BIOCOMPATIBILITY AND ANGIOGENESIS EVALUATIONS OF BIOMEDICAL GRADE CHITOSAN DERIVATIVE FILMS IN RABBITS

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by

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LIST OF ABBREVIATIONS

O-C	Oligo Chitosan
N-CMC	*N*-carboxymethyl chitosan
N,O-CMC	*N,O*-carboxymethyl chitosan
ANOVA	Analysis of Variance
dH_2O	Distilled water
EC	Endothelial cells
i.m.	Intra-muscular
vWF	Von Willebrand Factor
VEGF	Vascular Endothelial Growth Factor
H&E	Hematoxylin and Eosin
GlcN	*N*-glucosamine
GlcNac	*N*-acetyl-glucosamine
PMN	Polymorphonuclear
IL	Interleukin
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
mg/kg	Milligram per kilogram
S.E.M.	Standard Error of Mean
SD	Standard Deviation
v/v	Volume/volume
w/v	Weight/volume

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PENILAIAN BIOKOMPATIBILITI DAN ANGIOGENESIS BAGI FILEM TERBITAN KITOSAN GRED BIOMEDIKAL KE ATAS ARNAB

ABSTRAK

Kitosan terdiri daripada ‘glucosamine’ dan ‘*N*-acetylglucosamine’ yang merupakan elemen dalam tisu mamalia. Kitosan adalah polimer yang tidak toksik, biokompatibal dan boleh didegradasi, dan telah dicadangkan untuk digunakan sebagai agen topikal dalam penyembuhan tisu. SIRIM Berhad telah mengeluarkan tiga jenis filem terbitan kitosan yang baru iaitu ‘*N*-carboxymethylchitosan’ (N-CMC), ‘*N,O*-carboxymethylchitosan’ (NO-CMC) dan ‘Oligo Chito’. Memandangkan filem-filem ini merupakan ciptaan baru, kajian ini dijalankan untuk menilai biokompatibiliti dan angiogenesis bagi filem terbitan kitosan ini ke atas arnab menggunakan model implan dan luka separa tebal. Dalam model implan, poket kosong digunakan sebagai kawalan dan dalam model luka separa tebal, Aquacel® (pembalut komersial), digunakan sebagai kawalan. Keputusan histologi menunjukkan inflamasi yang berlaku di dalam semua implan terbitan kitosan adalah lebih tinggi daripada yang dilihat di dalam kawalan disebabkan oleh kehadiran implan di dalam tisu. Walaubagaimanapun, tindakbalas ini adalah teratur dan tidak menyimpang daripada inflamasi yang terlibat dalam proses penyembuhan luka seperti yang diperhatikan di dalam model luka separa tebal menjadikan terbiatan kitosan ini biokompatibal. Penilaian mata kasar ke atas filem terbitan kitosan dan luka menunjukkan kesemua filem ini iaitu O-C, N-CMC dan NO-CMC memiliki ciri-ciri asas yang diperlukan sebagai pembalut luka setanding dengan

pembalut komersial, Aquacel®. Dalam penilaian angiogenesis, keputusan kepadatan salur darah menunjukkan kesemua filem yang dikaji dapat merangsang angiogenesis memandangkan kepadatannya adalah lebih tinggi daripada kawalan bagi model implan dan setanding dengan Aquacel® bagi model luka separa tebal. Kesemua filem terbitan kitosan juga berupaya merangsang pengeluaran VEGF bagi kedua-dua model. Hubungan diantara pengeluaran VEGF bagi semua terbitan kitosan dan kepadatan salur darah dan ketebalan kapsul fibrus bagi model implan menunjukkan hubungan yang positif kecuali NO-CMC. Tiada hubungan dapat diperhatikan antara pengeluaran VEGF dan kepadatan salur darah dan indeks granulasi dalam semua luka yang dibalut filem terbitan kitosan kecuali Aquacel® dan O-C dalam indeks granulasi. Ini menunjukkan bahawa semua filem terbitan kitosan berupaya untuk merangsang angiogenesis dan proses ini mungkin dirangsang oleh VEGF atau faktor angiogenik yang lain.

BIOCOMPATIBILITY AND ANGIOGENESIS EVALUATIONS OF BIOMEDICAL GRADE CHITOSAN DERIVATIVE FILMS IN RABBITS

ABSTRACT

Chitosan is composed of glucosamine and *N*-acetylglucosamine, which are constituents of mammalian tissues. Chitosan is a non-toxic, biocompatible, and biodegradable polymer and has been proposed for use as a topical agent in tissue repair. SIRIM Berhad has developed three new types of chitosan derivative films which are *N*-carboxymethylchitosan (N-CMC), *N,O*-carboxymethylchitosan (NO-CMC) and Oligo Chito. As these films are novel inventions, this study was conducted to evaluate the biocompatibility and angiogenesis of these three types of chitosan derivative films on rabbits using implantation and partial-thickness wound models. In implant model, empty pockets served as control and in partial-thickness wound model, Aquacel® (commercial dressing), served as control. Histological examination revealed that inflammations elicited in all chitosan derivative implants were higher than that observed in control due to the presence of the implants in the tissue. However, these reactions were organized and did not deviate from the course of inflammation associated with healing process as observed in partial-thickness wound model, rendering these materials biocompatible. Macroscopic evaluations of the dressings and wounds demonstrated that all three types of chitosan derivative films, namely O-C, N-CMC and NO-CMC, possess the necessary basic attributes to be employed as wound dressing comparable to the commercial dressing (Aquacel®). In angiogenesis evaluation, results of the microvessel densities

demonstrated that the test materials were able to promote the angiogenesis as higher densities were observed in chitosan derivatives compared to control in implant model and comparable to Aquacel® in partial-thickness wound model. All chitosan derivative implants were also able to promote the endogenous expression of VEGF in both models. Relationship between VEGF expressions of the chitosan derivatives and their microvessel densities and fibrous capsule thickness in implant model showed positive correlations except for NO-CMC which showed no correlation in microvessel density. No correlation was observed between VEGF expression and microvessel densities and granulation index in all chitosan derivatives-treated wounds except for Aquacel® and O-C in granulation index. These signify that all chitosan derivatives do promote the angiogenesis and this process may be enhanced by VEGF or other angiogenic factors.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

The merit of attempting to isolate a wound from the environment has long been appreciated, at least in the sense of providing physical and aesthetic protection. A great increase in demand for dressing materials and confections, especially those based on natural products, has been observed for many years. During the healing process, the wound bed may be regarded as an open tissue-culture system threatened by bacterial spoilage and marked for death due to desiccation or asphyxiation for lack of blood vessels which provide oxygen and efficient gaseous exchange. A contact cover in the form of dressing is therefore required to keep invaders out and allow moist wound healing, which expedites keratinocyte migration and natural wound closure (Khan and Peh, 2003).

The wound cover or dressing should preferably be flexible, lightweight, odor-free and impermeable to microorganisms yet permeable for necessary water vapors and exudates leaving the wound. It would also be an advantage for the material to be hemostatic, transparent (to indicate the presence of infection), and biodegradable as it restores normal function to the skin (Watson & Dabell, 2006).

At present, there are various types of wound dressing available commercially. However, all of these dressings are not produced locally and they are imported from other countries at a considerably high cost. In an attempt to promote our local product,

SIRIM Berhad, in collaboration with Nuclear Malaysia Agency and Universiti Sains Malaysia, has developed chitosan derivative-based wound dressing in film form. Chitosan is obtained from chitin, a naturally occurring and abundantly available polysaccharide obtained from crustacean wastes, by *N*-deacetylation using strong alkali. It is composed of glucosamine and *N*-acetylglucosamine, which are constituents of mammalian tissues (Ravi Kumar, 2000, Hejazi and Amiji, 2003). It is a non-toxic, biocompatible, and biodegradable polymer. Various grades of chitosan are available commercially, which differ primarily in the degree of deacetylation and molecular weight. Muzzarelli *et al*. (1978) reported that the degree of deacetylation is one of the important characteristics that could influence the performance of chitosan in many of its applications. Chitosan has been shown to have hemostatic activity and proposed for use as a topical agent in tissue repair (Ueno *et al*., 2001).

Animal model plays a critical role in the development of biological devices pertinent to wound healing. It allows testing of a device's efficacy and safety in a living model prior to its use in humans. It is important that the model and experimental protocol chosen allow for controlled, reproducible, and quantifiable experimentation. Only under these circumstances can the results be considered valid and subsequently applied to human subjects (Saulis and Mustoe, 2001).

In this study, the chitosan was further manipulated and modified by SIRIM Berhad producing three novel types of chitosan derivative films which are *N*-carboxymethylchitosan (N-CMC), *N,O*-carboxymethylchitosan (NO-CMC) and Oligo Chito (O-C). Although there were other N-CMC, NO-CMC and Oligo chitosan produced and studied before, the types used in this study are considered to be novel invention as these three chitosan derivative films were produced in different

compositions, dosages, sources, and preparation methods which is currently in process of being patented. Thus, the biocompatibility of these products needs to be assessed prior to clinical application of these products. Two methods were used for the assessment, the implantation and wound dressing methods.

Implantation tests, as described by the ISO 10993-6, are designed to assess any localized effects and safety of a device in the human body. The wound dressing method was carried out to assess the performance of these three films as wound dressing. Through a macroscopic examination supplemented with microscopic analysis, the degree of tissue reaction for both methods is evaluated as a measure of biocompatibility to resolve whether the materials induce any toxicity in the host tissue as well as to determine the angiogenic activities promoted by these materials. These angiogenic activities were determined using immunohistochemical method.

Immunohistochemistry is a method by which the localization of proteins in the cells of a tissue section can be observed. Immunohistochemical staining is accomplished by using antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. Anti-von Willebrand Factor antibody, which specifically identify endothelial cells, was used for the detection of the blood vessels formed and anti-Vascular Endothelial Growth Factor antibody was used to determine the intensity level of angiogenesis.

1.2 HYPOTHESIS

Chitosan derivative films [*N*-carboxymethylchitosan (N-CMC), *N*,*O*-carboxymethylchitosan (NO-CMC) and Oligo Chito (O-C)] are biocompatible and able to promote angiogenesis.

1.3 GENERAL OBJECTIVE

The aim of this study is to evaluate the biocompatibility and angiogenesis effects of three types of biomedical grade chitosan derivative films which are *N*-carboxymethylchitosan (N-CMC), *N*,*O*-carboxymethylchitosan (NO-CMC) and Oligo Chito (O-C) in rabbit.

1.4 SPECIFIC OBJECTIVES

The specific objectives of this study are:

1.4.1 To determine the biocompatibility of three types of chitosan derivatives, namely O-C, N-CMC and NO-CMC.

1.4.2 To determine the basic attributes of chitosan derivative films as wound dressing in comparison with a commercial product, Aquacel ®.

1.4.3 To determine the intensity of angiogenesis in terms of microvessel density (MVD) of each type of chitosan derivative films in both wound dressing and implant models using immunohistochemical staining of endothelial marker.

1.4.4 To correlate the above findings with level of VEGF expression in each type of chitosan derivative films in both wound dressing and implant models.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOCOMPATIBILITY

For a biomaterial to be approved for use in contact with the human body, especially as a component of a medical device, the acceptance of the biomaterial by the human biological system has to be demonstrated. Thus, evaluation of chitosan derivatives biocompatibility is of considerable importance in resolving this material as wound management product. Biocompatibility can be defined as the ability of medical devices to perform their intended functions with an appropriate host response in a specific application during the whole life cycle of the device (Gad, 2002). It concerns the interactions that occur between biomaterials and host tissue. The effect of the biomaterial on tissues and *vice versa* has to be understood to ensure safety and performance of the device. Safety is primarily concerned with toxicity issues including degradation products while performance deals mainly with satisfying the role the medical device was designed for (Khor, 2001). No biomaterial can be considered for use in contact with human tissue unless it has been proven acceptable. What constitutes this acceptability is that the biomaterial must be demonstrated to meet these two conditions i.e. safe and able to perform the intended role.

The need to evaluate a medical device biologically depends on the material used in the device, the intended body contact, and the duration of that contact. For a material to be considered biocompatible, it must not irritate the surrounding structure, does not

provoke an abnormal inflammatory response and does not incite allergic or immunologic reaction or carcinogenic. In general, it demonstrates the acceptance of the biomaterial/device by the surrounding tissues and by the body as a whole (Park and Bronzino, 2003).

Generally, the ideal biomaterial should be biocompatible, and able to promote cellular interaction and tissue integration. They should be made from materials with controlled biodegradability. Such behavior of the biomaterial would avoid the risk of inflammatory or foreign-body responses that might be associated with the permanent presence of a foreign material *in vivo*. The degradation products should not provoke intense inflammation or toxicity. The degradation rate and the concentration of degradation products in the tissues must be at a tolerable level. They should be able to be removed from the body via physiological pathways (Khor and Lim, 2003).

In vivo biocompatibility describes the state of a biomaterial within a physiological environment/living body without the material adversely affecting the tissue or the tissue adversely affecting the material. This is demonstrated by tests utilizing toxicological principles that provide information on the potential toxicity of materials in the clinical application. Tests should be performed under conditions that simulate the actual use of the product or material as closely as possible and should demonstrate the biocompatibility of a material or device for a specific intended use (Gad, 2002). The design of testing procedures requires an understanding of the contact interface between technical materials and the biological system (Pariente *et al.*, 2000). ISO, International Organization for Standardization, is the world's largest developer and publisher of International Standards. This organization has developed the standard to evaluate a material/device that comes into direct or indirect contact with the human's

body. ISO 10993 describes the general principles governing the biological evaluation of medical devices, the categorization of devices based on the nature and duration of their contact with the body and the selection of appropriate tests. ISO 10993-6 specifies the test methods for the assessment of the local effects of an implant material in an *in vivo* environment, at both the macroscopic and microscopic level (ISO 10993-1:2003, ISO 10993-6:1994).

2.2 CHITIN AND CHITOSAN

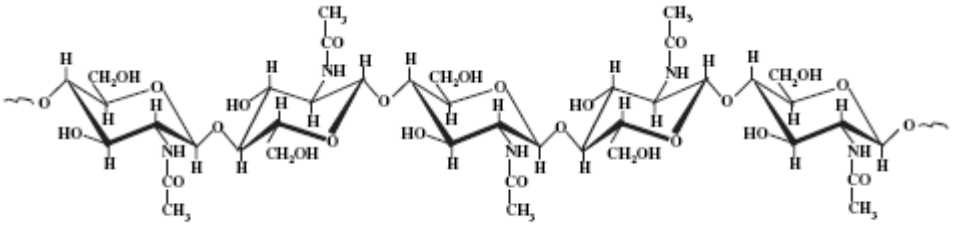
2.2.1 Chitin

Chitin was first discovered in 1811 by Henri Braconnot, a French scientist who isolated it from *Agaricus volvaceus* mushroom. In 1823, Odier found the same compound that forms the structure of the plant in the cuticles of insects. It was named chitin from the greek term χίτών (tunic, envelope) (Muzzarelli, 1978). Chitin is the second most abundant natural polysaccharides found in nature after cellulose (Mori *et al*., 2004). It is constituted of β-(1, 4)2-acetamido-2-deoxy-*D*-glucose units. This natural polymer that can be called poly-*N*-acetyl-*D*-glucosamine, can be formally considered a derivative of cellulose where the C-2 hydroxyl groups have been completely replaced by acetylamine groups (Muzzarelli, 1973). Chitin has intra- and inter-molecular hydrogen bonds and is water-insoluble due to its rigid crystalline structure (Sugimoto *et al*., 1998). The hydrogen bonding between adjacent polymers gives the polymer increased strength (Yamaguchi *et al*., 2003).

Chitin is widely distributed among invertebrates. Chitin is known to have three polymorphic solid-state forms designated as α-, β- and γ-chitin. It is found as α-chitin, which is by far the most abundant, in the calyces of hydrozoa, the eggshells of nematodes and rotifers, the radulae of mollusks and the cuticles of arthropods, and as β-chitin in the shells of brachiopods and mollusks, cuttlefish bone, squid pen and in the tubes of pogonophoran and vestimetiferan worms (Muzzarelli, 1999; Rinaudo, 2006). In the β-form all chains are aligned in parallel manner, whereas in α-chitin they are anti-parallel (Muzzarelli, 1999). However, little is known of the solid-state structure of γ -chitin except that it is a mixture of α- and β-chitin, with two parallel chains and for every anti-parallel stack that leads to water swelling properties intermediate between α- and β-chitin (Khor, 2001).

Chitin is also found in exoskeletons, peritrophic membranes and the cocoon of insects and is ubiquitous in fungi. The exoskeletons of shrimps, krill, crabs and lobsters have been a source of raw material for chitin production as the dry arthropod exoskeletons contain from 20 to 50% chitin. Chitin is a white, hard, inelastic, hydrophobic and highly insoluble nitrogenous polysaccharide which resembles cellulose in its solubility and low chemical reactivity (Ravi Kumar, 2000; Hejazi and Amiji, 2003). It is insoluble in water, dilute acids, cold alkalis of any concentration and organic solvents. Chitin is also insoluble in the usual solvents used for cellulose; it swells slightly in basic solvents and does not swell at all in the media used for esterification (Muzzarelli, 1973). Chitin can be dissolved with difficulty in liquid ammonia, and undergoes hydrolysis under limiting conditions in hydrochloric acid or sulphuric acid. Its derivatives therefore are formed with difficulty and their preparation requires drastic chemical operations, which in certain cases may lead to degradation (Muzzarelli, 1973).

Much attention has been paid to biomedical applications of chitin as it has many useful biological properties such as biocompatibility, biodegradability, non-toxicity, hemostatic activity, and wound healing property (Ravi Kumar 2000; Khor and Lim 2003). Examples are absorbable suture, antitumour agent, haemostatic agent and wound healing agent. It was also reported to be used in orthopedic/periodontal applications and tissue engineering (Khor and Lim, 2003; Rinaudo 2006). Chitin has been used in drug/gene delivery applications (Muzzarelli, 1973; Mi *et al*., 2002; Khor and Lim, 2003; Gentaa *et al.,* 2003; Rinaudo 2006) as enzymically decomposable pharmaceutical carriers because they are degraded by lysozyme, an enzyme present in human body and the degradation products do not introduce any disturbance. Pharmaceutical drugs may be incorporated into chitin membranes for delayed released. Media containing chitin are highly selective for the isolation of actinomycetes in water and soil (Muzzarelli, 1978). There were studies showing that chitin accelerated wound healing in many clinical cases. It was reported that there was an increase in tensile strength and healing rate of the wounded skin treated with chitin (Cho *et al*., 1999). Chitin and chitosan granules are noted to enhance reepithelialization and regenerate normal skins in open wounds (Okamoto *et al*., 1995). They are also found to have potent analgesic actions as they were able to reduce the inflammatory pain due to intraperitoneal administration of lactic acid in dose-dependent manner (Okamoto *et al*., 2002). However, because of its high molecular weight, high viscosity and low water solubility, the accelerating effects of chitin on wound healing could not be maximized due to the relatively low interaction between the wounds and the healing agents. For this purpose, more studies have been carried out on chitin modification which is still an active field of research in order to produce a ‘friendlier version of chitin’.



→4)-β*D*-GlcNAc-(1→ 4)-β*D*-GlcNAc-(1→ 4)-β*D*-GlcNAc-(1→

Figure 2.1: Primary structure of chitin (Prashanth and Tharanathan, 2007).

2.2.2 Chitosan

Chitosan is a cationic polysaccharide co-polymer constituted of β-(1, 4)2-amino-2-deoxy-*D*-glucose units (Srinivasa *et al*., 2007). It was firstly described by Rouget in 1859, but was formally named in 1894 by Hoppe-Seyler who fused potassium hydroxide at 180˚C with chitin and obtained a product with diminished acetyl content, that he called chitosan (Muzzarelli, 1973; Lubben *et al*., 2001; Khor, 2001). In chitin, the acetylated units prevail with degree of acetylation typically 0.90. Chitosan is the fully or partially *N*-deacetylated derivative of chitin where chitin is *N*-deacetylated to such an extent that it becomes soluble in certain solvents with a typical degree of acetylation of less than 0.35 (Ravi Kumar 2000). When the number of *N*-acetyl-glucosamine units is higher than 50%, the biopolymer is termed chitin. Conversely, when the number of *N*-glucosamine units is higher, the term chitosan is used (Khor and Lim, 2003). This deacetylation process releases amine groups (NH) and gives the chitosan the cationic characteristic where a majority of the other polysaccharides are usually neutral or negatively charged.

Chitosan is positively soluble by protonation of its amino groups when the solution pH is below 6 (Hsieh *et al.*, 2005). Converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The average molecular weight of chitin is 1.03×10^6 to 2.5×10^6, but the *N*-deacetylation reaction reduces this to 1×10^5 to 5×10^5 in chitosan (Ravi Kumar, 2000). Chitosan molecular weight may range from 300 to over 1000 kDa with a degree of deacetylation from 30% to 95% depending on the source and preparation procedure (Shi *et al.*, 2006).

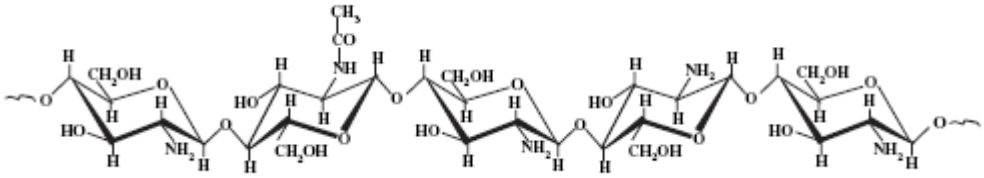
Chitosan is insoluble in water, organic solvents and alkali. It is also insoluble in mineral acids except under certain conditions. In the presence of limited amount of acid it is soluble in water-methanol, water-ethanol, water-acetone and other mixture i.e. dilute acids (Boucard *et al*., 2007). Chitosan is soluble in formic and acetic acids, and in 10% citric acid (Muzzarelli, 1975). Therefore chitosan is normally insoluble in neutral or basic pH conditions, while soluble in acidic pH. Chitosan has been the better researched version than chitin because of these characteristics rendering chitosan more accessible for utilization and chemical reactions.

Chitosan is able to promote cell growth and protein adsorption because most proteins are negatively charged. Chitosan also readily binds to negatively charged surfaces such as those on mucous membranes. The cationic nature of chitosan is also primarily responsible for the electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules (Madihally and Matthew, 1999). In the form of an acid salt, chitosan demonstrates mucoadhesive activity, thus making it an ideal candidate for a hemostatic agent (Wedmore *et al*., 2006).

Chitosan-based matrices have been widely used in the biomedical field. The ability of chitosan to form films may permit its extensive use in the formulation of film dosage forms (Kubota *et al*., 1991). Chitosan membranes have been proposed as artificial kidney membranes possessing high mechanical strength in addition to permeability to urea and creatine; they are impermeable to serum proteins (Muzzarelli, 1978). Chitosan was found to accelerate wound healing effectively (Diegelmann *et al*., 1996; Costain *et al*., 1997; Koide, 1998; Khan *et al*., 2000; Howling *et al*., 2001; Khan and Peh, 2003; Mori *et al*., 2004; Jayakumar *et al*., 2005; Rinaudo, 2006). Ishihara *et al*. (2002) has proposed the photocrosslinkable chitosan hydrogel as wound dressing and tissue adhesive as it could effectively stop bleeding from a cut tail of mice, and that the binding strength of the hydrogel with skin-slices of a mouse was found to be higher than that of fibrin glue. In China, chitosan is marketed by its Chinese manufacturer as 'Hyphecan' and has been proved to be effective in the management of de-epithelializing fingertip injuries and skin graft donor sites (Halim *et al*., 1998; Stone *et al*., 2000). Chitosan exhibits protective effects of the liver against drug-induced hepatitis as described by Santhosh *et al*. (2007) and intestinal cells from gliadin peptide toxicity (Silano *et al*., 2004). Similar to chitin, chitosan has also been proposed in controlled as well as site-specific drug delivery systems including oral, nasal, parenteral, transdermal administration, implants and gene delivery, stomach-specific and colon-specific (Yaku *et al*., 1973; Zhao *et al*., 2002; Gentaa *et al*., 2003; Hejazi and Amiji, 2003; Rinaudo, 2006; Gupta and Jabrail, 2007). Moreover, chitosan has been shown to be a potential penetration enhancer for the transmucosal (intestinal, buccal, sublingual and vaginal) absorption of hydrophilic drugs with a high molecular weight as it displayed good mucoadhesive and penetration enhancement properties (Sandri *et al*., 2004; Hejazi and

Amiji, 2003; Khor, 2001; van der Lubben *et al*., 2001). Chitosan was also reported to have procoagulant behavior (Benesch and Tengvall, 2002), antimicrobial activity (Koide, 1998; Sashiwa and Aiba, 2004; Jayakumar *et al*., 2005), blood compatible properties (Lee *et al*., 1995; Khor, 2001) and have been used in gene transfection (Rinaudo, 2006). The use of chitosan as a selective super antigen absorber has also been described (Khor, 2001).

Other than biomedical applications, chitosan has also been proposed in other field such as in the treatment of wastewater containing heavy metals and dyes where chitosan was used as adsorption agent and dyes removal (Jayakumar *et al.*, 2005; Krajewska, 2005; Sun and Wang, 2006; Ding *et al*., 2007). In agriculture, chitosan exhibits antivirus and antiphage activities where it inhibits the growth of bacteria and bacterial infection, and stimulates the natural defenses in plants (Rinaudo, 2006). A beneficial effect of chitosan as a food supplement is the reduction of plasma cholesterol and triglycerides due to its ability to bind dietary lipids, thereby reducing intestinal lipid absorption (Koide, 1998; Muzzarelli *et al*., 2006). Its use as a component of toothpaste, hand and body creams, shampoo, cosmetics and toiletries, as well as pharmaceuticals, has also been documented (Cai *et al*., 2006).



→4)-β*D*-GlcN-(1→4)-β*D*-GlcNAc-(1→4)-β*D*-GlcN-(1→4)-β*D*-GlcN-(1→

Figure 2.2: Primary structure of chitosan (Prashanth and Tharanathan, 2007).

2.2.3 Chitosan Derivatives

Chitosan usually has high molecular weight and strong network of intermolecular or intramolecular hydrogen bonds. Its poor solubility in water and common organic solvents has so far limited its widespread utilization (Fu *et al*., 2007). As a result, there have been many publications about the methods to enhance the solubility of chitosan, one of which was derivatization. Derivatization by introducing small chemical groups to the chitosan structure, for instance alkyl or carboxymethyl group can drastically increase the solubility of chitosan at neutral and alkaline pH values (Thanou *et al*., 2001).

Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-6, and C-3 positions, respectively (Prashanth and Tharanathan, 2007). These groups allow chemical modifications of chitosan that include acylation, *N*-phthaloylation, tosylation, alkylation, Schiff base formation, reductive alkylation, carboxymethylation, carboxyalkylation, silylation, and graft copolymerization (Krajewska, 2005; Shi *et al.*, 2006). Chemical modifications of these groups result in the production of numerous useful chitosan derivatives with improved solubility, microbiological properties and other features for various specific applications.

Carboxymethyl chitosan (CMC) is a chitosan derivative obtained by the carboxymethylation of chitosan where the carboxymethyl group is linked to the nitrogen or oxygen atom or both (Fu *et al*., 2007). The structure of CMC is similar to amino acids with amino group and carboxyl group in the molecule, and the difference from chitosan is the carboxymethyl group linked to the nitrogen or oxygen atom (Fu *et al*., 2007).

CMC has three types: *O*-Carboxymethyl chitosan, *N*-Carboxymethyl chitosan and *N,O*-Carboxymethyl chitosan (Majeti and Kumar, 2000; Baumann and Faust, 2001). Carboxymethyl-chitosan has many reactive functional groups, amino group (-NH_2), carboxyl group (-COOH), as well as both primary and secondary hydroxyl (-OH) groups at the C-3, C-6 positions, respectively (Sun and Wang, 2006; Sun *et al*., 2006). The solubility, viscosity, chemical reactivity and bioactivity of CMC are different from chitosan due to the minor difference in molecular structure of them (Cai *et al*., 2007; Fu *et al*., 2007). Hjerde (1997) reported that the CMC could be dissolved in acidic, neutral or basic aqueous solution when the substituting degree of carboxymethylation for chitosan is more than 0.60. The existence of carboxymethyl group (–CH2COOH) in the molecular structure conferred the CMC with better properties in becoming membrane, increasing viscosity, improving retentive moisture, flocculating properties, chelating properties (Cai *et al*., 2007) and antibacterial activity (Zhao *et al*., 2003). For these reasons, CMC has found itself more important and extensive applications in many other fields, such as medicine and health, industry, agriculture, biochemical industry and etc.

N,O-carboxymethyl chitosan is a chitosan derivative having carboxymethyl substituent on some of both the amino and primary hydroxyl sites of the glucosamine units of the chitosan structure (Chen *et al.,* 2004). The addition of carboxymethyl groups to chitosan's nitrogen and oxygen centers produces a water-soluble and negatively charged polymer, *N,O*-carboxymethyl chitosan, that is hydrophilic, lubricious and viscoelastic (Chen *et al.,* 2004; Kennedy *et al*., 1996; Costain *et al*., 1997). The swelling ratios of the *N,O*-carboxymethyl chitosan films at pH 7.4 were greater than that of chitosan films. Therefore, the *N,O*-carboxymethyl chitosan film has a less stereohindrance for penetration of lysozyme than the chitosan film, due to a greater

swelling ratio. Hence, the degradability of the *N,O*-carboxymethyl chitosan film was significantly greater than its chitosan counterpart. It was reported that *N,O*-carboxymethyl chitosan is nontoxic, either *in vitro* in fibroblast culture assays or *in vivo* in testing with intraperitoneal, oral, or subcutaneous treatments (Kennedy *et al*., 1996; Costain *et al*., 1997). Additionally, this derivative is suitable as an excipient in ophthalmic formations to improve the retention and bioavailability of drugs (Muzzarelli *et al*., 1999). It has also been proposed as polymeric carrier for site-specific protein drug delivery (Chen *et al.,* 2004) and adsorbing agent for wastewater treatment containing heavy metals (Sun and Wang, 2006; Sun *et al*., 2006) and congo red dye removal (Wang and Wang, 2008). *N,O*-carboxymethyl chitosan has also been formulated as a post-surgical lavage to wash wound areas thereby minimizing wound adhesion (Khor 2001) and was proved to be an effective anti-adhesion agent in clinical usage (Kennedy *et al*., 1996). In food industry, fruits could be stored for longer period after coating with *N,O*-carboxymethyl chitosan. *N,O*-carboxymethyl chitosan was reported to be effective in controlling the oxidation and flavor deterioration of cooked meat. The inhibition is thought to be related to chelation of free iron, which is released from hemoproteins of meat during heat processing, which, in turn, inhibit the catalytic activity of iron ions (Tharanathan and Kittur, 2003).

N-carboxymethyl chitosan is prepared by reaction with glyoxylic acid in the presence of a reducing agent (Ravi Kumar, 2000; Rinaudo, 2006). It is soluble in aqueous environment at neutral and alkaline pH values (Thanou *et al*., 2001). When prepared from a fully acetylated chitin, this derivative is water soluble in a wide range of pH (Rinaudo, 2006). *N*-carboxymethyl chitosan are mucoadhesive and lack systemic side-effects because they are not absorbed. An advantage of *N*-carboxymethyl chitosan

compared with conventional chitosan is that *N*-carboxymethyl chitosan are soluble above pH6.5 and compatible with polyanions (Ross and Toth, 2005). *N*-carboxymethyl chitosan is effective as an intestinal permeation/absorption enhancer *in vitro* and *in vivo* and it particularly increases the intestinal absorption of low molecular weight heparin across intestinal epithelia (Thanou *et al*., 2001). In food industry, *N*-carboxymethyl chitosan reduced aflatoxin production in *A. flavus* and *A. parasiticus* by more than 90%, while fungal growth was reduced to less than half. These results suggest that coating fruits with *N*-carboxymethyl chitosan may have some positive advantages for their long term storage. *N*-carboxymethyl chitosan was also documented to possess anti-oxidant property (Tharanathan and Kittur, 2003).

There has been an increasing desire to utilize chitin and chitosan of more uniform size particularly as oligomers. The benefits of oligomers are lower viscosity, low molecular weight, short chains and are soluble in neutral aqueous solutions. Subsequently, they seem to be readily absorbed *in vivo* (Pae *et al*., 2001; Khor, 2001). Two methods, namely chemical and enzymatic processes, are used for the preparation of chitin and chitosan oligomers, with different degrees of deacetylation, polymerization, and molecular weight. Chemical method involves the breakdown of the biopolymer chain by concentrated acids and alkali that can be slow and yielding oligomers with a wide distribution range (Khor, 2001). They are produced using concentrated acid solution, such as hydrochloric acid, nitrous acid, phosphoric acid and hydrogen fluoride followed by neutralization with alkaline solution. The chemical treatment is a very common and fast method to produce a series of the chitosan oligomer. However, this process has problems, including low yield, high cost, poor quality and production of acidic residual by use of concentrated acid (Lee *et al*., 2007; Prashanth and Tharanathan,

2007; Cai *et al.*, 2006; Choi *et al*., 2002; Dvorakova *et al*., 2001). Alternatively, chitin and chitosan derivatives can also be prepared through enzymatic preparation. In this case, certain enzymes are involved in the production of low molecular weight chitins and chitosans with high solubility such as chitinases, chitosanases, glucanases, lipases and some proteases (Choi *et al*., 2002; Robertus and Monzingo, 1999). In addition to this, lysozyme is also thought to be the primary enzyme responsible for *in vivo* degradation of chitosan through hydrolysis of the acetylated residues (Cai *et al.*, 2006; Ren *et al.*, 2005; Choi *et al.*, 2002; Dvorakova *et al*., 2001). In spite of the faster rate of chemical reaction, the enzymatic processes are generally preferable over chemical reaction because the use of enzymes automatically imposes mild reaction conditions and should permit better control of the oligomer distribution because of the selectivity of enzymes and also alterations are minimized in the chemical nature of the reaction product (Kim and Rajapakse, 2005; Khor, 2001; Ilyina *et al*., 2000). However, enzyme reaction on chitin progresses very slowly and higher oligomers are not obtained in good yields (Choi *et al.*, 2002).

Chitosan oligosaccharide was proved to be potent angio-inhibitory and anti-tumor compounds as it inhibits tumor-induced neovascularization. It also affects the mitogenic response and chemotactic activities of animal cells (Qin *et al*., 2002; Prashanth and Tharanathan, 2005; Kim and Rajapakse, 2005; Wang *et al*., 2007). Yoon *et al.* (2007) have reported the anti-inflammatory effect of chitosan oligosaccharide *in vitro*. *N*-acetylchitohexaose, water-soluble lower oligomers of chitin and chitosan was found to display a significant anti-metastatic effect of Lewis lung carcinoma transplanted into mice when it was administered intravenously (Tsukada *et al*., 1990). Chitosan oligosaccharide was found to inhibit the matrix metalloproteinase-2 activation

in primary human dermal fibroblasts (Kim and Kim, 2006), enhance certain macrophage functions in murine macrophage-like cell line (Yu *et al*., 2004; Feng *et al*., 2004; Han *et al*., 2005), induce complement activation via alternative pathway (Suzuki *et al*., 2003) and a positive regulator of primary rabbit neutrophils (Dou *et al*., 2007). Chitosan oligosaccharide was also described to have antimicrobial, antiviral, antioxidant, hypocholesteromic and immunostimulant effects (Kim and Rajapakse, 2005).

2.3 WOUND AND WOUND HEALING

2.3.1 General Aspect

A wound can be defined as the disruption of cellular and anatomic continuity of an organism or its parts, and wound healing may be defined as the restoration of the continuity (Castor, 1981). Whatever the trauma, the same tissue reactions are seen. One or another aspect may be magnified or inconspicuous after particular injuries, but the type and sequence of events is identical (Marks, 1981). Upon injury, a stereotypical sequence of events occurs, leading to the bridging of the defect and resurfacing. The depth of the injury determines the sequence of events (Irion, 2002). For cutaneous wound it can be divided into superficial, partial-thickness and full-thickness wound.

Superficial-thickness wounds involve only the epidermis and can be caused by shearing, friction or first degree burn. Healing occurs by regeneration of epithelial cells on the wound surface due to loss of contact inhibition, and migration of epidermal cells across the surface. Because no defect in skin continuity occurs, this type of healing does not cause scars, and accessory structures remain intact (Irion, 2002).

Partial-thickness wounds involve the epidermal layer and may also involve the superficial layer of the dermis. Healing in case of partial thickness wound progresses relatively quick with re-epithelialization by keratinocytes from wound edges and adnexial structures. Eschar may form on the wound (desiccated necrotic tissue, similar to a scab). The amount of inflammation, granulation tissue, wound contraction and scarring in the wound depends on the depth of dermal injury. Scarring may be minimal if injury is very superficial or clinically obvious if it involves deeper portion of the dermis (Gogia, 1995; Irion, 2002; Paddock *et al*., 2003).

Full-thickness wounds involve the complete epidermis, dermis and subcutaneous tissues. They may also involve muscles and bone. The healing of dermal wounds is highly complex as compared to both superficial and partial-thickness wound healing. In this wound, the essential mechanisms of inflammation, epithelialization, matrix synthesis and later scar formation all occur (Finley, 1981; Gogia, 1995; Irion, 2002).

Depending on the particular situation, wounds heal by one of three so-called intentions. The most direct method of wound healing is healing by first intention which applies to closed wound. It occurs in an uncomplicated setting usually in surgical wound or other incisions or lacerations that have clean, smooth edges and minimal subcutaneous tissue loss. It involves re-epithelialization of the surface and repair of underlying connective tissue. Healing by second intention applies to wounds with tissue loss, irregular edges, tissue necrosis, high microbial count or presence of other debris. It involves the formation of granulation tissue and wound contraction. Ultimately, the wound becomes totally closed by epithelialization of the residual defect. The time taken to achieve a totally epithelialized defect depends primarily on the initial size of the wound. Healing by second intention is frequently associated with deforming late scar

contractures. Healing by third intention also occurs with initially open wounds and is usually called ‘delayed primary closure’. It is favored when contamination, tissue loss or risk of infection is present. The wound is packed open for a few days to prevent infection and then closed after granulation tissue has formed. It can also be associated with substantial late scar contractures (Finley, 1981; Irion, 2002).

Cutaneous wound healing process goes through three phases before complete healing occurs which are the inflammatory phase, proliferative phase and remodeling phase. However, the process is continuous and the phases overlap (Cornacoff *et al*., 2008).

2.3.2 Inflammatory Phase

Inflammation is a nonspecific but predictable protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult (Mitchell and Cotran, 2003; Damjanov, 2006). The ultimate goal of inflammation is to bring to the invaded or injured area phagocytes and plasma proteins that can isolate, dilute, destroy or otherwise neutralize the harmful agents, remove debris, and eventually prepare for subsequent healing and reconstitution of the sites of injury (Sherwood, 2001; Mitchell and Cotran, 2003). Thus, inflammation is also intimately interwoven with repair processes whereby damaged tissue is replaced by the regeneration of parenchymal cells, and/or by filling of any residual defect with fibrous scar tissue/connective tissue (Mitchell and Cotran, 2003).

The Roman physician Celsus (circa 30 BCE to 38 CE) described the four cardinal signs of inflammation: *calor* (heat), *rubor* (redness), *tumor* (swelling) and *dolor*

(pain). Galen (130 BCE to 200 CE), another Roman physician, is credited for adding *functio laesa*, or loss of function, as the fifth classical symptom of inflammation (Damjanov, 2006).

Agents and means of provoking the inflammatory response include the physical agents (trauma, ionizing radiation, heat, cold), chemicals (corrosive chemicals, acids, alkalis, toxins), tissue necrosis (ischaemic infarction), microbial infections (viruses, bacteria, parasites) and hypersensitivity reactions (Underwood, 2004).

The inflammatory response has many players. These include circulating cells (neutrophils, eosinophils, basophils, lymphocytes, monocytes and platelets) and plasma proteins (clotting factors, kininogens and complement components), vascular wall cells (endothelial cells in direct contact with the blood and the underlying smooth muscle cells that impart tone to the vessels), connective tissue cells (sentinels to the invasion such as mast cells, macrophages and lymphocytes; and fibroblasts that synthesize the extracellular matrix and can proliferate to fill in a wound) and extracellular matrix of the surrounding connective tissue (fibrous structural proteins, gel-forming proteoglycans and adhesive glycoproteins that are the cell-ECM and ECM-ECM connectors). All these interact to resolve a local injury and restore normal tissue function (Mitchell and Cotran, 2003).

In an inflammation event, an initial inflammatory stimulus triggers the release of soluble chemical mediators from plasma/connective tissue cells. Acting together or in sequence, the chemical mediators amplify the initial inflammatory response and influence its evolution by regulating the subsequent vascular and cellular responses. Depending on the cause, damaged tissue is infiltrated with neutrophilic polymorphonuclear leukocytes and/or macrophages accompanied by fluid and exuded

proteins. These responses are terminated when the injurious stimulus is removed and the inflammatory mediators have been dissipated, catabolized or inhibited (Muller, 1981; Mitchell and Cotran, 2003). Inflammatory reactions are generally divided into two basic patterns; acute and chronic inflammation.

2.3.2 (a) Acute Inflammation

Acute inflammation is of relatively short duration, lasting from a few minutes up to a few days, and is characterized by fluid and plasma proteins exudation and a predominantly neutrophilic leukocyte accumulation. It is the immediate and early response to injury. This process has two major components, the vascular changes and cellular events. Vascular changes are alterations of the vessel caliber resulting in increased blood flow (vasodilation) and structural changes that permit plasma proteins to leave the circulation (increased vascular permeability). Cellular events involve the emigration of the neutrophil polymorphs from the microcirculation into the extravascular space and accumulation in the focus of injury (Mitchell and Cotran, 2003; Underwood, 2004).

Following tissue injury, there is a release of potent vasoactive mediators from tissue mast cells and components of the vascular wall causing a brief episode of vasoconstriction followed by dilation of precapillary arterioles and increased blood flow to the capillary beds (Sigal and Ron, 1994). As more blood flows into the area, local hyperemia occurs, accounting for the redness (erythema) and heat of the inflamed region (Marieb, 2001). Subsequently, the microvasculature becomes more permeable resulting in the movement of protein-rich fluid into the extravascular tissues. The clinical