvette.

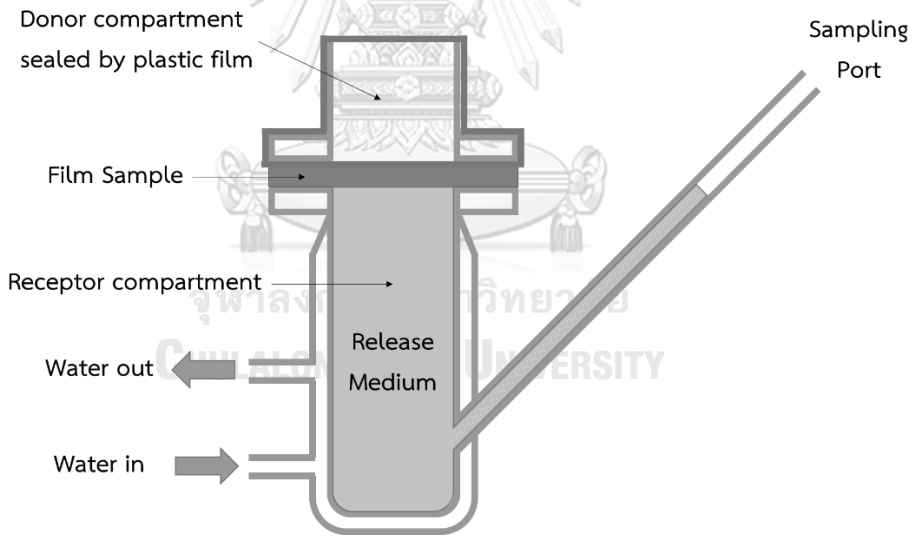

Figure 5: Schematic diagram of modified Franz cell system for the release assay

4.7 Preparation of samples for cell culture

BC film samples were cut by using metal borer, with a diameter of 15mm (the diameter of each of the wells in 24-well plate). 15mm diameter circular cover glass was used for control group. All of samples that subjected to cell culture assays were

then sterilized by autoclaving at 121℃ for 15 minutes. After that, the sterilized samples were then dried in hot air oven at 70℃ overnight.

4.8 Cell Culture

Human colon cancer (HT-29), human breast cancer (MCF-7), human dermal fibroblasts (HDF), human keratinocyte (HaCat), and monkey epithelial kidney cells (Vero) were cultured in DMEM. Human malignant melanoma skin cancer (A375) was cultured in RPMI-1640. The medium was supplemented with 10% v/v FBS and 1% of antibiotics. The culture was done in a CO_2 incubator at 37C with 5% CO_2 concentration.

4.9 Cell adhesion evaluation

The 24-well plates for this step were prepared by placing sanitized cover glass into each of the wells that was used for the experiment. After that, the film samples were placed over the cover glasses, then the metallic rings were placed after that into the wells that have film samples to keep the film samples in place. After that, the cell culture medium was added into each of the experimental wells. Then the system was incubated in CO_2 incubator for 1 hour. After that, discard all of the culture medium. Then the cells were seeded at $2x10^5$ cells per well (1mL working volume per well) for HT29 and MCF7 cells, and at $1x10^5$ cells per well (1mL working volume per well) for HDF, HaCat and A375, and then incubated for 24 hours, in case of 24-hour assay, and 48 hours, in case of 48-hour assay. When the incubation was finished, the culture medium and floating cells were discarded, leaving only samples intact. The samples were washed by 1mL PBS (pH 7.4) twice, and fixed in 2.5% glutaraldehyde in 0.1M PBS for at least 1-2 hours. Then the samples were rinsed twice with PBS and once with DI water. Each of the rinsing steps was done for 10 minutes. After rinsing, the samples were dehydrated by immersing them in series of ethanol solutions with increasing concentrations (30%, 50%, 70%, 95%, and 100% v/v), with 10-minute immersion duration per each concentration. For 100% (absolute) ethanol concentration, the immersion was done thrice. Then the samples were undergone critical point drying in

critical point dryer (Leica EM-CPD300, Austria). After that, the samples were mounted and coated with gold, by using sputter coater (Balzers SCD-040, Germany), before proceeding with observation under SEM (JEOL JSM-IT300, Tokyo, Japan).

4.10 Cytotoxicity Assay

Before proceeding with this step, the extracts of samples were produced by cutting samples, weighed, properly labeled, and then autoclaved separately at 121°C for 15 minutes. After that, the samples were then placed soaked in the medium and incubated at 37°C for 24 hours. The extract was then filtered through 0.45um syringe filter, before further dilution to 1000ug/mL prior to experiments if necessary.

Cells were seeded 10^4 cells per well into 96-well plate, with 100µl of working volume per well. The well plate with seeded cells was then incubated at 37°C, 5% CO_2 for 24 hours. Then the culture medium was removed from wells. After that, the extract of samples were then added into each of the wells, while the plain medium was used as control. Two sets of cell seeding and extract additions into 96-well plates were performed. One plate was a 24-hour assay, and another was 48-hour assay, in which they were incubated for 24 hours, and 48 hours respectively.

For Vero, HT29 and MCF-7 cells, after the incubation process, 5mg/mL MTT solution was added into each of the wells, then incubated again for 4 hours. Then the solutions in the wells were discarded, and 150uL of 9:1 DMSO:SDS solution was added into each of the wells. Finally, the well plates then were shaken for 5 minutes to homogenize the solution in the wells, and then the absorbance were measured at 570nm. The absorbance values were then processed to evaluate cytotoxicity values.

For HaCat, HDF, and A375 cells, after the incubation process, 10uL PrestoBlue™ solution was added into each of the wells, then incubated again for 2 hours. The well plates were shaken for 5 minutes to homogenize the solution in the wells, and then the absorbance were measured at 570 and 600nm. The absorbance values were then processed to evaluate cytotoxicity values.

CHAPTER V
RESULTS AND DISCUSSIONS

5.1 Absorption of curcumin into BC

Curcumin was loaded into BC films, which were synthesized by culturing *G.xylinus* in static conditions, by 24-hour direct immersion of the film in curcumin solution, because the absorption occurs rapidly in the initial 12 hours, then the absorption rate slows down, before eventually constant within 24 hours[133]. Absolute ethanol was used because of the good solubility of curcumin in ethanol[26]. The actual amount of curcumin in BCC films, in terms of unit area, were 0.19±0.03, 0.36.02±0.03, and 0.47±0.03 mg/cm^2 for BCC0.25, BCC0.5, and BCC1.0 films (with thickness of approx. 20 μm) respectively (Figure 6).

The morphology of cross section and surface of never-dried BC without curcumin under SEM is shown in figure 7. Similar never-dried morphology was also achieved by cultivation of *G.xylinus* on another type of culture medium such as Hestrin and Schramm medium with D-glucose, peptone, and yeast extract supplements[45]. Another study with another BC-producing strain such as *Gluconacetobacter sacchari* culturing in similar medium also achieve similar morphology of pellicles[134]. The air-dried films without loaded curcumin is shown in figure 8. It was found that the surface morphology of the air-dried BC films had well-organized microfibril network with thickness of 0.05-0.1 μm. The observation of the spacing between fibrils indicated the pore size of less than 0.1 μm. A previous study conducted by W. Woraharn revealed that when the air-dried pellicle is immersed in water, the pore diameter increases to 0.2-1 μm[131]. This means that water affects BC ultrastructure by relaxing the polymer chain. Therefore, BC is suitable for use as a release matrix of active substances (drugs). Figures 9-10 are the SEM images of air-dried BC loaded with curcumin, BCC0.5, and BCC1.0, respectively. It was found that, after 24-hour absorption and air-drying, curcumin aggregates were distributed uniformly on the surface of the films. However, between fibril layers, the appearance of aggregates was not significant, except in higher curcumin immersion concentration (BCC1.0), where some of curcumin aggregates could

be observed between fibril layers. W. Woraharn also further studied the samples by using FT-IR analysis and confirmed that there were shifts on peaks due to interactions between curcumin and BC[131]. This similar effect was also observed in ethanoic extract of mangosteen peel when it was loaded on the BC film[41]. Therefore, it can be discussed that curcumin entrapment is achieved by interactions between curcumin and BC microfibrils during air-dry process. Higher the concentration of curcumin solution used for immersion results in more curcumin molecules were diffused and absorbed into the BC pellicle.

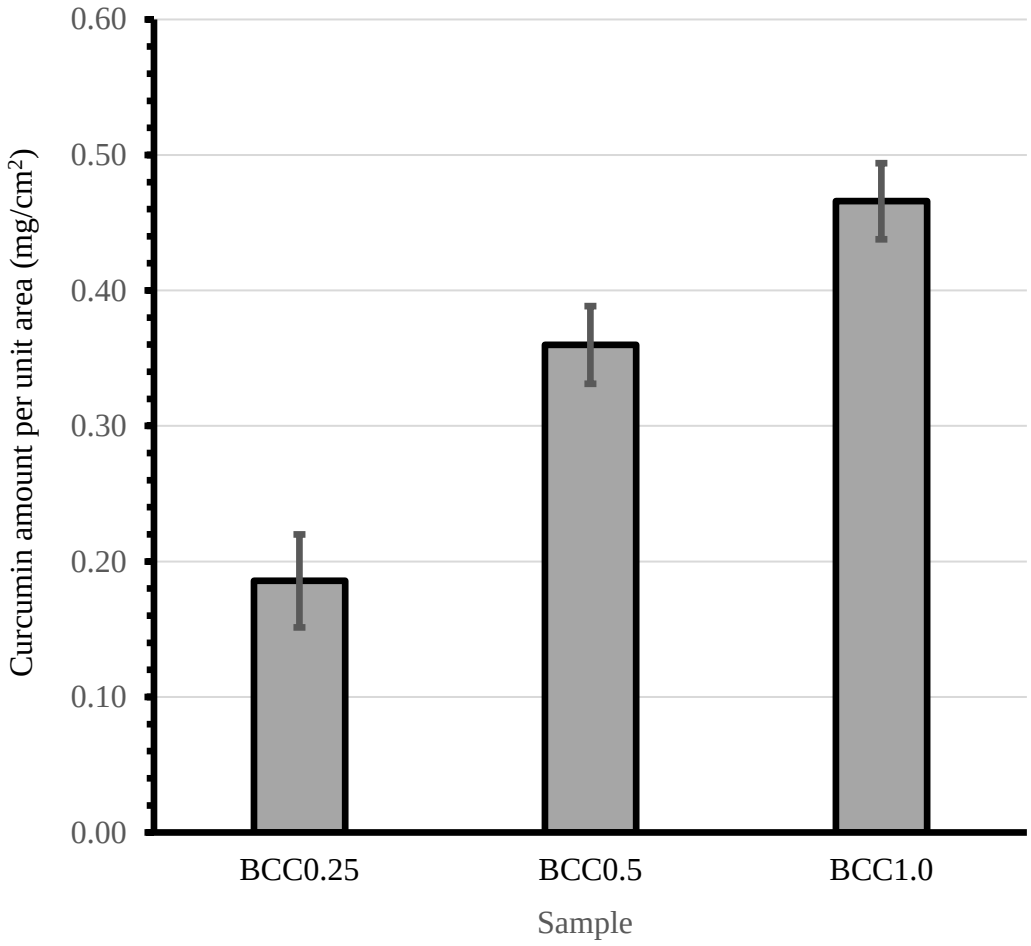

Figure 6: The actual amount of curcumin loaded in BC films in terms of amount of curcumin (mg) per unit area of the film (cm^2) (Values reported as mean ± SD; n=4)

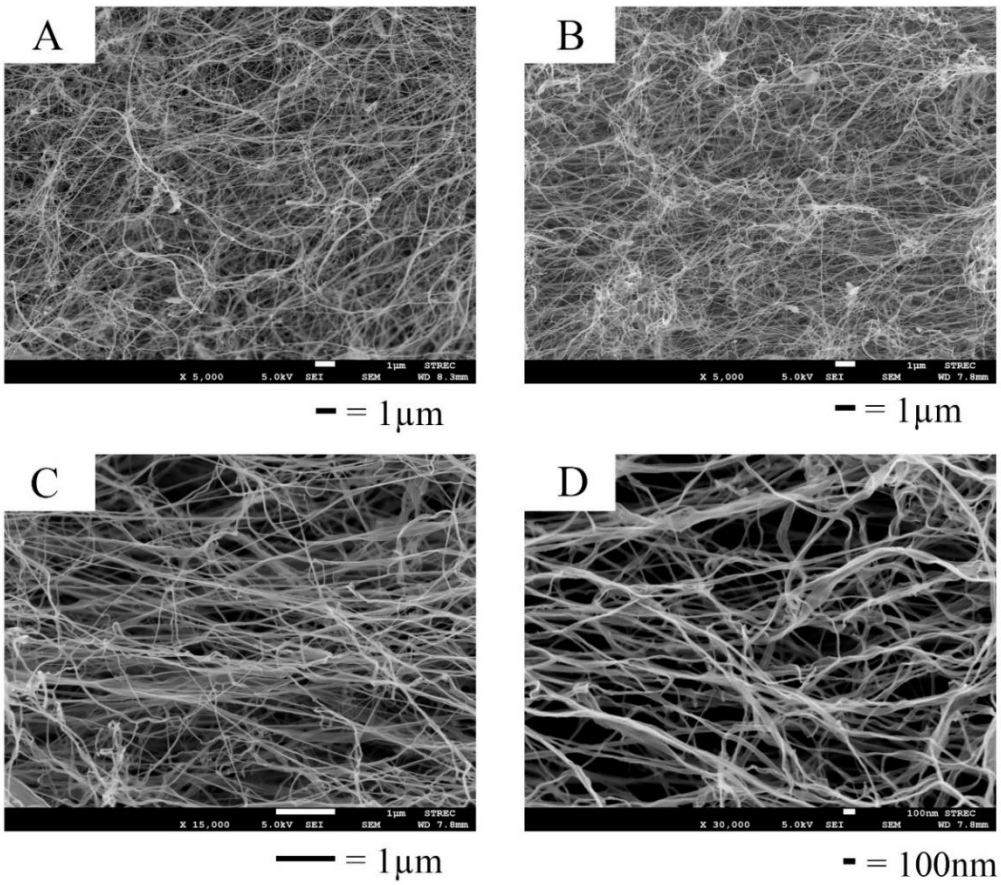

Figure 7: The SEM images showing surface of never-dried BC at 5000x (A) and cross-section of never-dried BC at 5000x (B), 15000x (C), and 30000x (D).

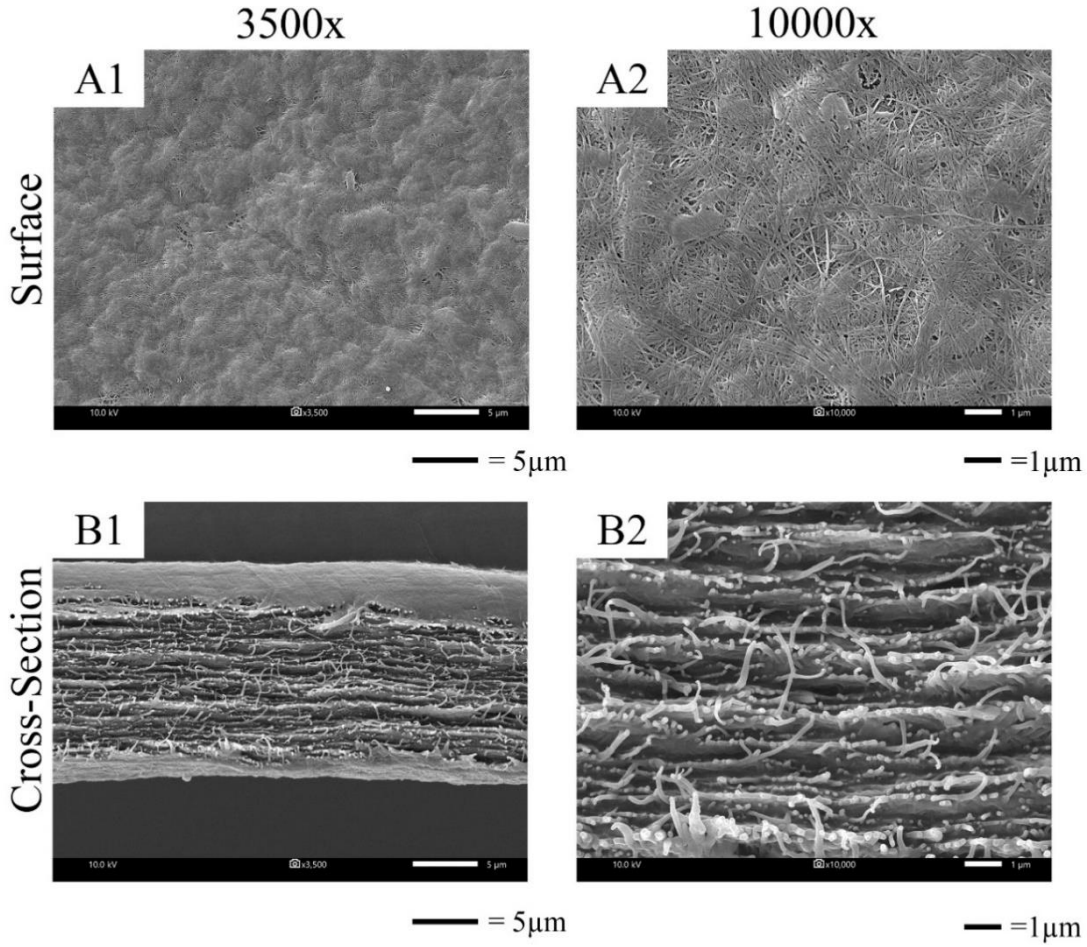

Figure 8: The SEM images showing surface (Row A) and cross-section (Row B) of air-dried BC in 3500x (Column 1) and 10000x (Column 2) magnifications

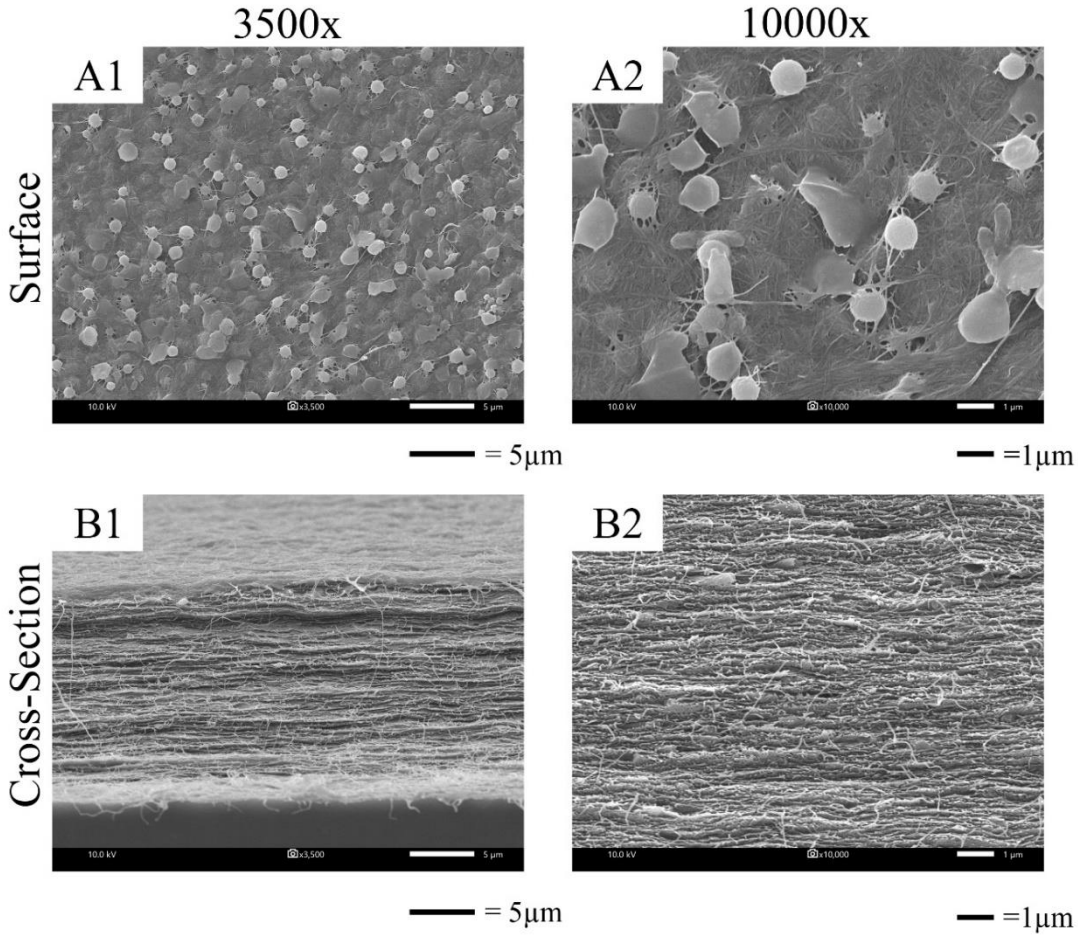

Figure 9: The SEM images showing surface (Row A) and cross-section (Row B) of BCC0.5 in 3500x (Column 1) and 10000x (Column 2) magnifications

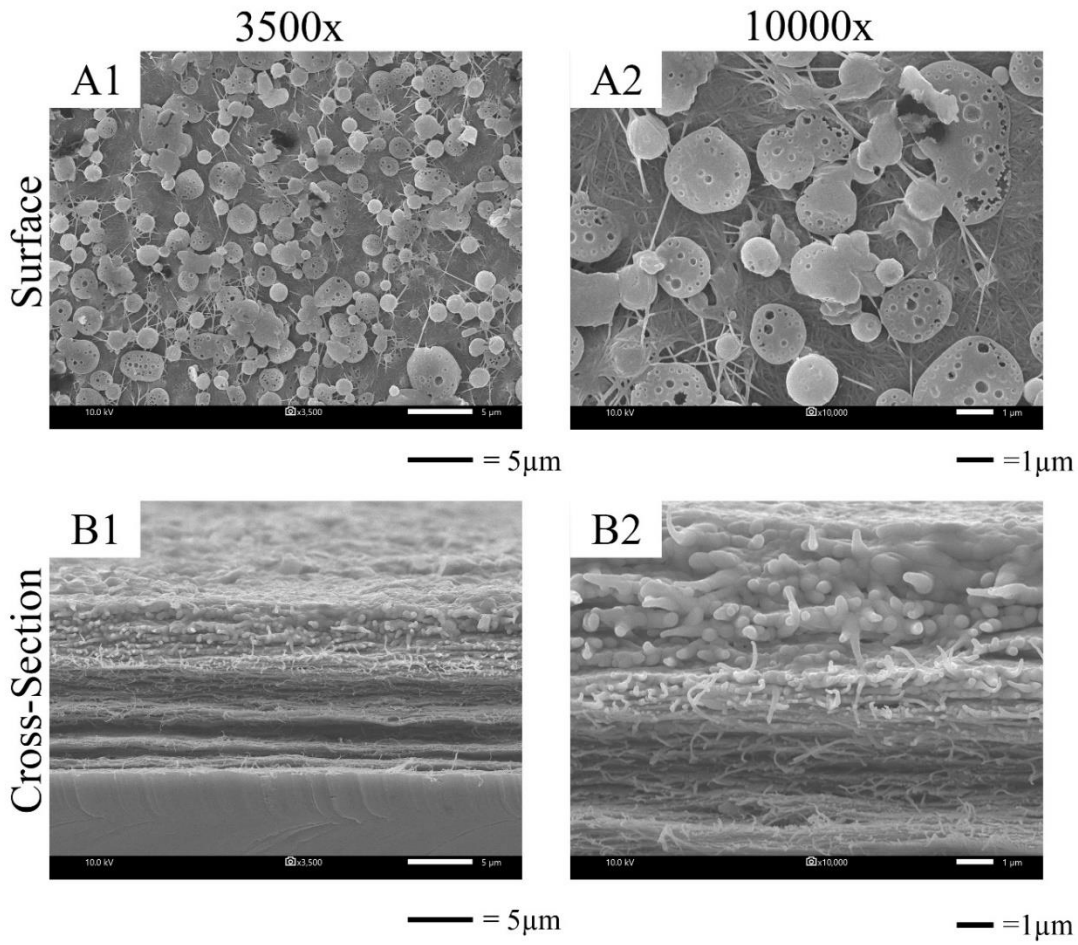

Figure 10: The SEM images showing surface (Row A) and cross-section (Row B) of BCC1.0 in 3500x (Column 1) and 10000x (Column 2) magnifications

According to S. Taokaew et al, loading of curcumin into BC pellicle is a post-biosynthetic modification step of BC[135]. Right after biosynthesis, the BC pellicle is in hydrated form. Therefore, the ultrastructure of BC is a 3D matrix formed by cellulose nanofibers, held by H-bonds between hydroxyl groups within and between fibers. When the hydrated pellicle is immersed into curcumin solution, curcumin molecules entered into the ultrastructure of BC pellicles through the pores between the fibers in the 3D matrix over the immersion period. (Figure 11) The process that is occurring during immersion is a bulk modification of BC by direct immersion of BC hydrogel in curcumin solution, which was then later dried in order to entrap curcumin, which is the active component. After that, the curcumin-immersed BC pellicles were undergone air-dry process, in which, despite being a simple evaporating process, but gave significant changes to BC ultrastructure. New hydrogen bonds were formed between fibers during drying, making the fiber network denser, leading to packing of curcumin molecules in BC densified matrix, due to shrunk ultrastructure. This makes the process non-reversible.

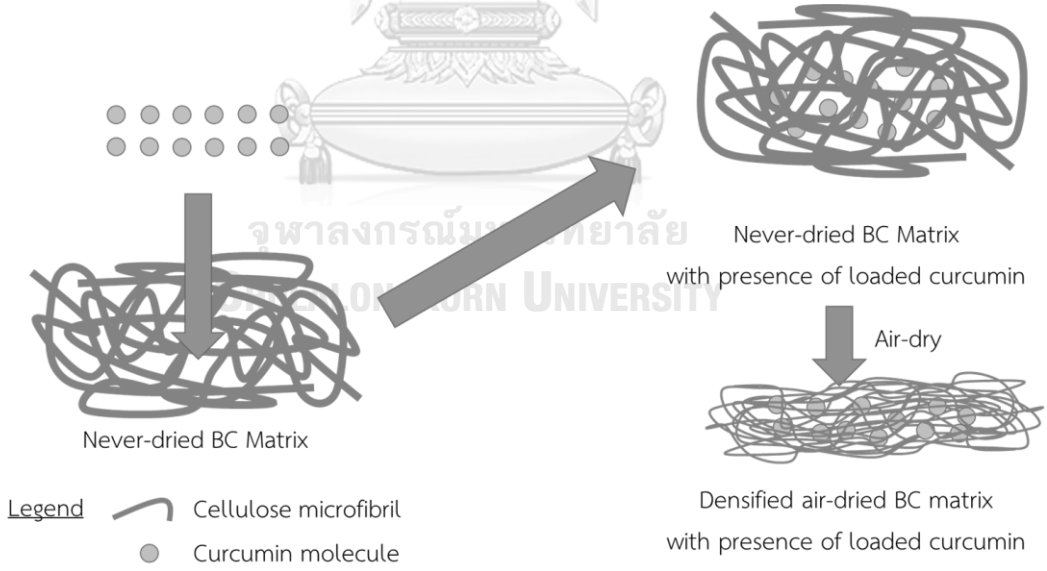

Figure 11: Illustration of mechanism of loading of curcumin in BC film

5.2 Release Assay

Figures 12-15 are the release profiles of curcumin from curcumin-loaded, air-dried BC pellicles under non-transdermal condition. The release assay was done by placing the BCC films, which were re-hydrated by immersion in DI water, on the surface of buffer solutions, which were acetate buffer (pH 5.5) and PBS (pH 7.4), along with respective media with Tween80 and methanol supplement, in modified Franz diffusion cells. The samples were taken hourly from hours 1-6, every 2 hours from hours 6-12, and every 12 hours from hours 12-48. The release profiles were expressed in terms of concentration of released curcumin in buffer solutions (Figures 12 and 14) and in terms of percentage of released curcumin based on actual amount of loaded curcumin (Figures 13 and 15).

When Tween80/methanol supplement was not used, the release of curcumin approached zero in both types of buffers, while significant release, with initial burst release, was observed. This was due to the hydrophobicity of curcumin[26], corresponding to the findings of Li et al (2015) that the release rates of curcumin from silk hydrogel films were significantly enhanced when Tween80 and Methanol supplement was added to the release medium[132]. This was due to the enhanced solubility of the release medium towards hydrophobic solute (curcumin) by the Tween80/methanol additive, the hydrophobic dissolution enhancer that allows the hydrophobic solute to dissolve throughout the polar solvent, which in this case was the release medium. However, the pH of the release medium was found to have less influence on curcumin dissolution. The burst release at the first hour occurred in all cases, with concentration at the first hour proportional to the initial loading concentration of curcumin in each of the samples. Higher the initial loading concentration, longer the initial burst period, with the period stretched up to 8 hours in case of BCC1.0. This was due to the dissolution of the curcumin adhered to the surface of the film, as observed in the SEM in section 5.1, into the release medium containing the hydrophobic dissolution enhancer (Tween80/methanol additive). Then the release occurred in a controlled manner, due to the loosening of the ultrastructure of air-dried BCC when immersed in an aqueous environment[41] (Illustration is shown

in figure 16). This also further confirmed by the SEM morphology of the air-dried BC films immersed in water studied by W. Woraharn (2009) that the pore diameter increases compared to the films that were not immersed in water[131],

The total amount of curcumin released was also proportional to the initial amount of curcumin, which was absorbed into the films. Higher the curcumin loading concentration into the film, more curcumin was released into the medium. Due to the instability of curcumin in aqueous solutions with pH higher than 7, the observed concentrations of curcumin released from BCC into acetate buffer were higher than that of PBS buffer. The reduction of curcumin concentration after the eighth hour was also observed when the release assay was performed in PBS, due to the degradation of curcumin caused by instability of curcumin as aforementioned. This correlates with the study from Y. Nimiya et al (2016) that curcumin concentration determined by absorbance measurement was reduced due to the degradation of curcumin, as the degradation products of curcumin do not have 420nm absorbance, with further confirmations provided by HPLC analysis[136]. Therefore, it can be suggested that degradation should occur slowly in lower pH solution (acetate buffer) and occur significantly faster in higher pH solution (PBS)[26, 137].

The similar release pattern was similar to the observations on some of the previous controlled release system developments. J. Shaikh et al (2009)[138] reported similar release pattern (initial burst) by suspending curcumin-loaded PLGA nanoparticles in deionized water inside dialysis membrane suspended in 50% v/v ethanol release medium. Abhishek Sahu et al, 2011[139] demonstrated similar release behavior of curcumin from Pluronic block copolymer micelles encapsulated with curcumin, by placing those micelles in a dialysis membrane. Lei Li et al (2014) also observed initial burst release of curcumin from functionalized PLGA-lecithin-curcumin-PEG nanoparticles by placing the nanoparticle suspension inside dialysis vessel, suspended in PBS containing 0.1% w/v Tween80[140].

The instability of curcumin in PBS is also confirmed by a publication by Y. Liu et al (2015), who found that free curcumin was unstable in pH 7.0 PBS buffer, at 4°C and 23°C, which was significantly less than the stability of curcumin which was loaded

in a controlled release matrix of their work, chitosan-coated liposome[71]. Another publication by Lei Li et al (2014) also demonstrated that free curcumin was highly unstable, as more than 95% of curcumin was degraded in six hours in pH 7.4 PBS at 37°C[140], which was more realistic than in aforementioned work. Therefore, the reduction of curcumin concentration after tenth hour is suggested to be caused by an instability of curcumin mentioned in aforementioned publications.

Therefore, this demonstrates that BC is a good candidate as a controlled release matrix for curcumin, in applications that require initial burst release followed by steady release. However, if an intended application requires different release profile, the re-design of matrix is necessary, and the measures of preserving curcumin stability are required.

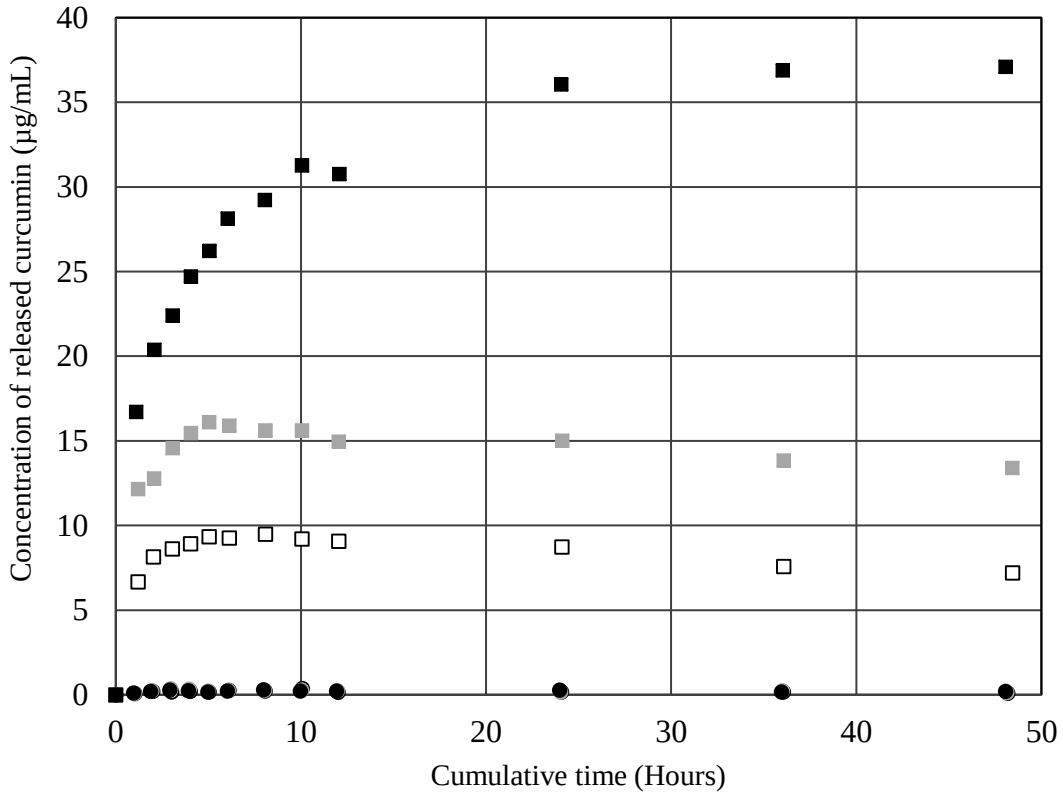

Figure 12: Release profiles of curcumin from BCC0.25 (white), BCC0.5 (gray), and BCC1.0 (black) into acetate buffer (pH 5.5) without Tween80/methanol supplement (circle) and with Tween80/methanol supplement (square) in terms of concentration of released curcumin

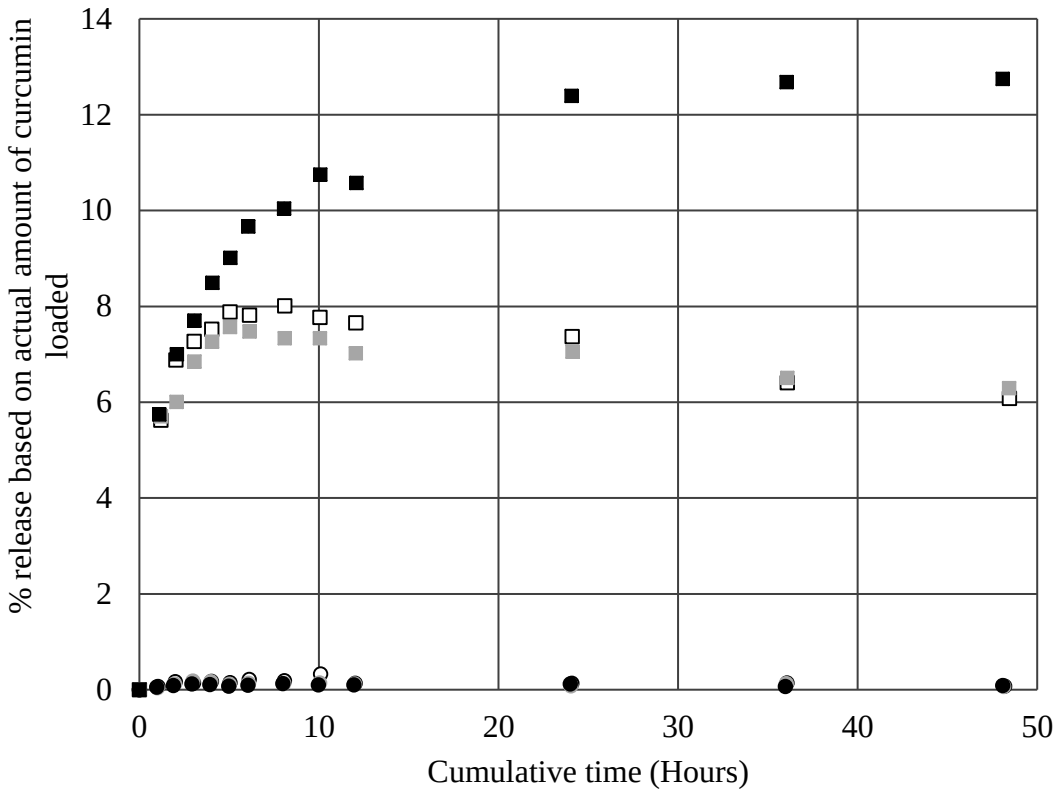

Figure 13: Release profiles of curcumin from BCC0.25 (white), BCC0.5 (gray), and BCC1.0 (black) into acetate buffer (pH 5.5) without Tween80/methanol supplement (circle) and with Tween80/methanol supplement (square) in terms of percentage of release based on actual amount of curcumin loaded in BC films

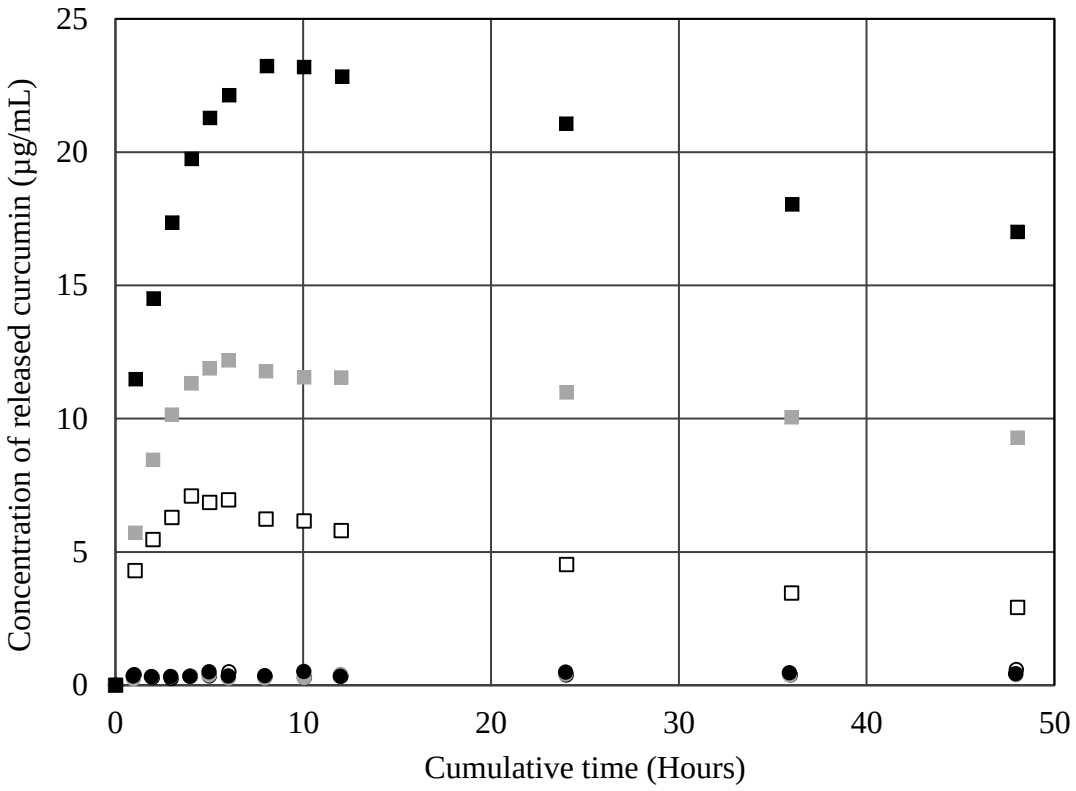

Figure 14: Release profiles of curcumin from BCC0.25 (white), BCC0.5 (gray), and BCC1.0 (black) into PBS buffer (pH 7.4) without Tween80/methanol supplement (circle) and with Tween80/methanol supplement (square) in terms of concentration of released curcumin

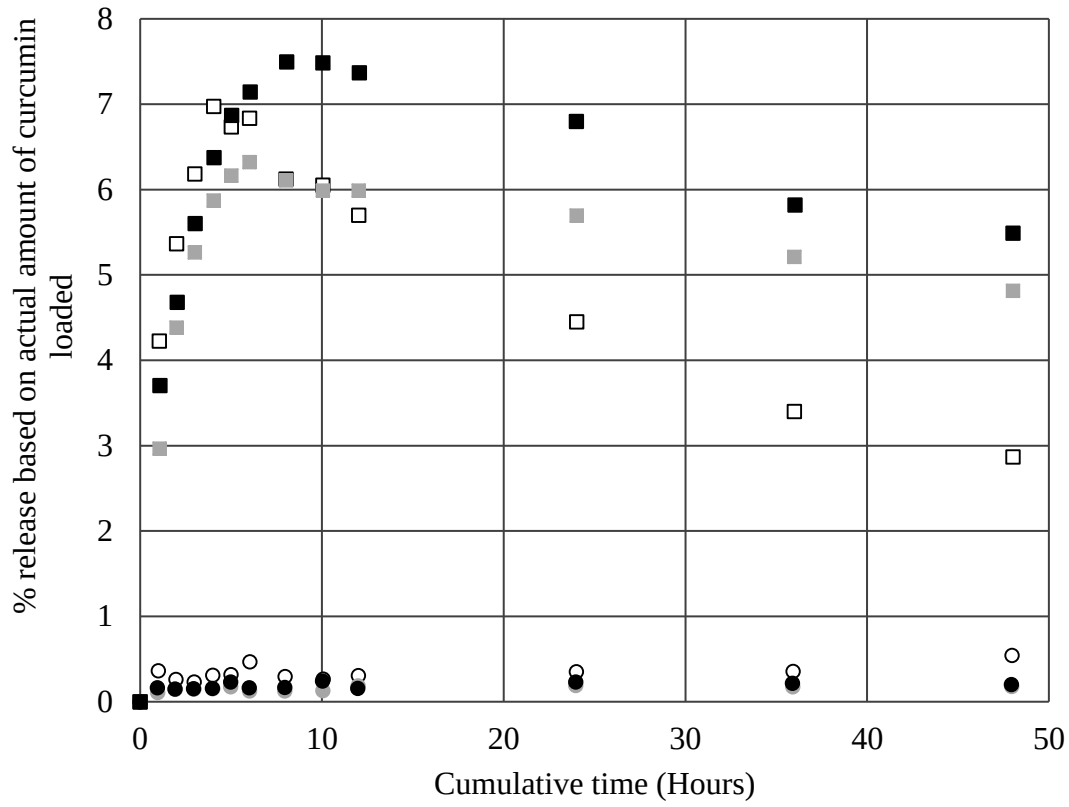

Figure 15: Release profiles of curcumin from BCC0.25 (white), BCC0.5 (gray), and BCC1.0 (black) into PBS buffer (pH 7.4) without Tween80/methanol supplement (circle) and with Tween80/methanol supplement (square) in terms of percentage of release based on actual amount of curcumin loaded in BC films

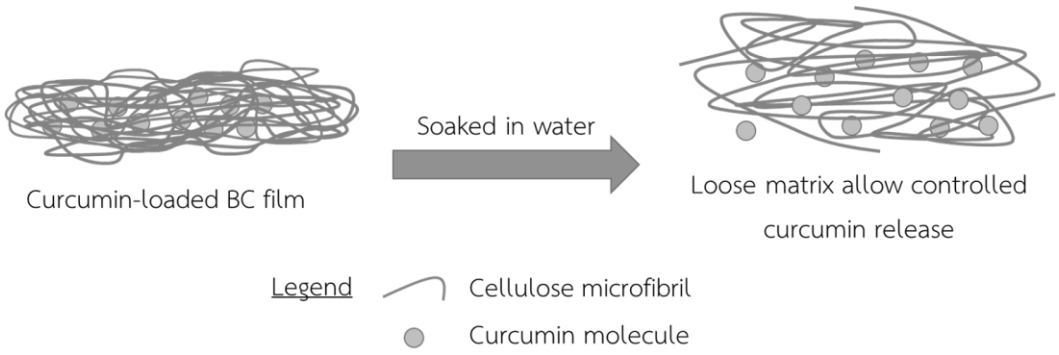

Figure 16: Illustration of release of curcumin from BC film

5.3 Cytotoxicity analysis of BC films

5.3.1 Cell adhesion analysis

The behavior of cells on BC/curcumin films were observed morphologically by observing the behavior of each of the cell types, HaCat, HDF, A375, HT29, and MCF7, in terms of adhesion on BCC0.5 and BCC1.0 films, on SEM at 1500x (Figures 16-25 column A) and at 5000x (Figures 16-25 column B), after 24 hours (Figures 17, 19, 21, 23, 25) and 48 hours (Figures 18, 20, 22, 24, 26) of incubation.

24-hour incubation causes the inhibition of growth of skin, colon, and breast cancer cells (Figure 20 for A375, Figure 22 for HT29, and Figure 24 for MCF7 cells), and more significant effects are observed when A375 cells were treated with BCC films for 48 hours (Figure 22). Similar effects were found on HT29 and MCF7, but not in an extent as strong as A375 cells (Figures 23, 25). This observation can be concluded that the cancer cells undergone apoptosis due to the presence of curcumin, which was sourced from the release from the BCC films. Due to the higher amount of curcumin released, the stronger effects can be observed when the cells were cultured on BCC1.0 than the same cells that were cultured on BCC0.5, and on progressed duration (48 hours vs 24 hours). This correlates with findings from Zhang et al (2015), who reported that curcumin caused shrinkage, contraction, and detachment of A375 cells, leading to abnormally round morphology[121]. However, this effect is not observed on normal cells. Therefore, this can be concluded that curcumin is selectively cytotoxic against cancer cells.

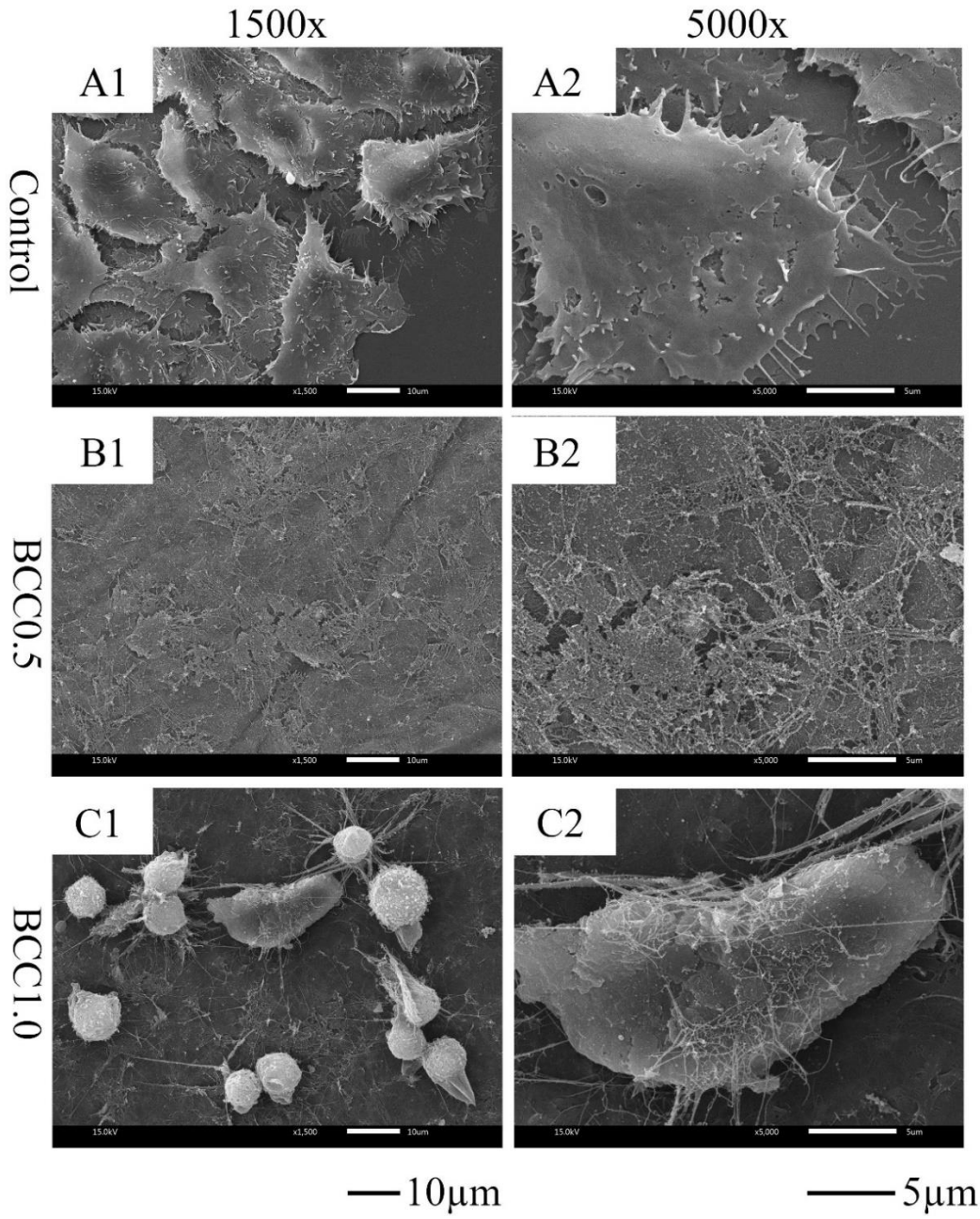

Figure 17: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HaCat cells after 24-hour treatment.

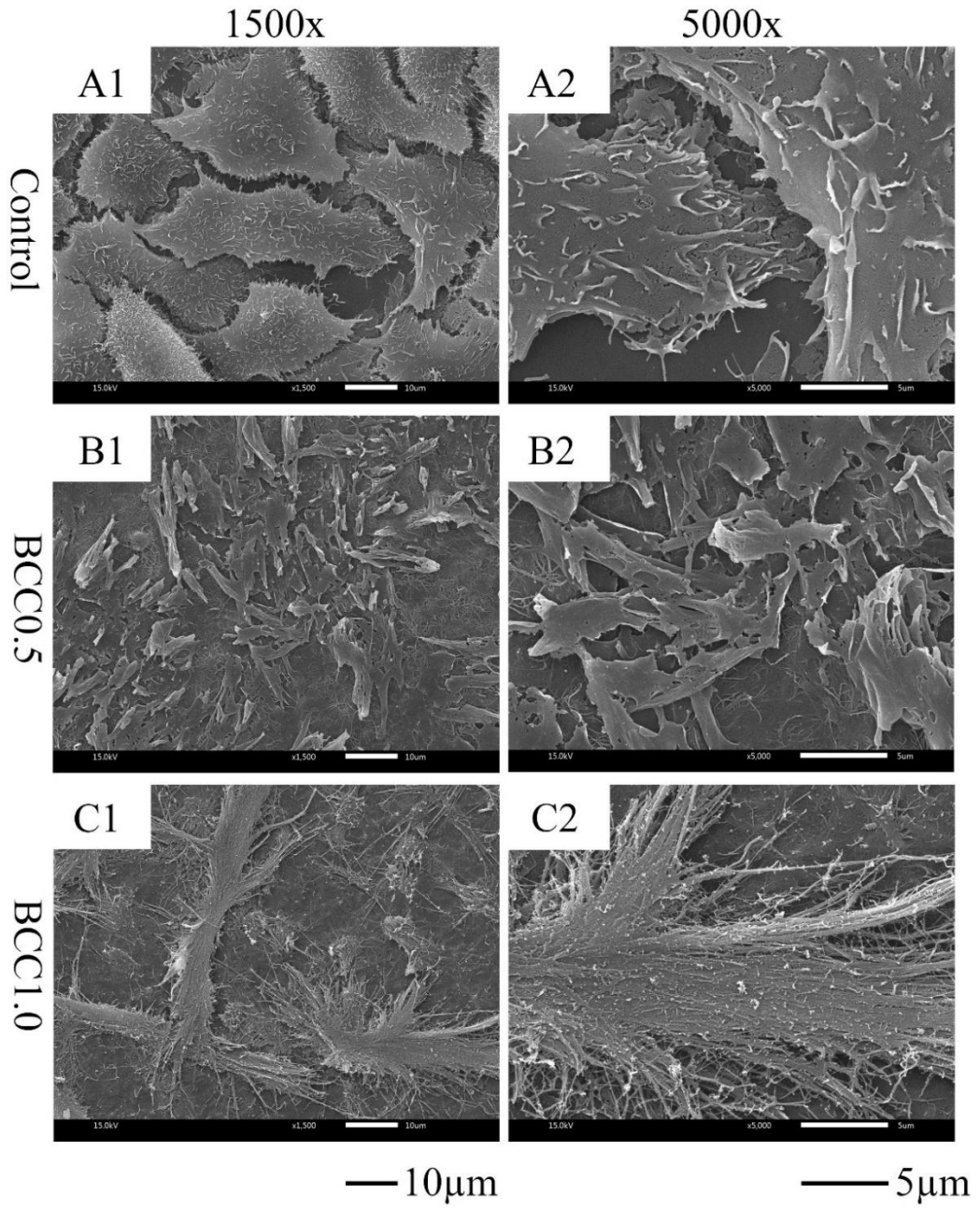

Figure 18: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HaCat cells after 48-hour treatment.

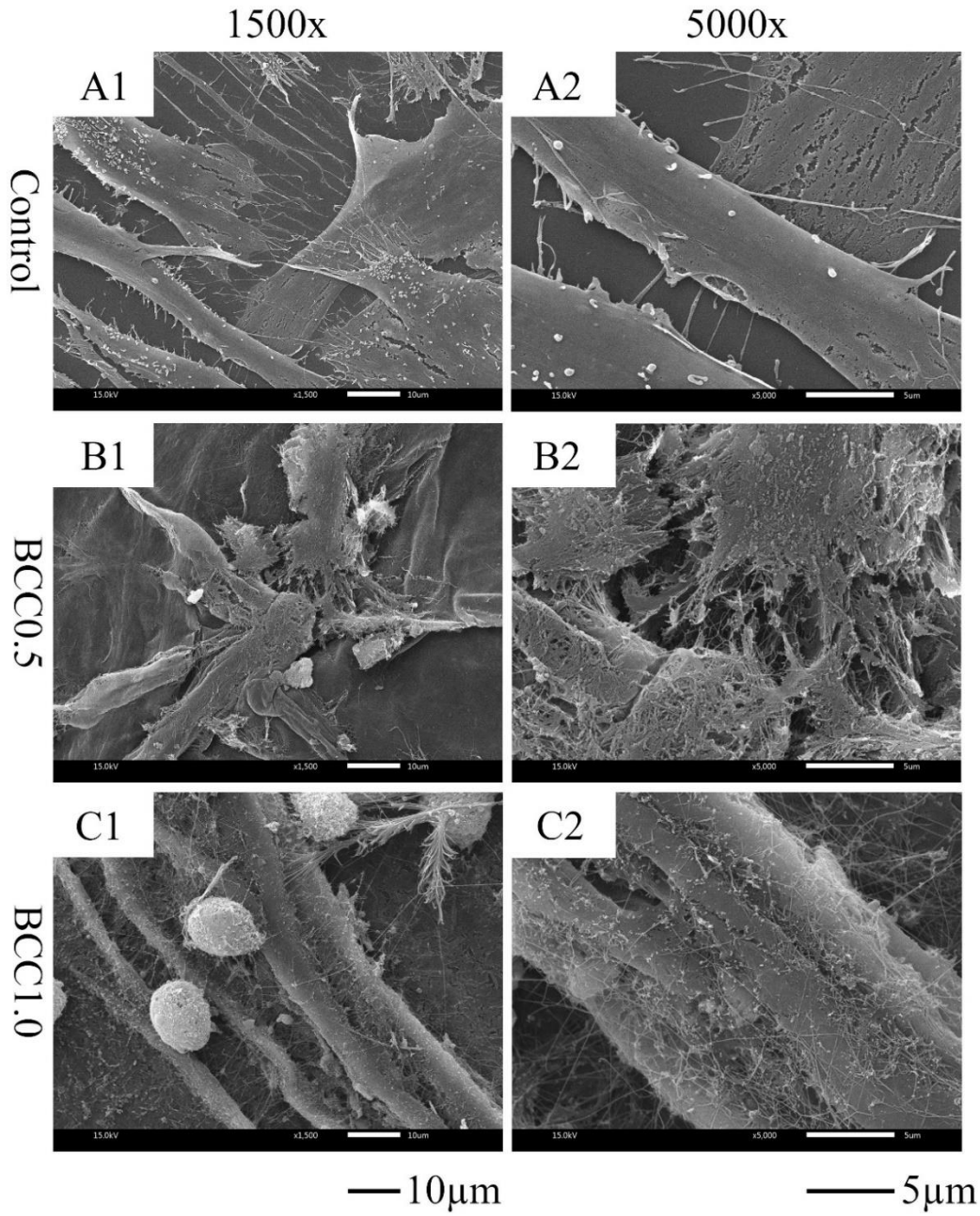

Figure 19: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HDF cells after 24-hour treatment.

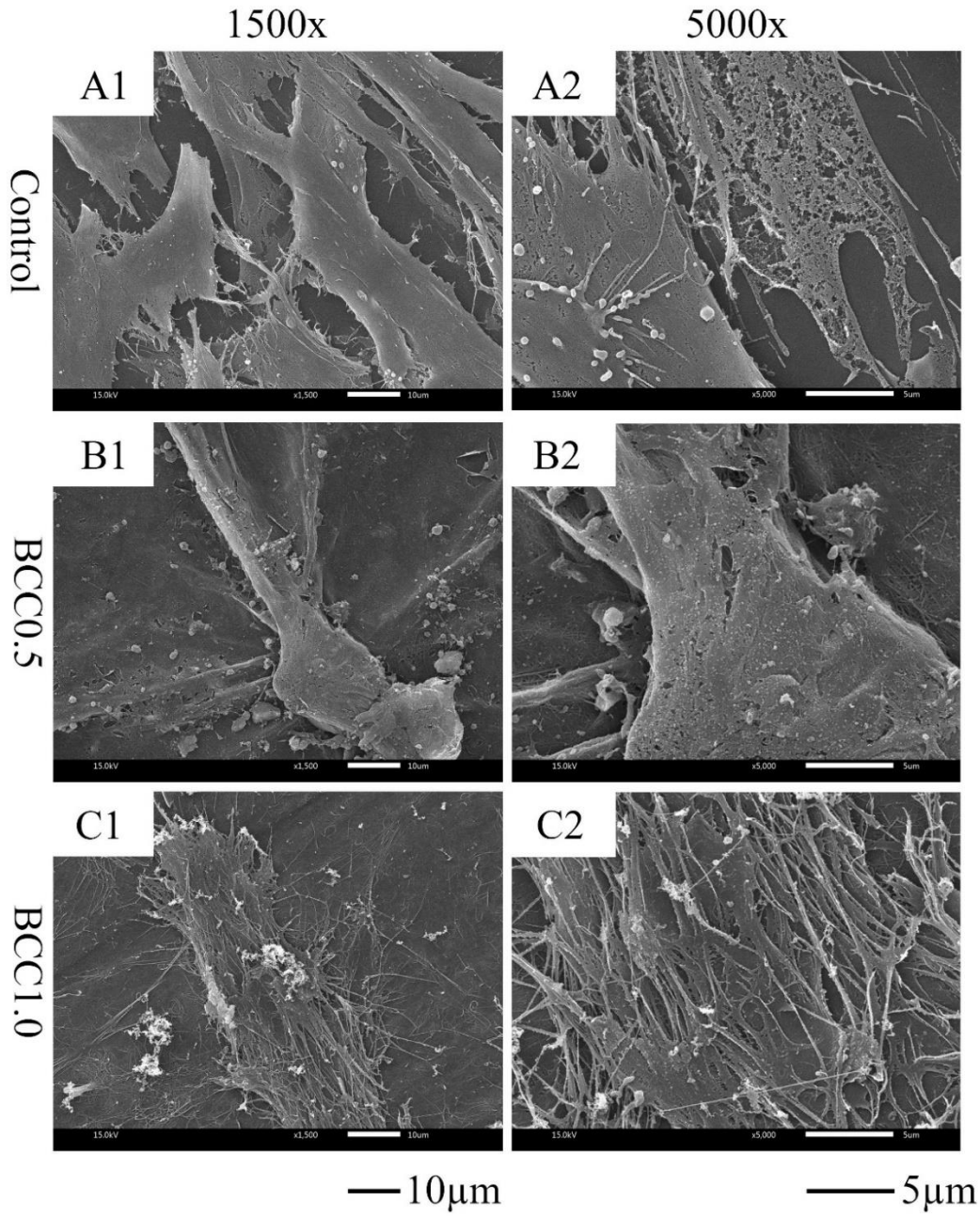

Figure 20: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HDF cells after 48-hour treatment.

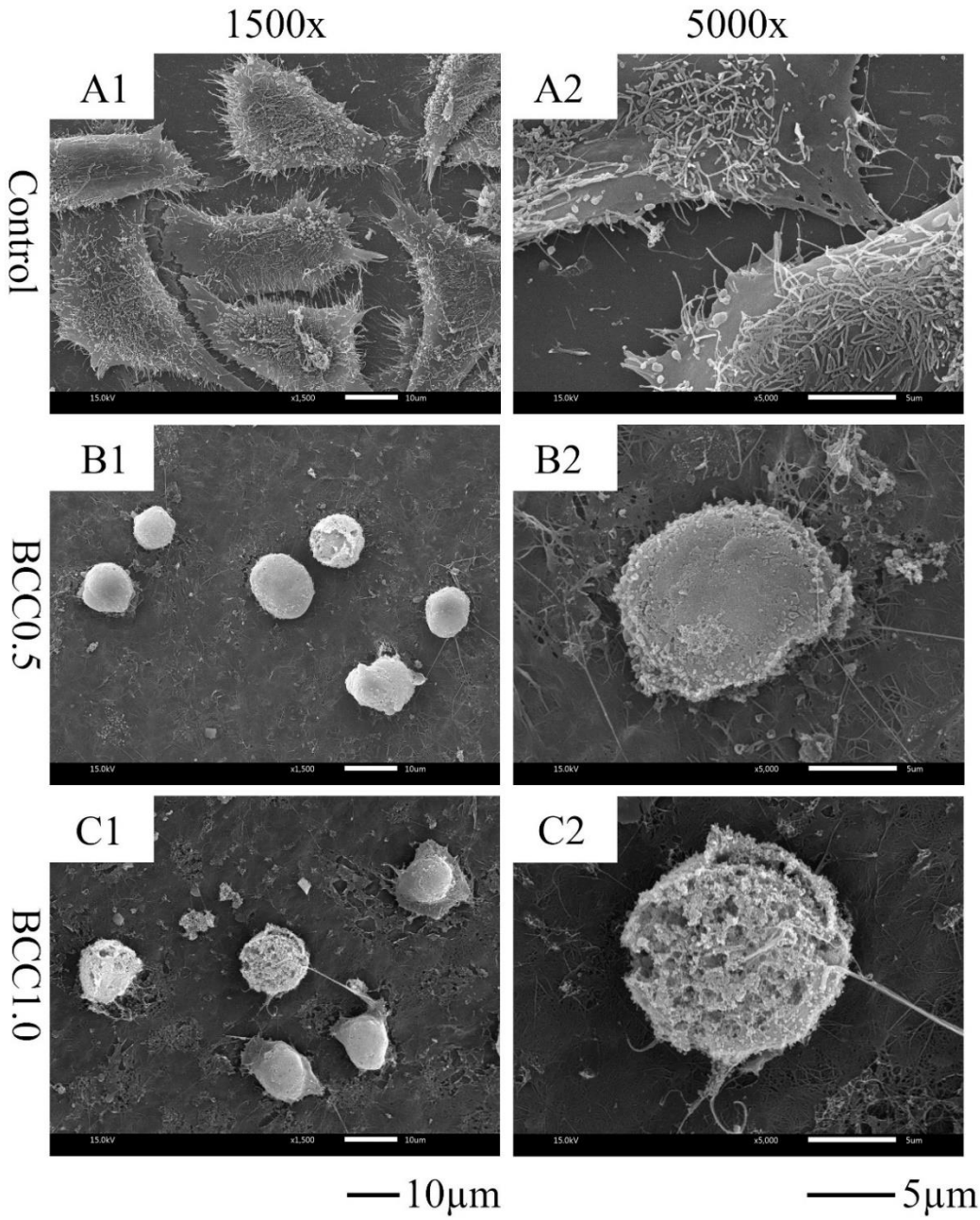

Figure 21: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on A375 cells after 24-hour treatment.

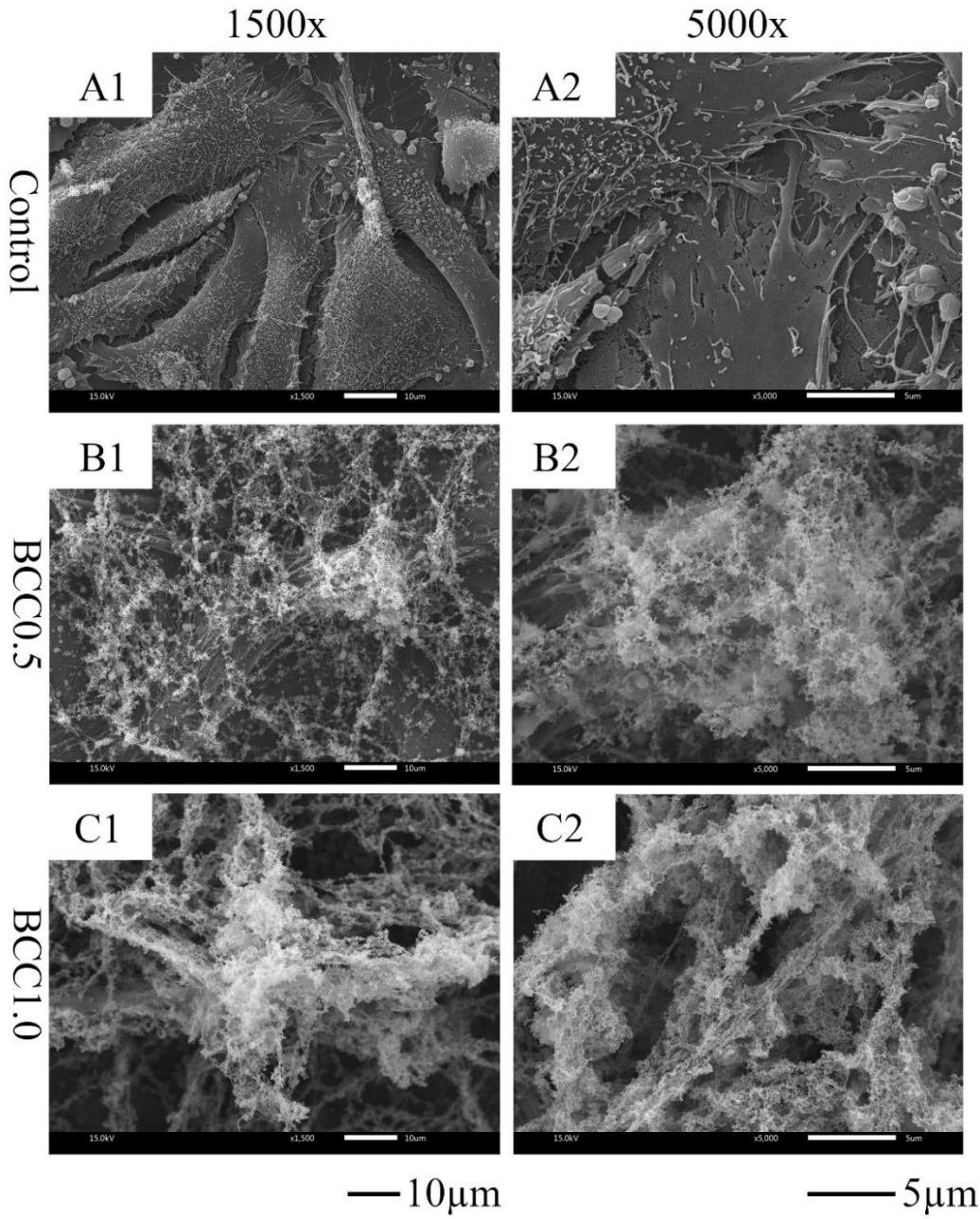

Figure 22: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on A375 cells after 48-hour treatment.

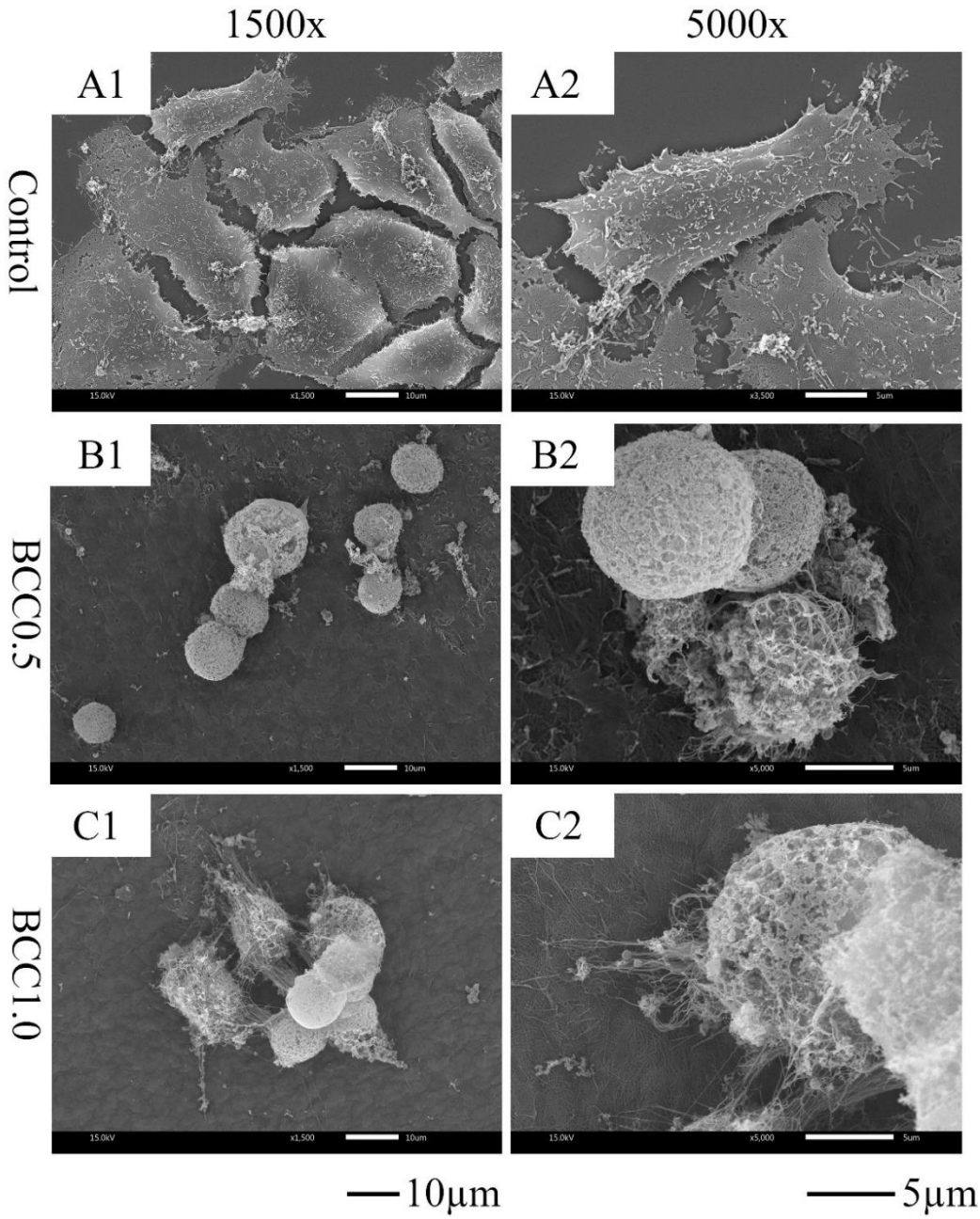

Figure 23: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HT29 cells after 24-hour treatment.

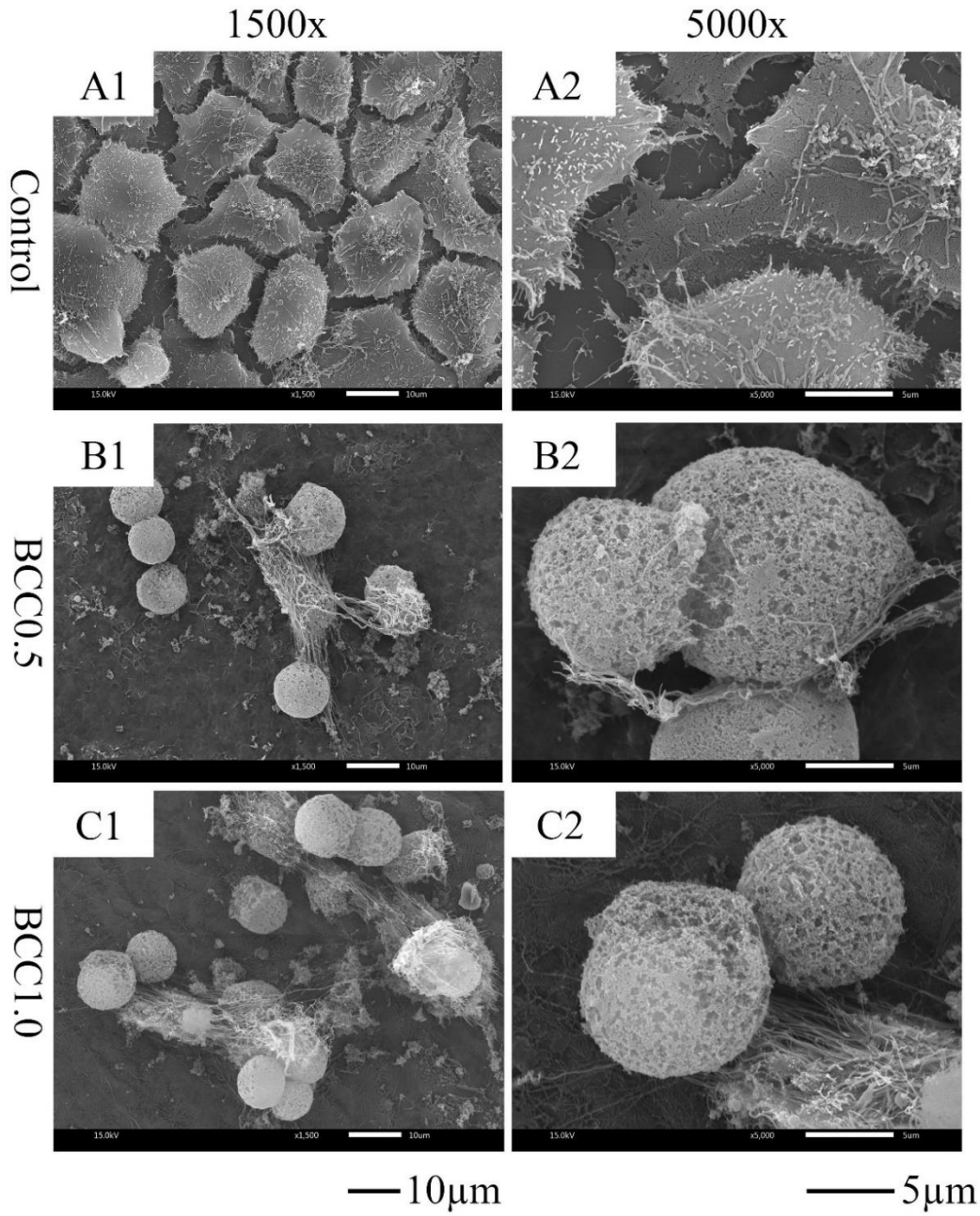

Figure 24: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on HT29 cells after 48-hour treatment.

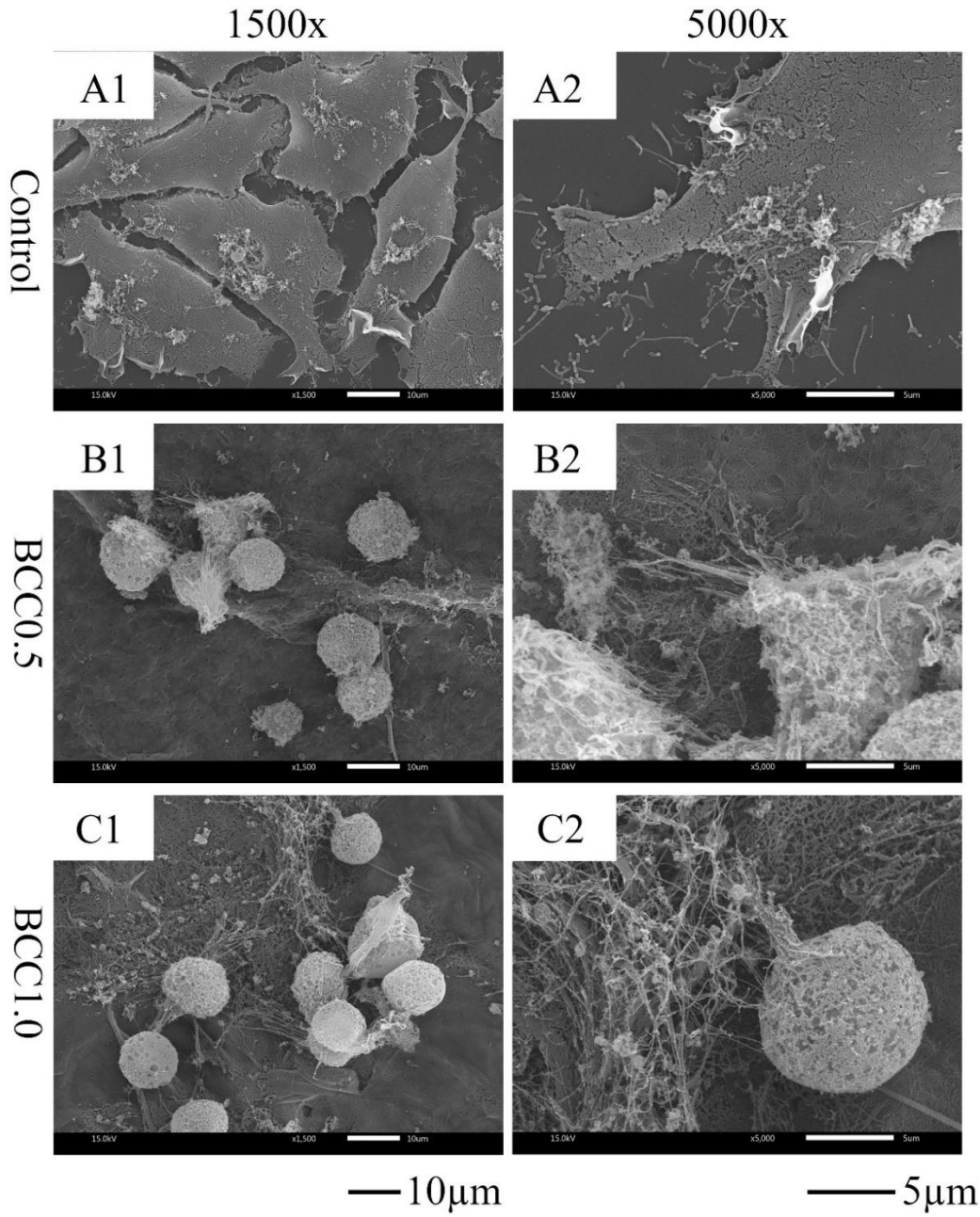

Figure 25: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on MCF7 cells after 24-hour treatment.

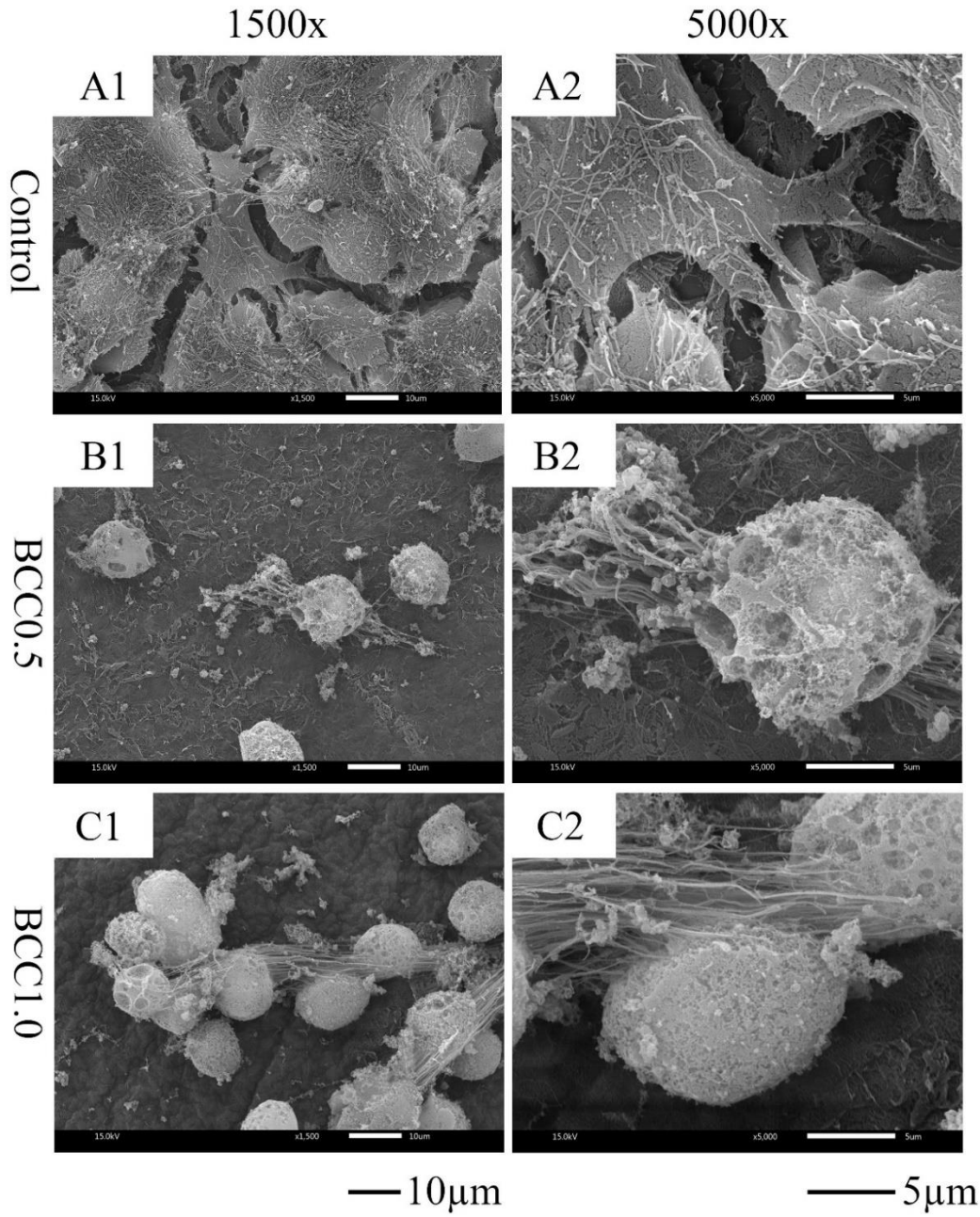

Figure 26: SEM images, at 1500x (Column 1) and 5000x (Column 2) magnifications, showing the cytotoxic effects of BCC0.5 (Row B) and BCC1.0 (Row C) against plain cover glass (Row A, control) on MCF7 cells after 48-hour treatment.

5.3.2 Cytotoxicity analysis

The cytotoxicity assay was performed by treating samples in a culture medium with sample extracts at a concentration of 1000µg/ml. Then the cells that were treated with sample extract were then measured for cytotoxicity. It was found that the viabilities of Vero, HaCat, and HDF cells, which were normal cells, were 102.53%, 107.61±7.09%, and 103.43±1.63% when treated with BCC0.5 sample extract for 24 hours, and 99.76, 102.28±3.38, and 98.42±0.17% when treated with the same extract for 48 hours. On the other hand, when the cells were treated with BCC1.0 sample extract, the viabilities of Vero, HaCat, and HDF cells were 108.00%, 83.90±5.80%, and 101.13±16.95% respectively when the cells were treated for 24 hours, and 104.59%, 92.71±3.95%, and 94.02±2.69% respectively when treated for 48 hours. (Figures 27, 28) The results were correlated with the SEM micrographs (section 5.3.1), which HaCat cells shrunk after 24 hour treatment on BCC1.0 film, but the cells were adhered to the same film when the treatment duration was increased to 48 hours. For HDF cells, the effects of curcumin on adhesion were insignificant, because the cells were adhered to the films throughout the incubation period. (Adhesions of HDF on BCC films can be seen on both 24-hour and 48-hour SEM micrographs)

However, in cancer cells, the viabilities of A375, HT29, and MCF7 cells were 88.04±1.36%, 103.74%, and 104.53% respectively when treated with BCC0.5 sample extract for 24 hours, and 72.78±1.81%, 96.22%, and 96.94% respectively when treated with the same extract for 48 hours. On the other hand, when treated with BCC1.0 sample extract, the cytotoxicity values of A375, HT29, and MCF7 cells were 82.43±6.80%, 93.30%, and 99.13% respectively when the cells were treated for 24 hours, and 70.89±0.69, 81.82, and 87.19% respectively when the cells were treated with the same extract for 48 hours. (Figures 27, 28) Therefore, curcumin has cytotoxic effects against A375, HT29, and MCF7 cells, with more effects observed on A375 cells, as they have both lower cell viability values and more intense effects were also revealed on SEM micrographs. In A375 cells, Y.P. Zhang et al (2015) found that curcumin affected the proliferation and cell invasion by increasing apoptotic activity [121]. This effect is also observed on other cancer cells, including colon cancer[113]

and breast cancer[141] cells which were also studied in this work. Other types of cancer cells that were reported to be killed by apoptosis were papillary thyroid cancer[112], pancreatic cancer[120], ovarian cancer[119], and prostate cancer[117]. Not only aforementioned apoptosis, but other effects were also observed on cancer cells, including chromatin condensation and nuclear shrinkage observed in cutaneous T-cell lymphoma cells[116], cell cycle arrest induction in prostate cancer cells[117], and ROS production that cause DNA fragmentation (indicator of apoptosis), altered mitochondrial membrane potential (inducing apoptosis via mitochondrial pathway), and cell cycle arrest in colon cancer cells[9]. Shahar Lev-Ari et al (2014) observed that curcumin suppressed NFkB-dependent inflammation mediator (NF-**K**B), which downregulates apoptosis and proliferation regulations, and promotes the activation of tumor growth, along with its associated activities[118]. On the other hand, there were studies that curcumin does not have cytotoxic effects on normal cells, including hepatocytes, lymphocytes, HDF, and rat dermal fibroblasts[142]. Not only in *in vitro* studies aforementioned, but *in vivo* confirmation was also conducted in mice models with colon tumors, which the tumors were significantly reduced when they were fed with diet with curcumin supplement[143]. The mechanism of the selectivity of cytotoxicity towards cancer cell of curcumin is that p53 induces G1 arrest, which allow cells to be repaired before proceeding further down the cell cycle in normal (healthy) cells[144]. This occurs in association with cyclin-dependent pathways that curcumin causes cell cycle arrest at G0 by downregulating Cyclin D1, and upregulating Cip1 simultaneously. Therefore, in healthy cells, cell cycle arrest at G0 phase occurs without apoptosis occurring. However, in cancer cells, the Cyclin D1 level is so high that despite curcumin-induced upregulation of Cip1, it is still insufficient to cause cell cycle arrest, causing apoptosis at G2 phase, which curcumin plays important role in induction of p53 in that situation. This makes healthy cells immune to curcumin-induced apoptosis which occurs at G2 phase[144, 145].

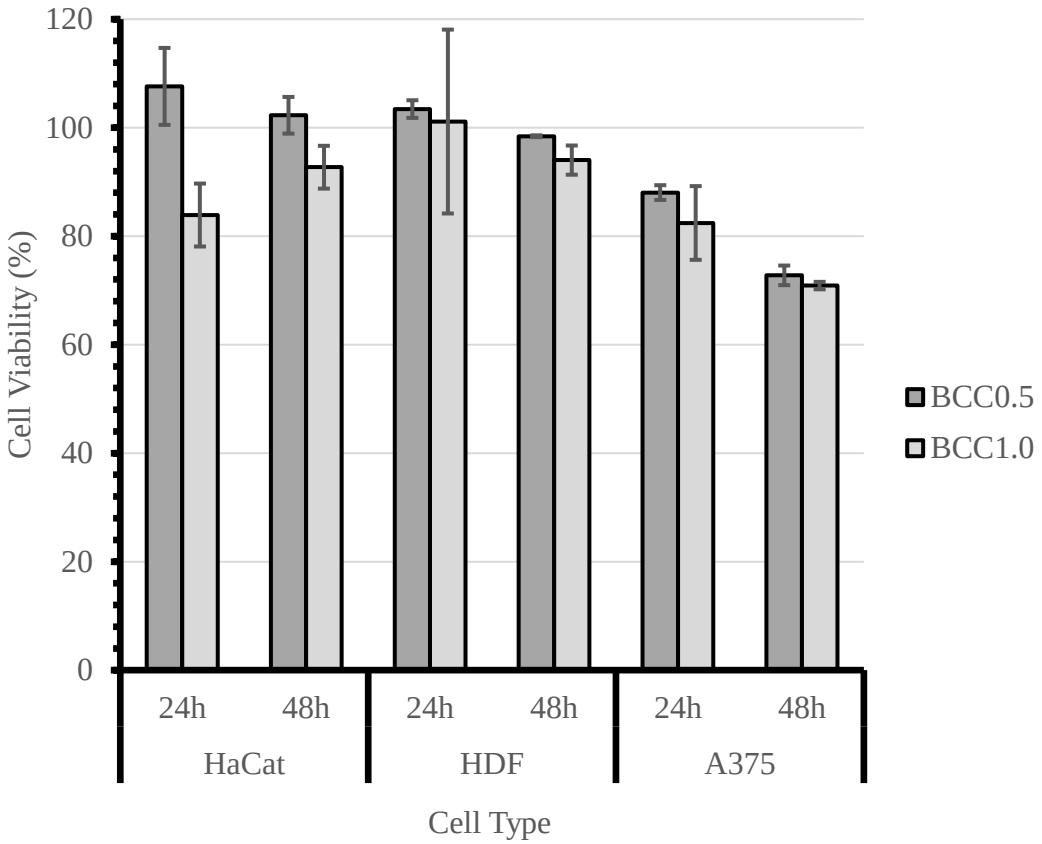

Figure 27: Cell viabilities of normal dermal (HaCat, HDF), and malignant melanoma (A375) cells when treated with 1000µg/mL sample extracts. (Values reported as mean ± SD; n=2; differences were declared statistically significant at the level of $p<0.05$, analyzed by Student's t-test. The differences of cell viabilities between all normal dermal cells and A375 cells were statistically significant except in case of 24-hour treatment with BCC1.0 extract; No statistically significant differences between both types of normal dermal cells for all cases)

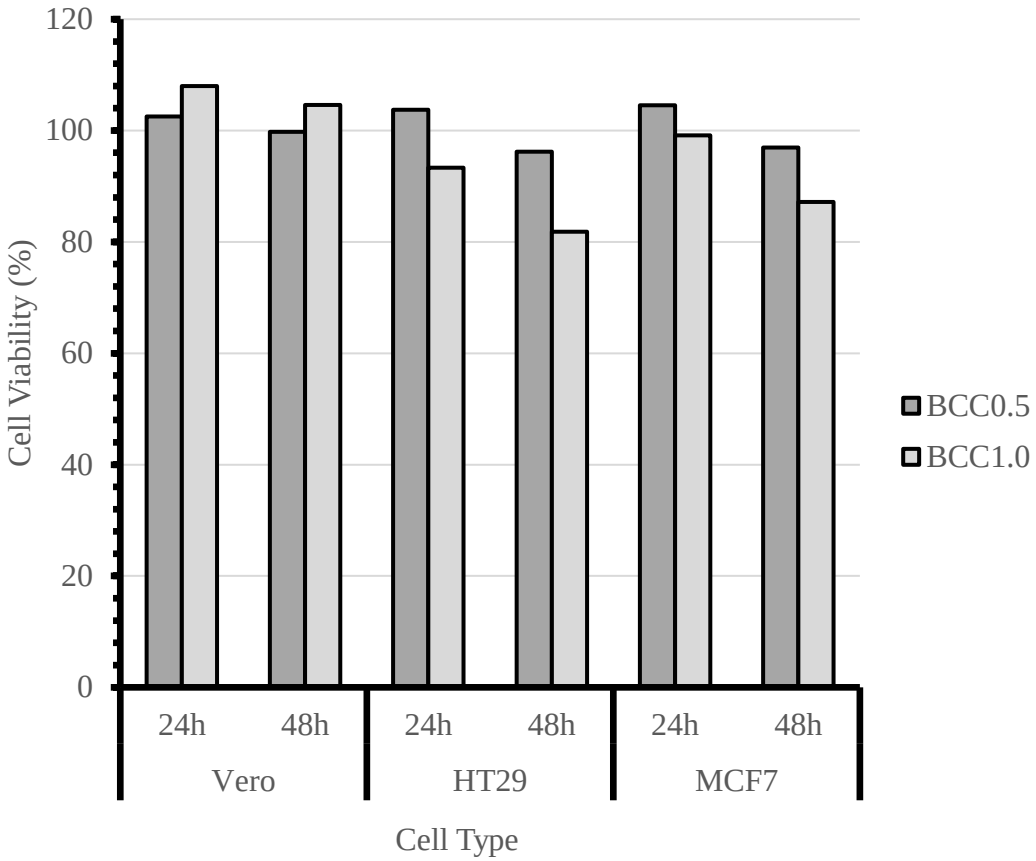

Figure 28: Cell viabilities of Vero, HT29, and MCF7 cells when treated with 1000µg/mL sample extracts.

CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study presents a new methodology for entrapment of curcumin in BC film, and the controlled release of the compound. Curcumin was absorbed into BC pellicles and then the pellicles were air dried. The non-transdermal controlled release of curcumin from BCC films was achieved in buffer solutions containing Tween 80 and methanol additives, at pH 5.5 and 7.4. Assessments of cytotoxicity and anticancer properties of BCC films revealed that curcumin released from BCC films was selectively cytotoxic toward A375, HT29, and MCF7 cells, but not toward normal cells.

6.2 Recommendations for future studies

The optimization of curcumin loading concentration and treatment time may be performed to improve the effectiveness of cancer treatment. Curcumin loading process and sterilization may be optimized by avoiding the severe sterilization condition when curcumin was loaded. Controlled release experiment may be performed by placing the films over the porcine skin in Franz diffusion cells to study the release of curcumin onto the real skin tissue. This would be beneficial when this film is to be developed into transdermal patch for skin applications. Non-cytotoxicity towards normal cells of the film should be performed on reciprocal normal cells of the cancer cells that are the main target of study.

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APPENDIX

Appendix I: Calibration curve of curcumin

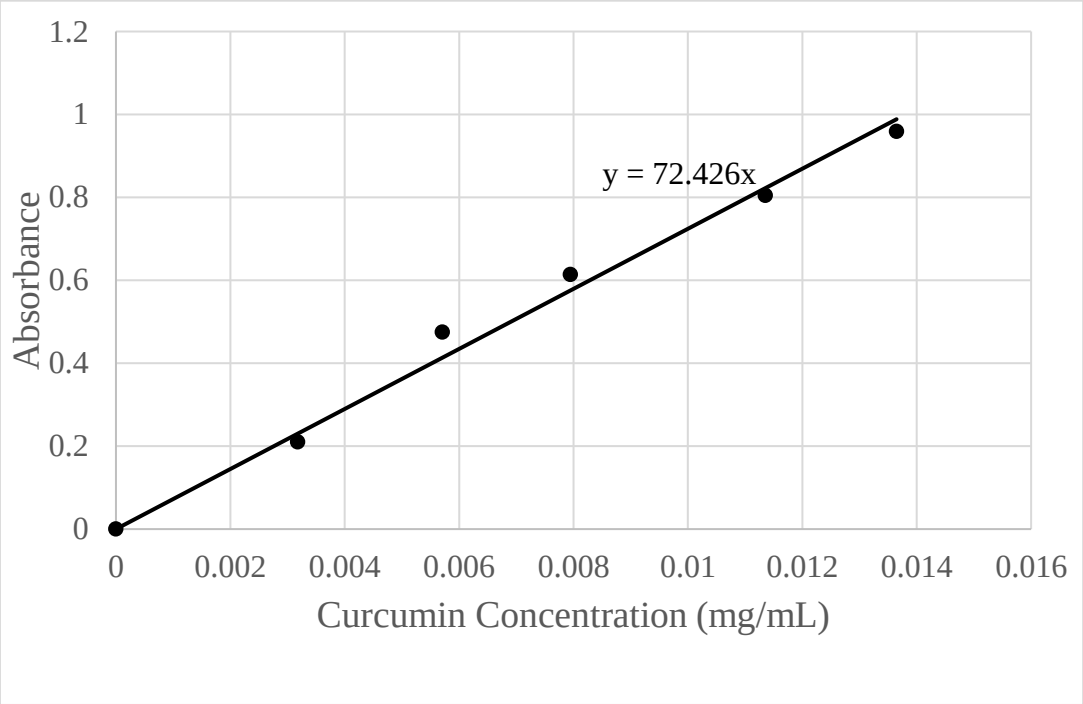

Figure A1: Calibration curve of curcumin

Table A1: Data plotted on calibration curve

Curcumin concentration at UV-vis (mg/mL)	Absorbance
0.003176471	0.21
0.005705882	0.475
0.007941176	0.614
0.011352941	0.805
0.013647059	0.959

Appendix II: Table of release assay data

Table A2: Release of curcumin from sample BCC0.25 when Acetate Buffer, pH 5.5, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
62	1.0333	0.0789	0.0665
120	2.0000	0.1992	0.1678
181	3.0167	0.1646	0.1387
241	4.0167	0.2079	0.1752
303	5.0500	0.1734	0.1460
367	6.1167	0.2561	0.2158
484	8.0667	0.2225	0.1875
605	10.0833	0.3852	0.3245
721	12.0167	0.1572	0.1325
1445	24.0833	0.1602	0.1349
2164	36.0667	0.1631	0.1374
2891	48.1833	0.0871	0.0734

Table A3: Release of curcumin from sample BCC0.25 when Acetate Buffer, pH 5.5, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
72	1.2000	6.6669	5.6237
122	2.0333	8.1587	6.8821
183	3.0500	8.6157	7.2676
242	4.0333	8.9211	7.5252
303	5.0500	9.3475	7.8849
368	6.1333	9.2665	7.8165
485	8.0833	9.4948	8.0091
603	10.0500	9.2111	7.7698
723	12.0500	9.0746	7.6547
1446	24.1000	8.7337	7.3671
2165	36.0833	7.5922	6.4042
2907	48.4500	7.2103	6.0821

Table A4: Release of curcumin from sample BCC0.25 when physiological PBS, pH 7.4, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
60	1.0000	0.3945	0.3616
118	1.9667	0.2853	0.2616
178	2.9667	0.2523	0.2313
240	4.0000	0.3368	0.3087
300	5.0000	0.3441	0.3155
362	6.0333	0.5093	0.4669
478	7.9667	0.3231	0.2962
604	10.0667	0.2901	0.2659
720	12.0000	0.3351	0.3071
1440	24.0000	0.3809	0.3492
2156	35.9333	0.3883	0.3560
2878	47.9667	0.5929	0.5435

Table A5: Release of curcumin from sample BCC0.25 when physiological PBS, pH 7.4, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
63	1.0500	4.3000	4.2246
120	2.0000	5.4616	5.3658
180	3.0000	6.2920	6.1817
243	4.0500	7.0989	6.9745
301	5.0167	6.8558	6.7357
361	6.0167	6.9589	6.8369
481	8.0167	6.2326	6.1234
603	10.0500	6.1575	6.0496
721	12.0167	5.8018	5.7001
1441	24.0167	4.5282	4.4488
2160	36.0000	3.4603	3.3996
2882	48.0333	2.9190	2.8678

Table A6: Release of curcumin from sample BCC0.5 when Acetate Buffer, pH 5.5, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
60	1.0000	0.0789	0.0397
117	1.9500	0.2385	0.1201
178	2.9667	0.3622	0.1824
238	3.9667	0.3307	0.1666
300	5.0000	0.2195	0.1106
365	6.0833	0.2634	0.1327
482	8.0333	0.2688	0.1354
602	10.0333	0.2741	0.1381
719	11.9833	0.2400	0.1209
1442	24.0333	0.1656	0.0834
2161	36.0167	0.2471	0.1245
2888	48.1333	0.1332	0.0671

Table A7: Release of curcumin from sample BCC0.5 when Acetate Buffer, pH 5.5, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
72	1.2000	12.1503	5.7067
124	2.0667	12.7921	6.0081
184	3.0667	14.5859	6.8506
243	4.0500	15.4642	7.2631
303	5.0500	16.1178	7.5701
368	6.1333	15.9108	7.4729
485	8.0833	15.6145	7.3337
603	10.0500	15.6217	7.3371
723	12.0500	14.9527	7.0229
1447	24.1167	15.0139	7.0516
2165	36.0833	13.8483	6.5041
2906	48.4333	13.4032	6.2951

Table A8: Release of curcumin from sample BCC0.5 when physiological PBS, pH 7.4, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
58	0.9667	0.2367	0.1075
116	1.9333	0.3213	0.1459
177	2.9500	0.3290	0.1494
239	3.9833	0.3367	0.1529
299	4.9833	0.3838	0.1743
361	6.0167	0.2741	0.1245
477	7.9500	0.2798	0.1271
603	10.0500	0.2856	0.1297
719	11.9833	0.4097	0.1860
1438	23.9667	0.4183	0.1900
2154	35.9000	0.3875	0.1760
2877	47.9500	0.3952	0.1794

Table A9: Release of curcumin from sample BCC0.5 when physiological PBS, pH 7.4, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
64	1.0667	5.7201	2.9653
120	2.0000	8.4572	4.3842
180	3.0000	10.1505	5.2620
243	4.0500	11.3265	5.8716
301	5.0167	11.8934	6.1655
361	6.0167	12.1915	6.3200
481	8.0167	11.7804	6.1069
603	10.0500	11.5510	5.9880
721	12.0167	11.5472	5.9860
1441	24.0167	10.9855	5.6949
2160	36.0000	10.0505	5.2101
2882	48.0333	9.2859	4.8138

Table A10: Release of curcumin from sample BCC1.0 when Acetate Buffer, pH 5.5, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
58	0.9667	0.1183	0.0511
113	1.8833	0.1997	0.0862
175	2.9167	0.2826	0.1220
235	3.9167	0.2487	0.1074
298	4.9667	0.1747	0.0754
362	6.0333	0.2173	0.0938
479	7.9833	0.3002	0.1296
598	9.9667	0.2269	0.0980
716	11.9333	0.2310	0.0997
1439	23.9833	0.2744	0.1185
2158	35.9667	0.1609	0.0695
2884	48.0667	0.2028	0.0875

Table A11: Release of curcumin from sample BCC1.0 when Acetate Buffer, pH 5.5, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
66	1.1000	16.7264	5.7473
125	2.0833	20.3809	7.0029
184	3.0667	22.4134	7.7013
244	4.0667	24.7185	8.4933
304	5.0667	26.2364	9.0149
363	6.0500	28.1317	9.6661
483	8.0500	29.2291	10.0432
604	10.0667	31.2850	10.7496
725	12.0833	30.7703	10.5728
1444	24.0667	36.0682	12.3931
2162	36.0333	36.8966	12.6778
2884	48.0667	37.0965	12.7465

Table A12: Release of curcumin from sample BCC1.0 when physiological PBS, pH 7.4, without Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
57	0.9500	0.3550	0.1633
115	1.9167	0.3230	0.1486
176	2.9333	0.3296	0.1516
238	3.9667	0.3363	0.1547
299	4.9833	0.5007	0.2303
360	6.0000	0.3528	0.1623
477	7.9500	0.3594	0.1653
602	10.0333	0.5238	0.2410
718	11.9667	0.3365	0.1548
1438	23.9667	0.5001	0.2300
2153	35.8833	0.4697	0.2161
2876	47.9333	0.4385	0.2017

Table A13: Release of curcumin from sample BCC1.0 when physiological PBS, pH 7.4, with Tween 80/methanol supplement, is used as release medium.

Cumulative time (minutes)	Cumulative time (Hours)	Concentration of released curcumin (µg/mL)	% release based on actual amount
0	0.0000	0.0000	0.0000
65	1.0833	11.4797	3.7042
122	2.0333	14.5037	4.6799
181	3.0167	17.3543	5.5997
244	4.0667	19.7497	6.3726
302	5.0333	21.2838	6.8677
363	6.0500	22.1332	7.1417
484	8.0667	23.2282	7.4950
603	10.0500	23.1936	7.4839
724	12.0667	22.8318	7.3671
1440	24.0000	21.0702	6.7987
2162	36.0333	18.0352	5.8194
2882	48.0333	17.0125	5.4894

VITA

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