FABRICATION OF BACTERIAL CELLULOSE, CHITOSAN AND ESSENTIAL OIL BIOCOMPOSITE FOR ANTIMICROBIAL EFFECT

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ABSTRACT

Chronic wounds represent a significant burden to patients, health care professionals and the health care system. Therefore healing process is very important to avoid inflammation to the wound area. The objectives of this study were to produce biocomposite film from bacterial cellulose, chitosan and essential oil and study the characterization of the biocomposite film by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), pycnometer, antimicrobial effect, biodegradability testing and water absorption. The film was synthesized by using mixing and casting process and bacterial cellulose is produced by *Acetobactor Xylinum*. The samples contain three different amount of chitosan which were (25%, 50%, 75%). The FTIR showed the interaction between components at 3000-3500 cm^{-1} which is OH bonding besides that it also show the , C=O at band 1651 cm^{-1} and also amine group at 1550-1580 cm^{-1}. From the antimicrobial test, the sample contain of 75% chitosan showed the higher percentage of effectiveness to kill the bacteria and good water absorption. Then from the density testing, the result showed the sample was contain 75% chitosan have high density and have lower degradable rate. Moreover the SEM analysis these film showed smooth, compactable and homogenous structure. In conclusion, the objectives in this study were achive and the biocomposite film contain bacterial cellulose, chitosan and essential oil had a potential in medical application especially for wound healing.

ABSTRAK

Masalah luka yang teruk ataupun kronik adalah satu beban yang akan ditanggung oleh pesakit, doktor dan juga kepada sistem kesihatan rawatan. Oleh sebab itu dengan memahami dan mempelajari proses penyembuhan biasa ia akan dapat mengatasi masalah ini. Tujuan kajian ini adalah untuk menghasilkan filem biokomposit daripada tiga bahan utama iaitu kitosan, selulose bacteria dan minyak esensial dan Filem yang terhasil akan diciri kan dengan mengunakan alat seperti spektroskopi inframerah transformasi Fourier (FTIR), mikroskopi pengimbasan elektron (SEM) dan gas pycnometer. Filem ini juga telah dicirikan dengan melihat kesan bakteria terhadap filem, penyerapan air dan kelembapan dan biodegradasi mengunakan kaedah timbus tanah. Filem ini dibuat dengan menggunakan process pencampuran dan proses tuangan, yang mana selulosa bakteria dihasilkan oleh *Acetobactor xylinum*. Kepekatan kitosan dalam filem ini ditetapkan pada tiga nilai iaitu 25%, 50% dan 75% kitosan, manakala nilai untuk bahan lain adalah sama. Ujian FTIR menunjukan terdapat ikatan antara bahan-bahan yang digunakan pada nilai 3000-3500 cm^{-1} iaitu ikatan OH, selain itu terdapat juga ikatan lain seperti, C=O pada ikatan 1651^{-1} amime pada 1550-1580 $^{-1}$. Filem yang mengandungi 75% kitosan menunjukan peratusan yang lebih tinggi keberkesanan untuk membunuh bakteria dan mempunyai kadar penyerapan air yang baik. Pada ujian ketumpatan pula filem ini mempunyai nilai kepadatan yang tinggi dan kadar biodegradasinya rendah. Ini disebabkan oleh structur filem tersebut sendiri. Kesimpulannya filem yang mengandungi kitosan, selulose bakteria dan minyak esensial mempunyai potensi dalam aplikasi perubatan terutama untuk penyembuhan luka

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%	-	percentage
SEM	-	Scanning Electron Microscopy
FTIR	-	Fourier Transform Infra-Red
°C	-	Celcius
BC	-	Bacterial Cellulose
µL	-	microliter
mL	-	milliliter
EAA	-	Effectiveness antibacterial activity
cm	-	centimeter
µm	-	micrometer
nm	-	nanometer

CHAPTER 1

INTRODUCTION

1.0 Background Of Study

In human body, skin is the biggest organ and it is a barrier against environment. When the skin is destroyed by damage or disease, the moisture content and protein in wound would be lost and the infection of wound area will be increase.

Chitosan is an N-deacetylated product of chitin and one of the most abundant polysaccharides in nature. Besides that chitosan known as biopolymer comprising between glucosamine and N-acetylglucosamine. Chitosan also have high molecular weight and a major component of insects and crustaceans shells. Chitosan is more suitable material for wound dressing. It is because chitosan has anti-infunctional activity and property to accelerate wound healing. Due to those reasons, chitosan has been one of the most important biomaterial for wound healing in the recent years.

Bacterial cellulose is known as polysaccharide and usually it been used traditionally in food industry and the latest it is used as a material for medical application. Bacterial cellulose has great characteristics such as good in mechanical properties, water sorption capacity, porosity, stability and conformability. Bacterial cellulose is nearly-purified cellulose and can be extracellulary synthesized into nano-sized fibrils by the *Acetobacter xylinum* by using glucose as a common substrate. In medical field, several application of bacterial cellulose have been reported such as an artificial skin for human with extensive burns, artificial blood vessel for microsurgery and wound dressing.

The essential oil also known by the names of aromatic oil, fragrant oil, ethereal oil and steam volatile oil. Basically, essential oil used in fragrance, flavor and pharmaceutical industries as well as in aromatherapy. Essential oil is concentrated, and volatile compound that have been made from plant. It is different with vegetable oil which it can evaporates when the oil is exposed to air. The formation of essential oil is by using distillation process. The essential oil also have special properties that make it popular as a natural agent for killing all three type of infection cause by organisms such as fungus, bacteria and virus.

Biocomposite consist of biodegradable polymers and natural fibers. Biocomposite can have complete degradation in soil without emitting any toxic or noxious component during degradation. Biocomposite or biodegradable polymer provide new evolution in industrial research.

1.1 Problem Statement

The effective wound dressing not only can protect the wound area from the surrounding environment but also effectively promote the healing process. An ideal wound dressing should have the following properties. Firstly it must be able to protect the wound from infection, secondly it must provide moisturized wound healing environment and lastly, it must be clean and dry. Thus this research will be concentrate on fabrication of biocomposite film that is the biodegradable, non-toxic and also effective as a wound dressing application.

1.2 Objective

The objective of this research is to produce biocomposite film from bacterial cellulose, chitosan and essential oil that has an antibacterial effect.

1.3 Scope Of Study

The scopes of the study are

i. to produce biocomposite film from bacterial cellulose, chitosan and essential oil
ii. to analyze the biocomposite film in different content of chitosan from 25% to 75 % in order to get the effective wound dressing
iii. to characterize the biocomposite film by using FTIR, SEM, gas pycnometer and to analyze the antimicrobial effect, biodegradable testing and water absorption.

1.4 Rationale And Significance

The production of biocomposite film from bacterial cellulose, chitosan and essential oil for wound healing can reduce the healing step. Besides that this film are biodegradable, non-toxic and also have antimicrobial effect which can promote on the healing process. It is because of the component that been used in this film.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Wound dressing is the most important thing in daily life. Most wound dressing today still in the traditional styles, such as using antibiotic and plaster. Thus it will take much time and cost, to be effective on wound healing process. This research will be focusing on the fabrication of biocomposite film by using bacterial cellulose, chitosan and essential oil.

2.2 Chitosan

Chitin is consists of 2-acetamido-2-deoxy-b-D-glucose. Chitosan is the N-deacetylated derivate of chitin. Chitin and chitosan have a range of current and potential applications in photography, cosmetics, artificial skin, dressing, food and nutrition, ophthalmology, water engineering, metal capture from wastewater, paper finishing, solid-state batteries, drug delivery system, biotechnology, and cell-stimulating materials (Ravi Kumar, 2000). In recent years, chitosan films have mainly been applied to the pharmaceutical field. Generally, chitosan is dissolved in acetic acid or hydrochloric acid, then the solvent is removed by drying; or chitosan is precipitated by using coagulating agent (e.g., sodium hydroxide), and finally made into film. The relevant applications include bone cell adhesion and growth (Hamilton et al., 2007), blood compatibility (Yang, Zhou, Chuo, Wang, & Yu, 2007) and cell adhesion (Freier, Koh, Kazazian, & Shoichet, 2005). Chitosan film is biocompatible, biodegradable, and nontoxic, thus it is applicable to pharmaceutical and food industries. However, chitosan cost is high and thus importance is attached to the research on the combination of chitosan and other macromolecules.

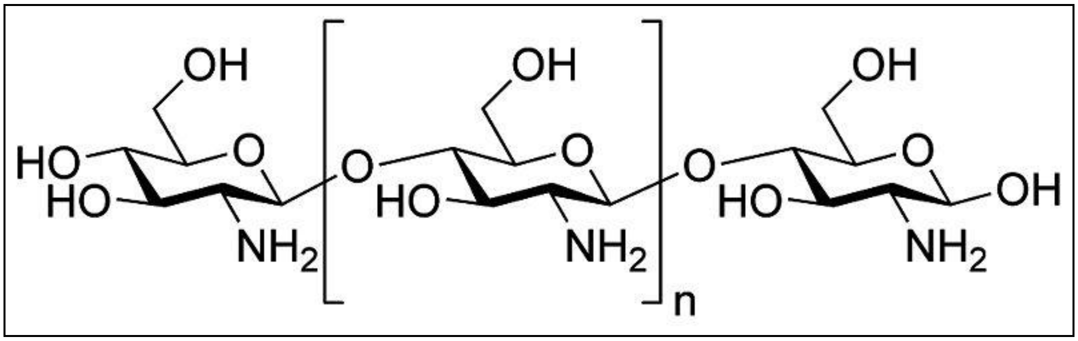

Figure 2.1: Chemical structure of chitosan

The main factors affecting the antibacterial activity of chitosan are molecular weight (MW) and concentration (Jeon et al., 2001; Liu et al., 2006). Chitosan is useful as a wound management aid to reduce scar tissue. Chitosan has also been found to be a good support material for gene delivery, cell culture, tissue engineering, drug delivery, anti-microbial agents and adsorption agents (Jayakumar, Prabaharan, Reis, & Mano, 2005).

The antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria. However, the antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions (e.g., substrate water activity or moisture or both). Although both native chitosan and its derivatives are effective as antimicrobial agents, there is a clear difference between them. Their different antimicrobial effect is mainly exhibited in live host plants. The fungicidal effect of *N*-carboxymethyl chitosan (NCMC) is also different in vegetable as compared to graminea hosts. In addition, oligomeric chitosans (pentamer and heptamer) have a better antifungal effect than larger units. The chitosan antimicrobial activity is more immediate on fungi and algae than on bacteria (Sarvard et al., 2002)

Chitosan inhibits the growth of a wide variety of bacteria4 (Table 2.1). Chitosan has been studied in terms of bacteriostatic/bactericidal activity to control growth of algae and to inhibit viral multiplication (Jolles et al.,1999)

Table 2.1: Minimum growth inhibitory concentration of Chitosan against Bacteria

Bacteria	Minimum growth inhibitory concentration (ppm)
Agrobacterium tumefaciens	100
Bacillus	1000
Xanthomonas campestris	500
Staphylococcus aureus	20
Pseudomonas fluorescens	500
Micrococcus luteus	20
Klebsiella pneumonia	700
Escherichia coli	20
Erwinia carotovora subsp	200
Erwinia sp	500
Corinebacterium michiganence	10

(Jolles eal.,1999)

Moreover, chitosan has several advantages over other type of disinfectants because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells.

The use of bioactive substances such as chitosan to control postharvest fungal disease has attracted much attention due to imminent problems associated with chemical agents, which include development of public resistance to fungicide-treated produce, an increasing number of fungicide-tolerant postharvest pathogens, and a number of fungicides that are still under observation(El Ghaouth et al.,2000).Chitosan (1 mg/mL) reduces the in vitro growth of numerous fungi with the exception of Zygomycetes, that is, the fungi containing chitosan as a major component of their cell walls. Hence, chitosan has potential as an edible antifungal coating material for postharvest produce.

Chitosan films are tough, long lasting, flexible, and very difficult to tear. Most of their mechanical properties are comparable to many medium-strength commercial polymers. The chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values. However, extremely good barriers were observed for the permeation of oxygen, while relatively low vapor barrier characteristics were exhibited.

2.3 Bacterial cellulose

Cellulose, the basic material of all plant substances, is the most abundant polysaccharide found in nature. Cellulose derived from plant is unpurified cellulose associated with other kinds of natural fiber like lignin and hemicellulose while bacterial cellulose (BC) is nearly-purified cellulose. BC can be extracellularly synthesized into nano-sized fibrils by the bacteria Acetobactor xylinum, using glucose as a common substrate. Plant-derived cellulose and BC have the same chemical structure. However, with an ultra-fine network structure, BC displayed advantages superior to the counterpart from plants with its physical and chemical properties: such as mechanical strength, crystallinity and hydrophilicity.(Muenduen et al.,2008)

Several applications of BC in medical fields have been reported such as artificial skin for humans with extensive burns, artificial blood vessels for microsurgery (Klemm, Schumann,Udhardt, & Marsch, 2001), scaffolds for tissue engineering of cartilage (Svensson et al., 2005) and wound-dressing (Czaja, Krystynowicz, & Bielecki, 2006).

BC shows high water content, good sorption of liquids, non-allergenic and can be safely sterilized without any change to its characteristics. Our previous report (Sanchavanakit et al., 2006) found that BC film supported the growth, spreading and migration of human keratinocytes but not those of human fibroblasts. To extend the field of the potential applications, modifications of BC in physical and biological properties need further studies. The modification by combination with other organic polymers could be an effective method to improve the characteristics and structure of the BC film.

Bacterial cellulose (BC) is a polysaccharide used traditionally in the food industry (M. Iguchi et al 2000), later in the fabrication of reinforced paper and recently it was investigated as a material for medical applications. Studies carried out in vitro and in vivo have demonstrated its biocompatibility (G Helenius et al.,2006). Due to its good mechanical properties, water sorption capacity, porosity, stability and conformability, BC has been used in tissue engineering of cartilage (A. Svensson et al., 2005), replacement of blood vessels in rats and in the wound healing process (Czaja et al.,2007)

BC is pure cellulose with no other components. Nanocomposites based on BC can be fabricated statically either by using the synthesized BC gel or modifying the cellulose biosynthesis. For instance, BC nanocomposites for biomedical applications with improved mechanical properties were created by soaking BC on polyacrilamide and gelatin solution (Yasuda et al.,2005) and (Nakayama et al., 2004). BC-hydroxyapatite scaffolds for bone regeneration have been developed by immersing the BC gel in simulated body fluid (SBF) or in both calcium and phosphate solution (Hong et al.,2006). Furthermore, BC-polyester and BC-PVA nanocomposites were developed for potential applications as vascular implant (Charpentier et al.,2006) Some researchers have introduced different materials into the culture media of BC. BC synthesized in the presence of collagen (Wiegand et al.,2006) chitosan has improved properties as wound dressing and for other biomedical applications. It has been reported that BC membranes produced in the presence of carboxymethylcellulose (CMC) have better adsorption capacity of metal ions than membranes of pure BC (Chen S ET AL.,2008)

However, the addition of some polymers can modify drastically the cellulose biosynthesis. The addition of CMC into the culture medium alters the crystallization and assembly of the cellulose fibrils A similar effect occurred when polyethylene oxide is added to the medium in the process for obtaining BC based nanocomposites (Brown et al.,2007).

In agitated cultures, it has been demonstrated that BC can be produced in the presence of solid particles (i.e. glass beads, paper fibres and $CaCO_3$) without affecting the rate of formation of the hydrogel. Recent studies report the inclusion of silica particles of 10–20 nm and multi-walled carbon nanotubes (20–40 nm outer diameter, 10–50 μm length) into the culture medium to produce BC-nanocomposites in static cultures (Yano, et al.,2008)

2.4 Essential oil

Essential oils also known as volatile or etheraeal oils or essences, are the mixtures of highly fragrant compounds found in aromatic plants and flowers that are the raw material of the flavors and fracrances. The essential oil producing plants are distributed widely across the plant kingdom and cover a large number of famililties including Lamiaceace (mints, basil, lavender, etc). Essential oils are secondary metabolites, often produced in special glands or secretory tissues, and are generally found to be most abundant in one particular plant organ such as flower, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, root/rhizomes, resin or exudates, depending on the species. Essential oils can be extracted from plant organs by crushing or by distillation in a heated aqueous or alcoholic solvent, and their active components subsequently isolated and characterized using HPLC and gas-liquid chromatography. Essential oils have been used by mankind from time immemorial for various purpose such as perfumes, flavors, and medicine. In the modern era, essential oil used in fragrances, cosmetics, soaps, foods, confectionary, preservatives, insect repellents, and pharmaceutical product. Many of essential oils possess antimicrobial, anticancer and other medicine properties.

The accumulation of essential oil in plants is developmentally regulated and depends on plant species as well as on plant organ tissue and cells. In plants such as menthe, ocimum sanctum, majorana hortensis, salvia officinalis and cymbopogon flexuosus, where leaves are the major source of commercially valuable oil, its accumulation is associated with early stages of leaf development, as was reported for palmarosa cymbopogon martini leaves The medicinal properties of essential oil have received increasing attention over the past 20 to 30 years but to date, still less than 10% of approximately 250000 of the world's flowering plant species have been analyzed for their pharmacological properties. Almost 25% of active, medical compounds currently prescribed in the USA and UK are isolated from higher plant (Anthony et al, 2005). Plant essential oils are of great value due to the anticancer, antiparasitic, and antimicrobial properties. Some aromatic compound of essential oil are important ingredient of food as flavoring, cosmetic as perfumes and aftershaves and oral healthcare.

Nowadays essential oils are gaining popularity in aromatherapy, one of the branches of phytotherapy, which uses the whole plants or part of plants for medical purpose. In aromatherapy pure essential oil from fragrant plant (such as lavender, jasmine, rose, sandalwood, rosemary, basil, thyme) are used to help relieve health problems and improve the quality of life in general During the treatment, essential oil are commonly used in oil burner, bath water, or massaged into the skin, thus the aroma of the essential oil evaporates and stimulates the olfactory sense. Aromatherapy is thought to be therapeutically effective due to both the psychological effect of the odor and the physiological effects of the inhaled volatile compound. The healing properties of aroma therapy are claimed to promote relaxation and sleep, relief of plain, and reduction of depressive symptoms, with the rationale that the essential oils have a claming and de-stressing effect. Although the pharmacokinetics and physiological effects of essential oils in aromatherapy are still unclear to date, used in maternal and child health, critical care environments, pain relief, cancer care, skin and hair condition, respiratory condition, digestive disorders, and some medical condition

2.5 Analysis Equipment

2.5.1 Fourier Transform Infra-Red (FTIR)

Fourier Transform Infra-Red (FTIR) absorption spectroscopy is an analytical technique based on the frequency at which chemical bonds vibrate when subjected to electromagnetic radiation passed through (transmission mode), or reflected off (reflection mode), a subject of interest. As functional groups and polar bonds of elements (e.g. Si, O, H, C and N) absorb radiation at specific wavelengths, FTIR spectroscopy can be used to both qualitatively and quantitatively measure these elements. FTIR analyses of geologic materials, particularly natural and experimental glasses, minerals and melt inclusions, have been undertaken for many years

These are transmission micro FTIR studies that utilise an attached microscope to analyse small areas (typically <200μm) of a sample. The result is a single high-resolution infra-red spectrum, providing an average water species content for the area analysed, with limited spatial resolution. An important advance in FTIR spectroscopy in the past decade has been the advent of spectroscopic imaging a technique that allows a map of an element or molecule of interest to be constructed over a large (sub mm) area from multiple spectra collected by transmission or reflection mode. More recent developments by Varian Inc. allow optical maps of a band of interest to be constructed from 4096 simultaneously collected high-resolution spectra, with a spatial resolution of just 5 μm. As well as improved spatial resolution, the simultaneous collection of spectra distinguishes this technique from image maps, which are a composite of individual spectra collected sequentially to form a large composite image. An increasing number of studies have utilised the spectroscopic imaging technique, however, with the exception of one study on cherts from Kanto Mountain, Japan (Ito and Nakashima, 2002),

2.5.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is a high resolution surface imaging technique. Many biological process and structures occur at surfaces and if antibodies are available, their components can be located within the surface structure. This is usually done in a similar way to immuno-fluorescence, using an unconjugated primary antibody followed by a tagged secondary antibody against the primary. A SEM can provide a wide range of magnification, from 30× to 100,000×, and the SEM chamber is large to work with (Li M et al.,2005) and (Liu et al., 2009) SEM images were digitally processed by DIC.

2.5.3 Gas Pycnometer

The gas pycnometer is one of the non-destructive techniques for density measurements. This device allows measurements of volume with high precision (accuracy of an order of 5×10^{-5}), so it is interesting to see if it is possible to determine swelling of delta plutonium alloys. The application on plutonium measurements entails some problems, caused by radioactive self heating While pycnometers (of any type) are recognized as density measuring devices they are in fact devices for measuring volume only. Density is merely calculated as the ratio of mass to volume; mass being invariably measured on a discrete device, usually by weighing. The volume measured in a gas pycnometer is that amount of three-dimensional space which is inaccessible to the gas used, i.e. that volume within the sample chamber from which the gas is excluded. Therefore the volume measured considering the finest scale of surface roughness will depend on the atomic or molecular size of the gas helium therefore is most often prescribed as the measurement gas, not only is it of small size, it is also inert and the most ideal gas

2.5.4 Antimicrobial testing

The number of tests for measuring antimicrobial susceptibility exist, they all follow 1 of 2 fundamental principles (diffusion or dilution of the antimicrobial agent), and these are available in a variety of formats (Walker, 2006). Tests generating quantitative data are the only ones currently used in countries with active surveillance programs For *Campylobacter* antimicrobial susceptibility testing, the agar dilution and microbroth dilution are considered the gold standard, the agar dilution method was not standardized until recently, and the microbroth dilution, although a very attractive alternative needs to be standardized by the Clinical and Laboratory Standards Institute (CLSI). This quantitative test, like the agar dilution method, uses a combination of dilution and agar diffusion principles in 1 single inert, nonporous thin plastic strip

The antimicrobial effect is most important test for that film, because this film must have an ability to stop the growth of bacterial. The chitosan and cellulose film demonstrated effective antimicrobial capability against *Escherichia coli* and *staphylococcus aureus*.

2.5.5 Biodegradable testing

Biodegradable polymers are a newly emerging field. A vast number of biodegradable polymers have been synthesized recently and some microorganisms and enzymes capable of degrading them have been identified. In developing countries, environmental pollution by synthetic polymers has assumed dangerous proportions. As a result, attempts have been made to solve these problems be including biodegradability into polymers in everyday use through slight modifications of their structures.

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Natural macromolecules, e.g. protein, cellulose, and starch are generally degraded in biological systems by hydrolysis followed by oxidation. It is not surprising, then, that most of the reported synthetic biodegradable polymers contain hydrolyzable linkages along the polymer chain; for example, amide enamine, ester, urea, and urethane linkages are susceptible to biodegradation by microorganisms and hydrolytic enzymes. Since many proteolytic enzymes specifically catalyze the hydrolysis of peptide linkages adjacent to substituents in proteins, substituted polymers containing substituents such as benzyl, hydroxy, carboxy, methyl, and phenyl groups have been prepared in the hope that an introduction of these substituents might increase biodegradability.

During degradation, the crystallinity of the sample increases rapidly at first, then levels off to a much slower rate as the crystallinity approaches 100%. This is attributed to the eventual disappearance of the amorphous portions of the sample. The effect of morphology on the microbial and enzymatic degradation of biodegradable polymer with a number of potential applications, has been studied. Scanning electron microscopy (SEM) has shown that the degradation of a partially crystalline polycaprolactone film by filamentous fungi proceeds in a selective manner, with the amorphous regions being degraded prior to the degradation of the crystalline region.

2.5.6 Water absorption

Water absorption is the amount of water absorbed by a composite material when immersed in water for a stipulated period of time. It is also the ratio of the weight of water absorbed by a material, to the weight of the dry materials. All organic polymeric materials will absorb moisture to some extent resulting in swelling, dissolving, leaching, plasticizing or hydrolyzing, events which can result in discoloration, embrittlement, loss of mechanical and electrical properties, lower resistance to heat and weathering and stress cracking

Moisture sensitive foods or pharmaceuticals are usually put into sealed packagingfilms with controlled water vapor permeability to obtain the required quality, safety and shelf-life. To be successfully applied as a biodegradable film in the medical field, chitosan, bacterial cellulose and essential oil film should resist moisture transfer through the inside and the outside of film.