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Biomedical-Grade Chitosan in Wound Management and Its Biocompatibility *In Vitro*

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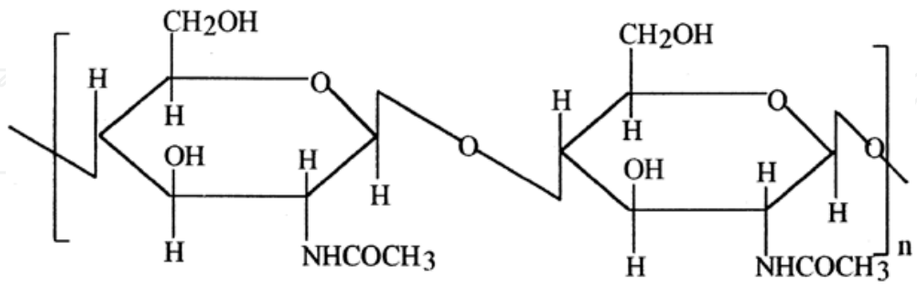
1. Introduction

Chitin (β-1,4-D-linked polymer of *N*-acetylglucosamine) is a naturally abundant mucopolysaccharide and is second to cellulose in terms of the amount produced annually by biosynthesis. Chitin is visually characterized as a white, hard, inelastic, nitrogenous polysaccharide, and approximately one billion tons are synthesized each year (Peter, 1997). Chitin is a common constituent of the exoskeleton in animals, particularly in crustaceans, mollusks and insects. Commercially sold chitin is usually extracted from shellfish waste (Skjak-Braek et al., 1989; Goosen, 1997). Chitin is structurally similar to cellulose; however less attention has been paid to chitin than cellulose, primarily due to its inertness. Hence, it remains an essentially unutilized resource. Deacetylation of chitin yields chitosan, which is a relatively reactive compound and is produced in numerous forms, such as powder, paste, film and fiber.

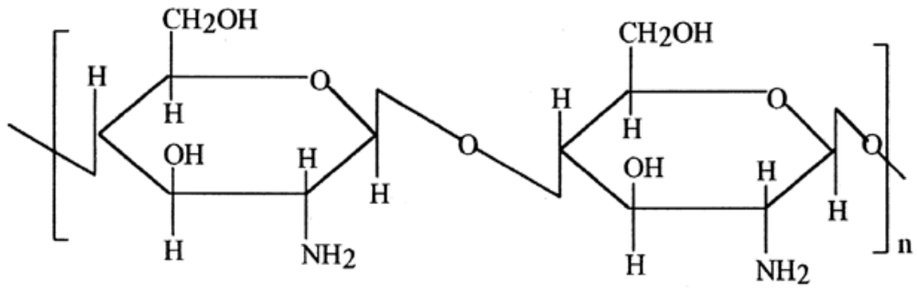
Chitosan is a poly-(β-1, 4-D-glucosamine) derived from the *N*-deacetylation of chitin (Figure 1). It is soluble in dilute aqueous acetic, lactic, malic, formic and succinic acids. Chitosan may be fully or partially *N*-deacetylated, but the degree of acetylation is typically less than 0.35. The acetylation ratio is defined by a variety of methods, including pyrolysis gas chromatography, gel permeation chromatography and ultra-violet (UV) spectrophotometry, titration, separation spectrometry and near-infrared spectroscopy (Kumar, 2000). Most commercial chitosans have a degree of deacetylation that is greater than 70% and a molecular weight ranging between 100,000 and 1.2 million Da (Li et al., 1997). Chitosans are of commercial interest due to their high percentage of nitrogen compared to synthetically substituted cellulose, rendering them useful for metal chelation and polyoxysalt and film formulations. Chitosan is polycationic at pH< 6 and it readily interacts with negatively charged molecules, such as proteins, anionic polysaccharides (*e.g.* , alginate and cargeenan), fatty acids, bile acids and phospholipids (Muzzarelli, 1996). Nonetheless, chitosan may also selectively chelate metal ions such as iron, copper, cadmium and magnesium.

Wound healing is defined as a tissue restoration and reparative process that is typically comprised of a continuous sequence of inflammation and repair, in which epithelial, endothelial and inflammatory cells, platelets and fibroblasts interact to resume their normal functions. The wound healing process is regulated by cytokines and growth factors and consists of four phases: the process is initialized by inflammation, followed by granulation, matrix remodeling and re-epithelialization. Research is currently being conducted to discover ways for humans to heal *via* regeneration and the use of a variety of dressing

materials to facilitate proper wound management. Significant advancement in wound care was pioneered by moist wound healing theory in the 1960's, which determined that occluded wounds healed faster than dry wounds and a moist wound healing environment increased the healing rates (Winter, 1962).



Chitin



Chitosan

Fig. 1. Chemical structures of chitin and chitosan.

Chitosan has been widely used as a biomedical application due to its excellent biocompatibility (Keong & Halim 2009; Lim et al., 2010). Chitosan is a benefit to wound healing because it stimulates hemostasis and accelerates tissue regeneration (Hoekstra et al., 1998). For a material to be used for biomedical research, it is more preferable a natural product because these materials are more biocompatible than synthetic materials. Chitosan is metabolized by certain human enzymes, such as lysozyme. Thus, chitosan is biodegradable. Chitosan is an attractive material for a tissue engineering scaffold because it has structural similarities to glycosaminoglycans and is hydrophilic. Chitosan's monomeric unit, *N*-acetylglucosamine, occurs in hyaluronic acid, an extracellular macromolecule that is important in wound repair. An effective approach for developing a clinically applicable chitosan is to modify the surface of the material to provide excellent biofunctionality and bulk properties. Surface modification techniques to blend various compound derivatives include coating, oxidation by low-temperature plasma and surfactant addition in order to blend with various derivatives. Furthermore, chitosan can be fabricated into a stable, porous bioscaffold *via* surface modification and lyophilization. However, blending with various additives may affect its biocompatibility. Therefore, evaluation of the biocompatibility of various biomedical-grade chitosan derivatives is necessary to engineer material that is of high quality and biocompatible for human wound management.

2. Chitosan and its derivatives

The practical use of chitosan is restricted to its unmodified forms in wound management, mainly due to its insolubility in water, high viscosity and its tendency to coagulate with proteins at high pH. Recently, modification of chitosan has been found to improve solubility. The introduction of various chemical side chains provides desired properties and expands the potential applications for chitosan use. Alteration of the molecular weight forms water-soluble chitosans, such as the randomly 50% deacetylated and partially depolymerized chitosans. Chitosan purification from proteins, carotenoids and inorganics produces a product of technical, food, pharmaceutical and medical grade, which is approved for use in many countries. Alkali treatment of chitin removes protein and deacetylates the chitin. Some soluble glycans can also be removed depending on the alkali concentration (Madhavan, 1992). In particular, processing of crustacean shells involves the removal of proteins and the dissolution of calcium carbonate, which is abundant in crab shells. Deacetylation of chitosan in 40% sodium hydroxide at 120 ^{o}C for 3 hours is approximately 70% efficient. However, it is necessary to perform additional modification on these polymers to improve the chemical properties.

Chitosan has one amino group and two hydroxyl groups in the repeating hexosamide residue. Chemical modification of these groups during a regeneration reaction creates various novel biofunctional macromolecular products that have an original or novel organization. Hence, the bioactivities of chitosan unmodified and in formulation with various drugs may have dual therapeutic effects. In its crystalline form, chitosan is only soluble in an acidic aqueous medium (pH < 6), such as acetic acid, formic acid and lactic acid, in which solubility is conferred by the protonated free amino groups on the glucosamine. Another limitation of sustained release chitosan systems is that they rapidly adsorb water and have a high swelling degree in aqueous environments, which causes rapid drug release to occur. Therefore, several chemically modified chitosan derivatives have been synthesized and examined to improve solubility and versatility (Jayakumar et al., 2005; Prabaharan & Mano, 2007). Chemically modified chitosan structures may result in improved solubility in general organic solvents (Qurashi et al., 1992). For example, phosphorylated chitosan is a water-soluble derivative of chitosan, which is potentially important for drug delivery systems. Non-covalent cross-linking is a useful method to prepare hydrogels from polymers for drug delivery. These gels are likely biocompatible due to the absence of organic solvents. The use of organic solvents may potentially lower drug absorption.

Chitosan derivatives are easily obtained under mild conditions and can be rendered as substituted glucans. The nitrogen content of chitin varies from 5% to 8% depending on the extent of deacetylation, whereas the majority of nitrogen in chitosan is in the form of aliphatic amino groups. Hence, chitosan undergoes reactions that are typical of amines, such as *N*-acylation and the Schiff reaction. *N*-acylation with acid anhydrides or acyl halides introduces amino groups on nitrogens in chitosan. Acetic anhydride fully acetylates chitins. Linear aliphatic *N*-acyl groups above propionyl allow prompt acetylation of hydroxyl groups. At room temperature, chitosan is able to form aldimines and ketimines with aldehydes and ketones, respectively. A reaction with ketoacids, followed by a reaction with sodium borohydride produces glucans that have proteic and non-proteic amino groups. For example, non-proteic amino acid glucans derived from chitosan are the *N*-carboxybenzyl chitosans obtained from *o*- and *p*-phthalaldehydic acids (Madhavan, 1992). *N*-carboxymethyl chitosan is derived from glyocylic acid. Chitosan and simple aldehydes produce *N*-alkyl

chitosan upon hydrogenation, where the presence of a bulky substituent can deteriorate the hydrogen bonds of chitosan. This compound swells in water in spite of the presence of hydrophobic alkyl chains (Muzarelli, 1973).

The Schiff reaction between chitosan and aldehydes or ketones yields the corresponding aldimines and ketimines, which can be converted to *N*-alkyl derivatives upon hydrogenation with borohydride. The film-forming ability of *N*-carboxymethyl chitosan imparts a pleasant feeling of smoothness to the skin and protects from adverse environmental conditions and consequences of detergent use. In addition, *N*-carboxymethyl chitosan is superior to hyaluronic acid in terms of its hydrating effects. Chitosan with several molecular designs, is presented when an alkyl or acyl chain is chemically introduced. For example, the introduction of an alkyl chain onto water-soluble modified chitosan (*N*-methylene phosphonic chitosan) introduces both hydrophobic and hydrophilic side chains. The presence of alkyl groups in *N*-lauryl-*N*-methylene phosphonic chitosan weakens the hydrogen bond and provides good solubility in organic solvents (Ramos et al., 2003). In addition, chitosan that is bound with sialic acid using *p*-formylphenyl-a-sialoside by reductive *N*-alkylation is a potent inhibitor of the influenza virus and is used as a blocking agent for acute rejection (Gamian et al., 1991).

Polyethylene glycol (PEG) is a water-soluble polymer that exhibits useful properties, such as protein resistance, low toxicity and immunogenicity. PEG is mixed with chitosan to produce the chitosan derivatives with improved biocompatibility. Chitosan-PEG enhances the protein adsorption, cell adhesion, growth and proliferation (Zhang et al., 2002). *N*-acylation of chitosan with various fatty acid (C_6-$C_{16)}$) chlorides may increase its hydrophobic character and make important structural changes and can be used as a matrix for drug delivery (Tien et al., 2003). Furthermore, chitosan may also be grafted with biomolecules similar to the chitosan derivatives. The conjugation of lipid groups to the chitosan molecule creates an amphiphilic self-aggregate molecule that is useful for drug delivery systems. One example of this is palmitoyl glycol chitosan (GCP), which is prepared by reacting glycol chitosan and sodium bicarbonate with palmitic acid *N*-hydroxysuccinimide in an ethanol solution (Uchegbu et al., 2001). In a different approach, the reaction of the amino group on chitosan and the carboxylic acid group on amino acids with glutaraldehyde may attach various amino acids (lysine, arginine, phenylalanine and aspartic acid) to a chitosan molecule. These amino acid-functionalized chitosan moieties are entrapped on poly L-lactide (PLA) surfaces (Figure 2).

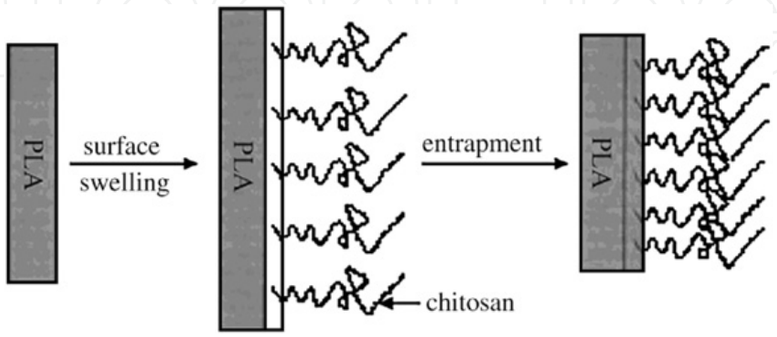


Fig. 2. Entrapment of functionalized chitosan on a PLA. In this process, the solvent swells the surface of the PLA to allow penetration of the amino acid-chitosan derivatives. These adhere upon addition of a non-solvent chitosan solution (Chung et al., 2002).

3. Chitosan in wound management

A wound is defined as the disruption of the anatomic structure and function of a body part. This may be the result of a simple cut, burns and any other injuries. Wounds are generally classified as wounds without tissue loss (*e.g.*, surgical incision) or wounds with tissue loss, such as burn wounds, wounds due to trauma, abrasions or secondary events to chronic ailments (*e.g.*, venous stasis, diabetic ulcers and iatrogenic wounds, such as skin graft donor sites and dermabrasions). In contrast, wound healing is a process of restoration by which tissue repair takes place and usually is comprised of a continuous sequence of inflammation and tissue repair during which epithelial, endothelial, inflammatory cells, platelets and fibroblasts briefly interact to restore normal function. The ordered sequence of healing events is accomplished and regulated by cytokines and growth factors. Soon after the elimination of macrophages, which appear during the inflammatory phase, wound healing is impeded and the tensile strength of the scar is diminished.

The use of chitosan has advantages due to the biocompatibility and biodegradability of the molecules, which does not harm the environment. When chitosan is applied to the body, besides being biocompatible, it is then slowly biodegraded by lysozymes, chitinase and chitosanase to harmless oligomers and monomers (amino sugars), which are completely absorbed by the body. Chitosan embodies analgesic, bacteriostatic and fungistatic properties, which are particularly useful for wound treatment. Additionally, chitosan modulates macrophage function and the secretion of numerous enzymes (*e.g.*, collagenase) and cytokines (*e.g.*, interleukins and tumor necrosis factor) during the wound healing process (Majeti & Ravi, 2000). The degradation of chitosan into monomers and oligomers at a wound site significantly accelerates the wound healing process (Minagawa et al., 2007). In addition, clinical studies have shown an absence of scar formation at the wound site in the presence of chitosan (Okamoto et al., 1993; Okamoto et al., 1995). Chitosan structurally resembles glycosaminoglycans (GAG), which have long-chain, unbranched, repeating disaccharide units and are important for maintaining cell morphology, differentiation and function (Nishikawa et al., 2000). GAG and proteoglycans are widely distributed throughout the human body and may bind and modulate numerous cytokines and growth factors, including heparin and heparan sulfate. Hence, the cell-binding and cell-activating properties of chitosan are crucial for wound healing.

Various chitosan derivatives have been produced for wound management, particularly to enhance wound healing. For example, oligo-chitosan (O-C) and *N, O*- carboxymethyl-chitosan (NO-CMC) derivatives have been fabricated into films for wound dressing (Lim et al., 2007). *N*-carboxybutyl chitosan has also been used in patients undergoing plastic surgery to promote tissue regeneration. The use of *N*-carboxybutyl chitosan improves cutaneous tissue regeneration with good histoarchitecture and vascularization at the wound site (Biagini et al., 1991). Additionally, 5-methylpyrrolidinone chitosan is compatible with other polymer solutions (*e.g.*, gelatin, polyvinyl alcohol, polyvinyl pyrrolidone, and hyaluronic acid), which are beneficial for the treatment of wounded meniscal tissues, decubitus ulcers, depression of capsule formation around prostheses, scar formation and retraction during wound healing (Muzzarelli, 1995).

3.1 Analgesic, antimicrobial and anti-inflammatory effects of chitosan in wound healing

Chitosan treatment reduces inflammatory pain due to intraperitoneal administration of acetic acid in a dose-dependent manner. Studies suggest that chitosan has potent analgesic actions. Bradykinin is one of the main substances related to pain. Okamoto et al. (2002)

reported that the bradykinin concentration during administration of a chitosan-acid acetic solution in the peritoneal lavage fluid was lower than during the administration of a 0.5% acetic acid solution, suggesting that chitosan has analgesic effects. Open wounds are often associated with severe pain in patients. Chitosan that is formulated for wound management may induce analgesia by providing a cool, pleasant and soothing effect when applied to an open wound. Excellent pain relief is conferred by chitosan when it is applied as a topical agent to open wounds, such as burns, skin abrasions, skin ulcers and skin grafted areas (Ohshima et al., 1987).

Chitosan-dependent antimicrobial activity has been observed against various microorganisms, such as fungi, algae and bacteria. These antimicrobial effects are controlled by intrinsic factors, including the type of chitosan, the degree of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates and the environmental conditions (*e.g.,* substrate water activity or moisture or both). The antimicrobial activity of chitosan differs mainly in live host plants. For example, the fungicidal effects of *N*-carboxymethyl chitosan (NCMC) are different in vegetable and graminea hosts. The antimicrobial activity is more immediate on fungi and algae than on bacteria (Savard et al., 2002). Furthermore, in the presence of more than 0.025% chitosan, the growth of *Excherichia coli*, *Fusarium*, *Alternaria* and *Helminthosporium* is inhibited (Hirano, 1995). The cationic amino groups of chitosan bind to anionic groups in these microorganisms, resulting in growth inhibition. During the infectious period of a burn wound, bacterial infection may delay the healing and probably cause serious complications, such as sepsis. Chitosan that is incorporated with minocycline hydrochloride (CH-MH) was therefore developed to achieve both wound healing enhancement and antibacterial effects (Aoyagi et al., 2007).

Chitosan has anti-inflammatory effects that are beneficial for the treatment of prolonged inflammation at the wound site. Water-soluble chitosan (WSC) significantly suppresses the secretion and expression of proinflammatory cytokines (*e.g.,* tumor necrosis factor-α and interleukin-6) and inducible nitric oxide synthase (iNOS) in astrocytes, the predominant neuroglial cells in the central nervous system, and is actively involved in cytokine-mediated inflammatory events (Kim et al., 2002). Moreover, *N*-acetylglucosamine is an anti-inflammatory drug and is synthesized in the human body from glucose. It is incorporated into glycosaminoglycans and glycoproteins. Chito-oligosaccharides (COS), which have a molecular weight of 5 kDa, are better anti-inflammatory agents than indomethacin, a non-steroidal anti-inflammatory drug (Spindola et al., 2009). Chitosan exerts anti-inflammatory effects by inhibiting prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2) protein expression and attenuating the pro-inflammatory cytokines (*e.g.,* tumor necrosis factor-α and interleukin-1β). However, chitosan treatment increases the expression of the anti-inflammatory cytokine, interleukin-10 (Chou et al., 2003).

3.2 Chitosan-based wound dressings

Wound dressings are generally classified by their mechanism of action. They are termed passive products, interactive products and bioactive products. Wound dressings before the 1960s were considered passive products minimally affected the wound healing process. Gauze and tulle dressings accounted for the largest market segment. Polymeric films, which are mostly transparent, permeable to water vapor and oxygen but impermeable to bacteria, are commonly recognized as interactive products. Bioactive dressings are important for the

delivery of substances for wound healing, for which the delivery of bioactive compounds or dressings is constructed from material having endogenous activity, such as proteoglycans, collagen, non-collagenous proteins, alginates or chitosan. The pioneering research by Winter (1962) initiated the concept of a wound dressing that establishes an optimal environment for wound healing. Therefore, the development of wound dressings from traditional passive materials was replaced by active dressings that create and maintain a moist, healing environment. An ideal wound dressing must be biocompatible, able to protect the wound from bacterial infection and should provide a moist, healing environment (Purna & Babu, 2000).

Some wound dressings are prepared from aqueous solution of 5-methylpyrrolidinone chitosan, which is dialyzed and laminated between stainless steel plates and freeze-dried to yield fleeces. The material can be fabricated into many forms, such as nonwoven fabrics, filaments and so forth. 5-methylpyrrolidinone forms oligomers when applied to a wound site due to lysozyme-derpendent degradation. Flexible, thin, transparent novel chitosan-alginate polyelectrolyte complex (PEC) membranes accelerate the healing of incision wounds in a rat model in comparison to a conventional gauze dressing. The closure rate and appearance of wounds treated with a PEC membrane were comparable with wounds treated with Opsite (Wang et al., 2002). In addition, the chitosan-based Hyphecan cap is useful in the management of deepithelializing fingertip injuries, achieving shorter healing time (Halim et al., 1998). A chitosan bilayer derived from sulfadiazine has excellent oxygen permeability, water vapor transmission rate and water-uptake capability, which benefits the wound dressing (Mi et al., 2001). Chitosan complexed with gelatin has been useful as a surgical dressing. It is prepared by dissolving the chitosan in an acidic solution before addition to gelatin at a ratio of 3:1 chitosan and gelatin (Sparkes & Murray, 1986). The stiffness of the resulting chitosan-gelatin dressing is reduced by the addition of plasticizers such as glycerol and sorbitol. Additionally, chitosan gels may be used in surgery and dentistry as a biological adhesive to seal wounds and to improve wound healing.

3.3 Chitosan as a tissue engineering scaffold for artificial skin

Individuals who suffer from extensive skin loss are in danger of succumbing to either massive infection or severe fluid loss. Patients often cope with problems of rehabilitation arising from deep, disfiguring scars and crippling contractures. Tissue repair requires a complex biological process, where inward cell migration and the proliferation of various types of neighboring cells concertedly restores tissue function.

Tissue engineering is a recent, advanced technology to develop living tissue substitutes and replace diseased or damaged tissues and organs in the human body. Tissue engineering applies the development of polymeric scaffolds, that, among other characteristics, are biodegradable and biocompatible. These scaffolds may be used simultaneously as a carrier matrix for bioactive agents and as a support for primary undifferentiated cells *in vitro*. The three-dimensional (3D) framework of a scaffold must be able to promote adherence, proliferation and differentiation of cells, which ultimately are guided to form the desired tissues. Biological scaffolds are mostly biodegradable and biocompatible, and, with the appropriate growth factors, induce cell growth. In addition, a biological scaffold must also fill space with optimal mechanical strength and control the release of bioactive molecules.

Current tissue engineered systems cover every tissue and organ, with skin and cartilage constructs for repair of skin loss and joints already clinically performed (Pomahac et al.,

1998; Kuo et al., 2006). Acute, chronic, and more extensive wounds or skin loss would be inevitable unless some skin substitutes are applied. The primary role of skin substitutes is to promote wound healing by stimulating the host to produce various cytokines, which may promote the formation of granulation tissue during the wound healing process. Cultured skin from human cells is extremely thin and needs mechanical support from biopolymer complexes, such as collagen, fibrin or chitosan. Hence, skin tissue engineering produces a construct that offers the complete regeneration of functional skin. It restores normal functions, such as barrier formation, pigmentory defence against UV irradiation, thermoregulation and mechanical and aesthetic functions. During the past couple decades, xenografts, allografts and autografts have been used as skin substitutes for wound healing. However, skin substitutes occasionally do not provide skin recovery and cause antigenicity at the donor site. Therefore, these are not widely used.

In general, the substrate material upon which the cells are cultured enhances cellular organization in 3D and provides the initial mechanical integrity for the cell-polymer construct. Chitosan-based scaffolds are of current interest for tissue engineering because these natural products are mostly biocompatible and biodegradable. Moreover, the natural components of living structures have biological and chemical similarities to tissues, in which formation of the native extracellular matrix (ECM) is crucial. One of chitosan's most promising features is its excellent ability to form porous structures for use in tissue transplantation or as template for tissue regeneration. Chitosan scaffolds are commonly porous-structured by freezing and lyophilizing a chitosan solution (Figure 3). Alternatively, the creation of porous chitosan scaffolds may also be achieved through an internal bubbling process (IBP). In this process, calcium carbonate ($CaCO_3$) is added to a chitosan solution to generate a chitosan-$CaCO_3$ gels in a specific shape of a mold (Chow & Khor, 2000). The interconnected porous structure is crucial, and numerous cultured cells can be seeded onto it. Cells proliferate and migrate within the scaffold and ultimately form a tissue or organ.

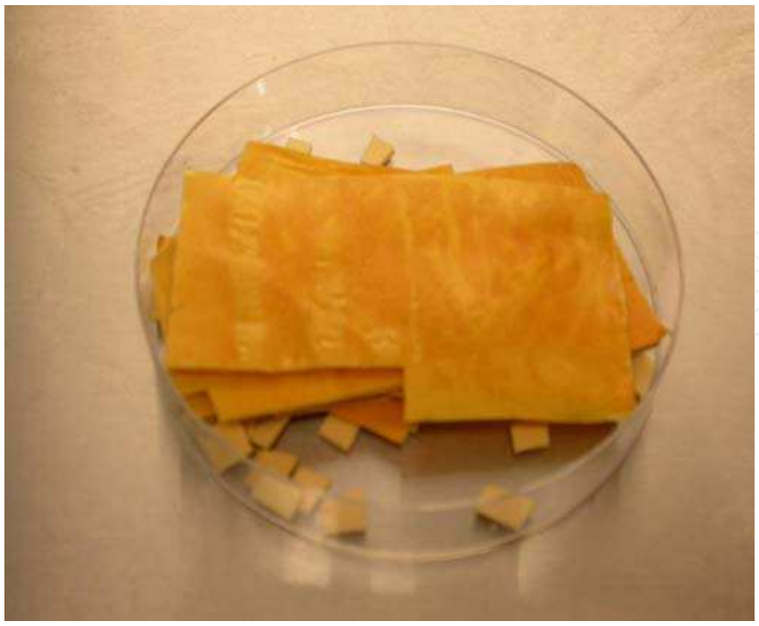
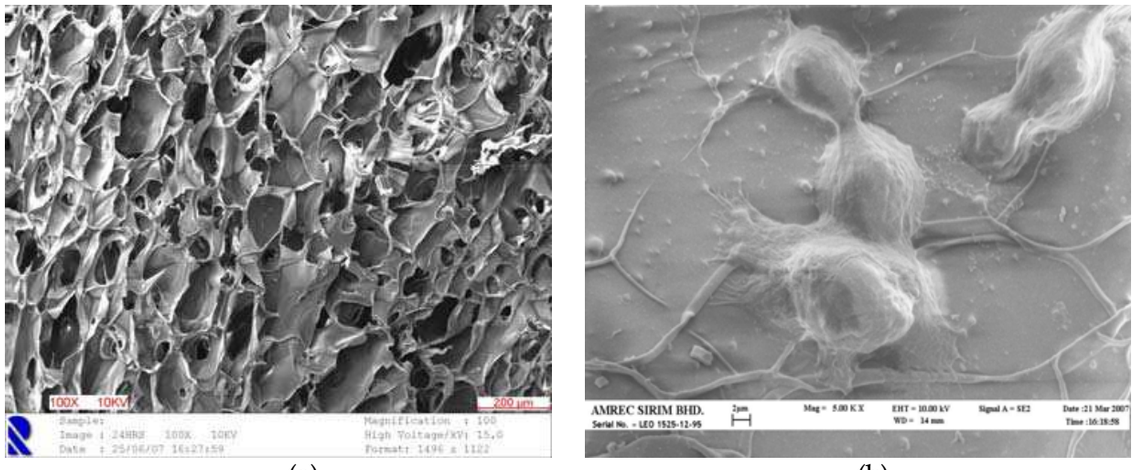


Fig. 3. A chitosan porous skin regenerating template (CPSRT) produced by lyophilization process in a freeze dryer for 24 hours.

Because chitosan is positively charged, the negatively charged cell surface binds electrostatistically with chitosan and grows in the presence of a medium *in vitro* (Figure 4). For example, a CPSRT that is seeded with skin cells, such as keratinocytes or fibroblasts, may form a skin sheet-like tissue. However, regulation of porosity and pore morphology of a chitosan-based scaffold is particularly important to control angiogenesis, the cellular colonization rate and organization within an engineered tissue *in vitro*. The mechanical properties of chitosan scaffolds formed by the lyophilization technique are primarily dependent on pore size and pore orientation. Tensile testing of hydrated samples shows that porous membranes can greatly reduce elastic moduli compared to non-porous chitosan membranes. Their mean pore size is typically controlled by varying the temperature, whereas the pore orientation can be directed by controlling the geometry of the temperature gradients during freezing and thermal gradients. The freezing and lyophilizing process generates an open microstructure with a high degree of interconnectivity within the inner layer compared with that of the surface layer. In addition, chitosan-gelatin scaffolds have also been used to construct an artificial skin bilayer *in vitro* that consists of co-cultured keratinocytes and fibroblasts (Mao et al., 2003).



(a) (b)

Fig. 4. CPSRT viewed using a scanning electron microscopy (SEM). (a) Porous structures of a CPSRT without cultured cells. (b) Proliferating cells in the CPSRT.

3.4 Sterilization issues for chitosan as wound dressing

Chitosan products intended for parenteral administration or in contact with bodily fluids (*e.g.,* wounds) must be sterilized before use. Sterilization using dry heat, saturated steam autoclaving, ethylene oxide (EO) and gamma irradiation are among the current methods used for most pharmaceutical and medical products. It is often assumed that the existing sterilization technologies are adequate for chitosan material. A focus on the efficacy of the strerilization process in terms of killing microorganisms, the nature of the residuals formed and the properties of the chitosan must not be ignored. Deleterious effects of a sterilization method on the chitosan material should be minimal.

Before a sterilization method for chitosan products is approved, the effects of sterilization on the properties and performance of the biopolymer must be evaluated and documented. Sterilization methods either chemically or physically result in lethal alteration of the

structure or function of the biomolecule microorganisms. Therefore, various forms of sterilization may also affect the chitosan biopolymers by similar mechanisms, resulting in hydrolysis, oxidation, chain scission, depolymerization or cross-linking of the polymer. For example, saturated steam autoclaving may not be suitable to sterilize chitosan that is complexed with proteins, growth factors or enzymes. This is because the high temperature may completely denature the biomolecules and result in poor biopolymer performance. In addition, heat may alter the physical properties of chitosan, affecting its aqueous solubility, rheology and appearance. Exposure to dry heat resulted in lower chitosan aqueous solubility and insolubility in some acidic aqueous media (Lim et al., 1999). This may be related to the interchain crosslink formation that involves the amino (NH_2) group in chitosan, causing a reduction in the tensile strength and strain at the break point.
Gamma irradiation causes main chain scission events in chitosan (Lim et al., 1998). Irradiation with 2.5 Mrad in air improved the tensile strength of the chitosan film, which is probably due to changes in chain interaction and rearrangement. Additionally, gamma irradiation may have depolymerized chitosan at a radiation dose of 10 kGy (Yang et al., 2007). However, applying anoxic conditions during irradiation did not affect film properties, in part due to the pre-irradiation application of negative pressure that may minimally affect the structure of the chitosan film. Hence, gamma irradiation at 2.5 Mrad under anoxic conditions may provide suitable sterilization for chitosan products. In addition, sterilization using saturated steam autoclaving is recommended for chitosan products becasue it retains the tensile strength of the chitosan film (Rao & Sharma, 1997). Nonetheless, saturated steam autoclaving causes darkening of chitosan to a yellow color, which may result from the Maillard reaction between NH_2 and OH groups (Yang et al., 2007). However, sterilization of chitosan derivatives and porous-structured chitosan scaffolds using EO was also reported to retain the biocompatibility of the porous chitosan (Lim et al., 2007; Lim et al., 2010). Chitosan that is sterilized using EO must be quarantined and saline-irrigated prior to use to remove EO residues. Chitosan sterilized by EO that was quarantined under aeration for 10 days was void of EO residues. Additionally, the chemical properties and structure of chitosan were not affected after EO, as determined by Fourier transform infrared spectroscopy (FTIR) (Yang et al., 2007). Hence, the sterilization method used for chitosan derivatives may depend greatly upon the type of application.

4. *In vitro* biocompatibility evaluations of chitosan as a wound dressing

The application of *in vitro* model systems to evaluate toxicity significantly enhances our understanding of the mechanisms of drug- and chemical-induced toxicity. Biocompatibility of a biomaterial refers to the extent to which the material does not have toxic or injurious effects on biological systems. This means that patient's tissue, that comes into contact with the material does not suffer from any toxic, irritating, inflammatory and genotoxic effects. The United States Food and Drug Administration (FDA), the International Organization for Standardization (ISO) and the Japanese Ministry of Health and Welfare (JMHW) require that manufacturers conduct adequate safety testing of their finished devices through pre-clinical and clinical phases as part of the regulatory clearance process. *In vitro* models for testing the biocompatibility of chitosan and chitosan derivatives are useful to evaluate the toxicity, particularly from the leachability of chitosan as residual monomers or oligomers. Moreover, current *in vitro* toxicity models are preferred to *in vivo* models as the preliminary method to evaluate newly developed dressing materials. These models examine materials

outside the body, and data are more reproducible. The use of these methods eliminates concerns about animal ethical issues and subsequently reduces the number of animals used in the *in vivo* biocompatibility tests. Because thousands of drugs and chemical compounds are synthesized every year, the cost of animal testing, which may be expensive, needs to be reduced. The use of animals to evaluate materials may also be very time consuming. Moreover, *in vivo* models are complicated due to the presence of structural and functional heterogeneity, and these models do not clearly define or evaluate drug mechanism.

Analysis of the effects of newly developed chitosan in cell culture systems is useful as a screening tool for their potential activity *in vivo* as wound healing agents. However, it is important to select appropriate cell lines for *in vitro* biocompatibility screening. If chitosan and its derivatives were meant to be used to treat bone injuries, osteoblast or chondrocyte cell cultures would be appropriate for the experiment. In addition, fibroblast and keratinocyte cell culture systems are more reasonable for biocompatibility *in vitro* experiments for chitosan in wound management. Various *in vitro* cell culture systems have been used to investigate and evaluate cellular processes, such as fibroblast and keratinocyte proliferation and cell migration toward growth factors present in a wound (Kawada et al., 1997). However, normal cell or non-transformed cell culture models are of particular interest for in vitro biocompatibility studies of cutaneous toxicity. Studies of new wound dressings, new drugs, cosmetic products and other chemicals require phenotypically normal cell systems. In addition, correlation of *in vitro* biocompatibility testing with *in vivo* irritation potential has been used with normal keratinocyte cultures rather than transformed cell lines (Korting et al., 1994). These *in vitro* models are simple methods to assess material biocompatibility and the ability of chemicals and biomaterials to promote cell proliferation during wound repair. Model compounds, which are known to be toxic (positive control) or non-toxic (negative controls), must be included to determine the validity of the *in vitro* system. The effects of unknown agents are compared to the effects of the controls. For example, organotin-polyvinylchloride (PVC) and high density polyethylene (HDPE) are used as positive and negative controls, respectively, in a direct-contact *in vitro* biocompatibility assay for chitosan wound dressing materials.

Numerous biocompatibility *in vitro* tests must be performed prior to the approval of chitosan products for human use. Otherwise, side effects and tissue toxicity will cause long-term effects, such as alteration of the immune system and development of malignancies, due to genetic damage induced by drug treatment. Optimal *in vitro* biocompatibility must mimic the biological response to materials when they are in contact with tissue. Therefore, *in vitro* biocompatibility testing of newly developed chitosan should measure cellular and molecular responses in cultured cells.

4.1 Cellular assessment: cytotoxicity *in vitro*

In vitro cytotoxicity is considered the most preliminary procedure in the biocompatibility *in vitro* assay. Cultured cells may undergo necrosis, a disruption of membrane integrity, or apoptosis (molecularly-controlled cell death) following treatment with cytotoxic compounds.

Cytotoxicity assays are commonly used to measure the response of cells to toxic substances. These measurements are of either an end-stage event (*e.g.,* permeability of cytoplasmic membranes of dead and dying cells) or some metabolic parameter (*e.g.,* cell division and enzymatic reaction). For example, trypan blue and propidium iodide dye exclusion assays are relatively simple assessment of cell membrane integrity, an end-stage event. These dyes

are normally excluded from the interior of healthy cells. Cell membranes become compromised, when exposed to cytotoxic compounds, allowing trypan blue or propidium iodide dyes to cross the membrane and stain intracellular components. The staining is visible under light microscopy. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) are substrates that are reduced enzymatically only in viable cells, to form formazan crystals, which are either dissolved in organic solvent (MTT assay), or water (MTS assay). The formation of formazan results in a purple color that is used as an indicator of viable cells. The absorbance of the colored solution is measured (*e.g.*, 500 to 600 nm) using a spectrophotometer to generate quantitative data. MTT is a proven, reliable and cost-effective method to measure cell viability *in vitro* (Lim et al., 2007; Keong & Halim, 2009; Lim et al., 2010). In addition, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in most cell types and is instantly released into cell culture medium upon rupture of the cell membrane. The LDH concentration in the medium is proportional to the number of dead or damaged cells. Measurement of LDH is a useful cell-mediated and drug-mediated cytotoxicity assay *in vitro*.

4.2 Genetic assessment: genotoxicity *in vitro*

Even though biocompatibility is typically measured by cytotoxicity, there is a growing concern that newly developed chitosan wound dressings may exert genotoxic effects. Hence, the safety assessment of a newly developed chitosan intended for body contact or permanent implantation would be incomplete without genotoxicity assays. When substances impose a genotoxic effect by damaging and mutating the deoxyribonucleic acid (DNA) of the cells, the growth of viable cells is abnormal or fully retarded.

The Ames test and the *in vitro* micronucleus assay are among the oldest genotoxicity assays and are commonly used. The Ames test, a salmonella point mutation assay that assesses five strains of bacterium *Salmonella typhimurium*, is used to determine the mutagenic potential of chemical compounds. The basis of the Ames test is that the salmonella bacteria cannot reproduce in growth medium unless bacteria undergo mutation. The Ames test is a high throughput genotoxicity screen that requires a small amount of test substance (approximately 2 mg). This test is relatively sensitive and accurate and is an immediate indicator of chemical mutagenic activity. The *in vitro* micronucleus assay, on the other hand, tests the effects of a compound on the induction of chromosomal breakage or clastogenesis. This assay evaluates the induction of micronuclei, which is the product of chromosomal breakage using Chinese hamster ovary (CHO) cells. Micronuclei occur in the cytoplasm following cell division. Therefore, the cellular replication kinetics and the percentage of binucleated cells with micronuclei are measured. This high throughput assay requires relatively little compound (3 mg). These early genotoxic tests may predict the toxicity potential and identify compounds for further tests.

The single-cell gel electrophoresis (SCGE) or Comet assay also has been a useful approach for assessing DNA damage. This technique is relatively more sensitive and is less expensive than other genotoxicity assays, that measure low levels of DNA damage. In addition, it requires few eukaryotic cells and test substance. The term "comet" describes the migration pattern of fragmented or unwound DNA caused by genotoxicity (Figure 5). The first comet assay was originally developed two decades ago using neutral conditions (Ostling & Johanson, 1984). In this method, cells are embedded in agarose and lysed by detergents. The liberated DNA is electrophoresed under neutral conditions. However, the measurement of

the breaking potency (*e.g.*, comet tail length and comet tail intensity) is limited to DNA with double-strand breaks (DSB). Therefore, a more sensitive comet assay has been introduced, which uses alkaline electrophoresis conditions (pH > 13) to detect DNA damage in single cells (Singh et al., 1988). This new method not only detects DNA with DSB, but also detects single-strand break (SSB) and alkali-labile sites (ALS) which may result from genotoxic agents. Furthermore, the production of DNA strand breaks correlates with the mutagenic and carcinogenic properties of environmental pollutants (Mitchelmore & Chipman, 1998).

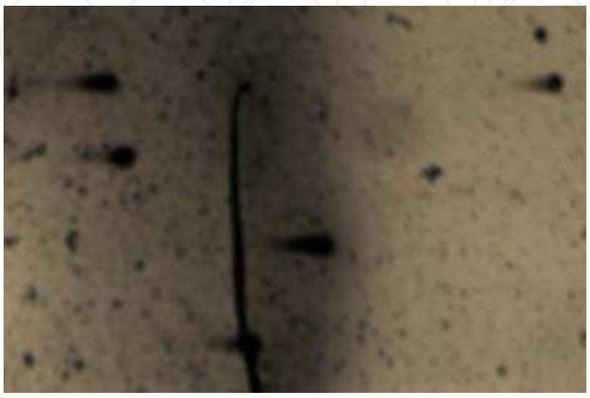


Fig. 5. Cultured human skin keratinocytes treated with a newly developed CPSRT that imposes a genotoxic effect, leading to DNA breakage. Subsequently, a comet-like shape is produced after electrophoressis at a constant voltage.

4.3 Human skin pro-inflammatory cytokine assessment: skin irritation *in vitro*

It is necessary not only to assess the cytotoxicity and genotoxicity of these materials, but also to determine the inflammatory potential *in vitro*. Despite being inert and non-toxic, newly developed chitosan wound dressings trigger adverse foreign body reactions, such as inflammation. Various cells in the dermis and epidermis are involved in these responses and they secrete various cytokines, particularly pro-inflammatory cytokines that cause skin irritation *in vitro*. Cytokines are low molecular weight glycoproteins that are produced by immune and non-immune cells. They are pleiotropic and interact with various receptors expressed on the surface of target cells. The binding of cytokines to cell surface receptors triggers intracellular signaling, protein synthesis and the production of other cytokines. The induction of other cytokines is regulated by autocrine, juxtacrine or paracrine pathways in response to micro-environmental stimuli. Cytokines mediate the interaction between various cells, and cytokine dysregulation indicates disease pathogenesis (Lazutka, 1996). Pro-inflammatory cytokines are detected at low levels in body fluids and in tissues under normal circumstances. Elevated expression may indicate activation of cytokine pathways associated with inflammation or disease progression.

The skin is the primary target tissue for exogenous noxes, which protect against harmful environmental hazards, UV-radiation and endogenous water loss. The skin epidermis consists mainly of keratinocytes, in which the cornified keratinoyctes in the outermost layer is an effective barrier against a vast number of substances. Upon stimulation, keratinocytes conduct immune surveillance of the epidermis and stimulate inflammatory responses (Steinoff et al., 2001). Harvell et al. (1995) defined skin irritation as a local, non-immunogenic inflammatory reaction that appears shortly after stimulation and usually disappears after a

few days. Skin irritation is one of the most common adverse responses to cutaneous inflammation. The presence of erythema, oedema, dryness of the skin, fissures, desquamation, itching and pain are attributed to both irritant contact dermatitis and allergic contact dermatitis. Testing for skin irritation in animals can potentially cause them pain and discomfort. The results are not always reflective of effects in humans (Nixon et al., 1975; York et al., 1996). Hence, several alternative *in vitro* tests were developed, of which the *in vitro* reconstructed organotypic skin equivalents is the most favored, because of its resemblance to the structure of human skin. There are two different kinds of reconstituted skin equivalents available: 1) epidermal equivalents that consist of multilayered, differentiated human keratinocytes grown on a synthetic matrix, and 2) full skin equivalents consisting of multilayered, differentiated human keratinocytes grown on fibroblasts containing collagen matrices. Currently, various companies provide reconstituted human epidermal *in vitro* skin equivalents, such as EpiDerm (MatTek, Ashland, MA, USA), Episkin (Episkin, Chaponost, France), Apligraf (Organogenesis Inc., MA, USA) and Skinethic (Skinethic, Nice, France). In addition, because keratinocytes initiate and regulate skin irritation (Coquette et al., 2000), keratinocyte cultures may also serve as indicators for skin irritation *in vitro*.

Keratinocytes, the principle epidermal cell, is also a major contributor to epidermal cytokine production, a fact that is not well recognized by the immunology community (Tizard, 2000). Nevertheless, numerous cytokines are produced by keratinocytes (*e.g.*, Interleukin-1, -6, -7, -8, -10, -12, -15, -18 and -20 and tumor necrosis factor-α), either constitutively or upon induction by stimulants (Grone, 2002). These cytokines trigger multiple biological events, such as the migration of inflammatory cells, systemic effects on the immune system, keratinocyte proliferation and differentiation and the production of other cytokines. Lim et al. (2010) reported that non-biocompatible chitosan wound dressings increase the production of tumor necrosis factor-α and interleukin-8 in an experiment using keratinocyte cultures in an *in vitro* assay. Regardless of the chemical class or mechanism of drug action, the onset of skin irritation by chemicals and newly developed chitosan derivative wound dressings is in accordance with the general principles of toxicology. These biologic effects depend on various factors, such as the concentration of the test substance, the duration and frequency of exposure, the rate of penetration and the intrinsic toxic potential of the substance.

5. Conclusion

Chitosan and chitosan-based derivatives have various medical applications. It is well-known that chitosan possesses medicinal properties that accelerate wound healing and tissue regeneration. Chitosan is a natural product. It is biocompatible and biodegradable, enabling it to be used for wound dressing material. However, the practical use of chitosan is restricted to the unmodified forms, as these are water-insoluble and have high viscosity and the tendency to coagulate with proteins at high pH. Thus, chemical modification of chitosan may ultimately enhance its solubility and potential use for wound dressings. Chitosan has analgesic, antimicrobial and anti-inflammatory effects, which are beneficial for wound treatment. Chitosan is widely applied for the development of various chitosan-based wound dressings and biological scaffolds for tissue engineering. Nevertheless, each chitosan product that is intended for parenteral administration or for wound dressings comes in contact with bodily fluids, must be sterilized prior to application. The most frequently used

sterilization methods are autoclaving, EO and gamma irradiation. The choice of sterilization method depends on the intended application. *In vitro* biocompatibility using newly developed chitosan wound dressings should be measured at the cellular and molecular level. These assays measure cytotoxicity, genotoxicity and skin irritation. *In vitro* model systems have made significant contributions to our understanding of the mechanisms of toxicity and carcinogenicity. They are indispensable resources to determine toxicology and identify potentially toxic compounds in chitosan wound dressings for human health risk assessment.

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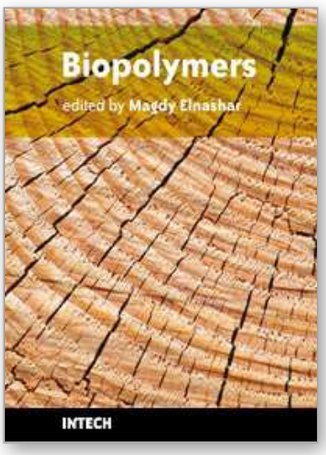

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Biopolymers are polymers produced by living organisms. Cellulose, starch, chitin, proteins, peptides, DNA and RNA are all examples of biopolymers. This book comprehensively reviews and compiles information on biopolymers in 30 chapters. The book covers occurrence, synthesis, isolation and production, properties and applications, modification, and the relevant analysis methods to reveal the structures and properties of some biopolymers. This book will hopefully be of help to many scientists, physicians, pharmacists, engineers and other experts in a variety of disciplines, both academic and industrial. It may not only support research and development, but be suitable for teaching as well.

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