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Applications of Chitin and Chitosan for Biomaterials

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Introduction

A biomaterial has recently been defined as an 'interactive material' able to establish an appropriate interaction with the surrounding tissue without inducing an adverse host response (Palapura and Kohn, 1992; Zhao *et al.*, 1990). In recent years, much attention has been paid to various biological dressings derived from natural products because of their high compatibility. Chitin and chitosan exhibit various biological activities and have been used in agriculture, industry, and medicine (Nishimura *et al.*, 1984; Suzuki, *et al.*, 1986). Chitin, a neutral(1–4)-linked polysaccharide and a natural mucopolysaccharide that consists of 2-acetamido-2-deoxy-β-D-glucopyranose residues (N-acetyl-D-glucosamine units), was first described in 1811 by Braconnot (Braconnot, 1811). Chitin is distributed widely in nature as the skeletal material of crustaceans and insects, and as a component of cell walls of bacteria and fungi, and is water-insoluble due to its rigid crystalline structure and intra- and inter-molecular hydrogen bonds (Muzzarelli, 1977; Nagai, Sawayanagi and Nambu, 1984). Chitin – the biopolymer that, among other things, forms the exoskeleton of insects and crustaceans – is the second most abundant polymer occurring in nature, after cellulose, with some 150×10^3 metric tons produced annually. Like cellulose, it is a glucose-based, unbranched polysaccharide. It differs from cellulose at the C-2 carbon, where instead of a hydroxyl group chitin has an acetamido residue.

Various measures are required to make water-soluble chitin derivatives. Chitosan, on the other hand, a polymer of D-glucosamine that was discovered by Rouget (Rouget, 1859) in 1859, composes the cell wall of mucor rouxii (Bartnicki-Garcia, 1968) and is easily obtained by deacetylation of chitin. Chitosan, furthermore, is readily soluble in water as a result of salt formation by the C-2 amino group of its glucosamine residue with various acids. They are known to be biodegraded into oligomer in vivo (Tokura *et al.*, 1983), despite similarity of its chemical and crystalline structure to that of cellulose (Berger and Wiser, 1957).

Chitin was found to promote biological activities such as an increase in the tensile strength of the suture site in rats (Prudden *et al.*, 1970; Yano *et al.*, 1985) and rabbits

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rabbits (Nakajima, Atsumi and Kifune, 1985) which indicate that unmodified chitin has been able to accelerate wound healing. Furthermore, there are generally no adverse effects (Nakajima *et al.*, 1986; Sapelli *et al.*, 1986).

Chitosan, introduced by Rawls (Rawls, 1984), is an attractive candidate for treating major burns. It forms a tough, water-absorbent, biocompatible film that can be formed directly on the burn by immersing the limb or body in an aqueous solution of chitosan acetate. 'The solution doesn't sting,' as acid solutions usually do. In fact, it feels 'quite pleasant.' The resultant film is permeable to oxygen and has a strong ability to absorb water, both useful properties in a burn covering. It is also slowly degraded by the enzyme lysozyme, which is present in wound areas, raising the possibility that the film might not need to be periodically removed from the wound.

Chitosan is a non-acetylated or partially deacetylated chitin (a linear homopolymer of β(1–4)-linked NAGA), that has already been proposed as a biomaterial because of its apparent satisfactory biocompatibility. Indeed, chitosan appears to have no adverse effects after implantation in tissue and for this reason it has been used for a wide range of biomedical applications such as artificial skin substitute (Muzzarelli *et al.*, 1988).

When chitosan is used as a biomaterial, it is very important to test its toxicity toward organisms. All tests have shown the safety of chitosan, including those for mutagenicity, acute and subacute toxicity tests, chronic toxicity test, pyrogens, hemolysis, and sensitization (Seo, 1990). In chitosan, furthermore, the accelerating effects on wound healing in small animals such as rat and dog were well documented in some cases. For example, burn therapies of rat (Suzuki, 1986), as a hemostatic agent for vascular grafts (Muzzarelli, 1977; Nagai, Sawayanagi and Nambu, 1984) and dermal and bone wound repairs of dog (Nagai, Sawayanaga and Nambu, 1984). The bioactivities of chitosan have also been studied to establish the influence of the degree of deacetylation. Chitin deacetylated to about 70% (DAC-70) was the most potent in immunological activity, such as macrophage activation, cytokine production, suppression of growth of Meth-A tumor in syngeneic mice, and anti-infectious activity. These activities were not observed for native chitin (Nishimura *et al.*, 1984). The DAC-70, furthermore, showed the highest lysozyme susceptibility among various deacetylated chitins (DACs, with degrees of deacetylation of 45, 66, 70, 77, 84, 91, and 95%). This susceptibility provides a measure of in vitro digestibility (Sashiwa *et al.*, 1990)..

These facts suggest strongly that chitin and chitosan are excellent candidates for biomaterials, and many attempts have been made to develop chitin and chitosan biomaterials. However, previous products have not completely achieved functionality as biomaterials and there are few commercialized biomedical materials such as Beschitin W (Unitika Co., Ltd., Kyoto, Japan). One of the reasons is difficulty in manufacturing chitin and chitosan because of their poor solubility in appropriate solvents under mild conditions. Hence, further investigations have been conducted on the materials, their shape, and so forth.

Achieving the following objectives were particulary important in the application of chitin and chitosan for biomaterials: to administer freely, to decrease the treatment frequency, to simplify operation, to avoid the use of antibiotics for food animals, and to produce inexpensive products. We have developed effective chitin and chitosan biomaterials for various kinds of animal. On the other hand, wound healing is an important factor in surgical treatments. Especially in human cosmetic surgery, mate-

rials such as amniotic membrane (Walker, Cooney and Allen, 1977), lyophilized porcine skin (Bromberg, Song and Mohn, 1965), and polyvinyl formal sponge (Jewett and Chardack, 1963) have been developed to promote the healing of skin wounds. However, clinical applications of all these materials suffer from some drawbacks related to antigenecity, cost, fitness, and post-operative scar formation. Development of an ideal artificial skin that is pain-free and devoid of post-operative scar formation has been sought. For several years, we have applied wound healing materials derived from chitin and chitosan to pets, large domestic animals, and zoo animals, and demonstrated positive and biological effects on various infectious wounds, histological responses *in vivo*, inflammatory cells *in vitro*, and so on. In this review, we would like to summarize the application of chitin and its derivatives for biomaterials around our studies.

Preparation of biomaterials

FLAKE

Flake-type chitin (Nippon Suisan Co., Ltd., Tokyo, Japan) was sterilized with ethylene oxide gas before use (Okamoto *et al.*, 1993).

POWDER

Commercial squid pen chitin (Nippon Suisan Co., Ltd, Tokyo, Japan) which was β-chitin purified from Neon flying squid (*Ommastrephes bartrami*) and 9% deacetylated and had an average molecular weight of over 100 000 and chitosan flake (Flonac C, Kyowa Tecnos Co., Ltd., Japan), which was comprised of 82% deacetylated α-chitin purified from crab shell and had an average molecular weight of 80 000, ash of maximum1.2%, and heavy metals as Pb, Cd and As of maximum 5 ppm, was pulverized into several grades between 3 and 90μm with a mill (Ube Industries, Ltd., Japan, CF-400). This biomaterial packed into a Medic Roll (Sanko Chemical Industry Co. Ltd., Japan) was sterilized with ethylene oxide gas (EOG) for 12 h at 60°C with a EOG sterilizer (Ikiken Co., Ltd. Z-601), prior to use in surgical operations (Tanioka *et al.*, 1993).

SUSPENSION

Chitin and chitosan powders pulverized into less than 30μm in particle size were suspended and irradiated with supersonic wave, followed by sterilizing at 1.7 atm and 121°C (105°C for chitosan) for 20 min in an autoclave prior to use (Tanioka *et al.*, 1993).

Chitosan of 300 000 molecular weight having a degree of deacetylation of 85% was solubilized in 1% acetic acid, filtered through Whatman GF/A filters, and precipitated by adding 1 M NaOH until a pH of 9 was reached. The pellet was then centrifuged at 8000 rpm for 5 min and washed several times with distilled water till neutral values of pH were obtained. The neutralized chitosan suspension was finally lyophilized and stored at 4°C (Peluso *et al.*, 1994).

COTTON

The cotton-like chitin product was produced by pulverizing squid pen chitin with the use of an ACM Pulverizer 10 manufactured by Hosokawa Micron Co., Ltd. (Osaka, Japan). The pulverization was conducted using a bar-shaped hammer and a grooved liner for 25 min at a rotational frequency of 6800 rpm with a rated current of 24 A. The obtained cotton-like chitin had an apparent specific gravity of 0.05 to 0.13 g/cm^3 and was in the form of fibers 0.1 to 0.8 mm in length and 10 to 120 μm in width. This biomaterial was packed into a Medic Roll (Sanko Chemical Industry Co., Ltd., Japan) was sterilized with ethylene oxide gas (EOG) for 12 h at 60°C with a EOG sterilizer (Ikiken Co., Ltd. Z-601), prior to use in surgical operations (Tanioka *et al.*, 1993).

The cotton-like chitosan product obtained was composed of fibers 2–20 mm in length, 20–50 μm in width and 3–15 μm in thickness, and had an apparent specific gravity of 0.1–0.2 g/cm^3. Chitosan was dissolved with stirring in a mixture of water and acetic acid and filtered twice by the application of pressure, and then defoamed by letting the solution stand overnight. The material was spun by extruding from a spinneret with 500 capillaries (0.1 mm diam.) first into a coagulating bath containing a mixed solution composed of ethyleneglycol, ice and potassium hydroxide. After coagulation, the spun threads were passed into a mixed solution of methanol and water. The resulting threads were stretched 1.15-fold in air and washed with water overnight, then treated with hot water at 70–80°C for 3 to 5 h and immersed in methanol overnight. The material was reeled with a reeling machine and dried. The resulting chitosan threads were cut to a length of 1 to 2 cm and treated with a mixer and then dried, resulting in a cotton-like chitosan product. This biomaterial was packed into a Medic Roll (Sanko Chemical Industry Co., Ltd., Japan) was sterilized with ethylene oxide gas (EOG) for 12 h at 60°C with a EOG sterilizer (Ikiken Co., Ltd. Z-601), prior to use in surgical operations. Commercial products: Chitopack C, Eisai Co., Ltd., Tokyo, Japan.

SPONGE

A 1.5% (w/v) dispersion of chitin in water was frozen at –20°C, followed by freeze-drying for 24 h, resulting in a sponge-like chitin product. This biomaterial was packed into a Medic Roll (Sanko Chemical Industry Co., Ltd., Japan) was sterilized with ethylene oxide gas (EOG) for 12 h at 60°C with a EOG sterilizer (Ikiken Co., Ltd. Z-601), prior to use in surgical operations (Tanioka *et al.*, 1993). Commercial products: Chitipack S, Eisai Co., Ltd., Tokyo, Japan.

MEMBRANE (FILM)

Many articles have dealt with the use of chitosan membranes, for the removal of toxic metal ions, hemodialysis, treatment of brines, immobilization of enzymes and other purposes (Muzzarelli, 1977; Muzzarelli *et al.*, 1980; Hirano *et al.*, 1980; Hirano, Tobetto and Noishiki, 1981). Membranes are usually cast from chitosan solutions in acetic acid or other suitable acids. These manufacts are neutralized with sodium hydroxide or ammonia before drying.

Chitosan having a degree of deacetylation of 87% and average molecular weight of

191 000 was used. One gram of chitosan was dissolved in 100 ml of aq. 1% acetic acid with or without the water-soluble chemicals and, after removing dissolved air under vacuum, poured into a glass vessel, followed by leaving overnight at 60°C. The membrane obtained was transparent, 2.5 cm diameter, and 80 to 100 μm in thickness (Muzzarelli *et al.*, 1988; Yomota, Komuro and Kimura, 1990).

Rawls (1984) reports that chitosan forms a tough, water-absorbent, biocompatible film that can be formed directly on the burn by immersing the limb or body in an aqueous solution of chitosan acetate.

A solution of chitosan (2g) in water (98g) containing acetic acid (2g), di(hydroxyethyl)sulfoxide (1g) and lithium chloride (1g) was allowed to evaporate on polyethylene film. The soft flexible film obtained was molded into a lens in a steel mold at 90°C under a platen pressure. The formed material was removed from the mold, immersed in a 10% aqueous NaOH solution for 20 min and then repeatedly washed with distilled water to afford a soft, hydrophilic, water-absorbent lens which had an oxygen permeability (Allan *et al.*, 1984).

The film-forming ability is a characteristic property of chitosan derivatives: when a chitosan membrane is acetylated, a regenerated chitin (N-acetylchitosan) membrane is obtained (Muzzarelli, 1983).

STICK

Chitosan threads of 18 pieces were wetted with water and twisted together to form a twisting stick of 5 mm diameter. The twisting chitosan sticks thus obtained were immersed in aqueous 7%(w/v) poly(vinyl alcohol) or aqueous 2%(w/v) sodium carboxymethylcellulose. These materials were attached to the sticks in amounts of 0.5–1.0 ml/10 cm stick, and subsequently dried in vacuo. The chitosan sticks of 3 mm diameter were cut into length of 5–6 cm, followed by putting a silicon tube of 4 mm diameter and 5 mm in width on the end of the stick, which was sterilized with EOG at 60°C for 12 h prior to use (Tanioka *et al.*, 1993).

TABLET

Oxytetracycline (OTC) releasing tablet: Chitin powder (shrimp shell, 200 mg) and OTC (200 mg) were mixed and pressed into a tablet under 300 kg/cm^2 at ambient temperature for 1 min with a Riken MODEL P-16B press. These core tablets were successively immersed in aqueous solutions of various chitin derivatives (covering agent) and acetone (coagulator) successively, then air dried to give OTC releasing tablets covered with polysaccharides (Shigemasa, Sashiwa and Saimoto, 1993; Tanioka *et al.*, 1993).

PAPER

Chitin paper: Squid pen chitin flakes were crushed in water in a Waring Blendor to less than about 200 mesh. The final chitin concentration was 0.5 to 1.0%(w/v). Paper was then prepared according to the JIS instruction book. The paper was dried at ambient temperature under pressure to avoid shrinkage (Tanioka *et al.*, 1993). Commertial product of chitin paper: Beschitin W (Unitika Co. Ltd., Kyoto, Japan).

COMPOSITE

Squid pen chitin was disaggregated (dispersed and swollen) in water at 40°C or lower by use of a homogenizer or a mixer. The mixture was poured into a predetermined amount of water to achieve 0.5–4.0 g/l chitin. The chitin suspension was poured onto a reinforcing material (a non-woven fabric of poly(ethyleneterephtalate) (Du Pont Co. Ltd., USA) by use of a suction-type paper-making apparatus of the batch style, resulting in a composite sheet having a chitin layer. The thickness of the composite sheet could be adjusted by adjusting the concentration of the starting chitin suspension. This biomaterial (Chitin-NWF) was packed into a Medic Roll (Sanko Chemical Industry Co., Ltd., Japan) was sterilized with ethylene oxide gas (EOG) for 12 h at 60°C with a EOG sterilizer (Ikiken Co., Ltd. Z-601), prior to use in surgical operations (Tanioka *et al.*, 1993). Commercial products: Chitipack P, Eisai Co., Ltd., Tokyo, Japan.

CATHETER

Polyethylene catheters were surface primed either with chromic acid solution or with oxygen plasma treatment, then dipped into a 0.6% chitosan solution in 1% acetic acid. After drying, the catheters were exposed to ammonia and then soaked in a pH 7.0 phosphate buffer containing 1% heparin. About 40 μg of chitosan were deposited per square cm. The heparinization procedure added 2–3 units of heparin per square cm (Muzzarelli, 1983).

GEL

Chitosan ascorbate gels were prepared by mixing finely-ground chitosan powder with a mixture of sodium ascorbate and ascorbic acid and then adding enough water to reach the desired consistency. The pH of the thixotropic gels thus obtained was adjusted by properly selecting the ascorbate ascorbic ratio and made to suit the physiological conditions (Muzzarelli *et al.*, 1988).

Applications of biomaterials

A biological filling agent for the promotion of wound healing in tissue defect is required not only in the treatment of abscesses, but also in the treatment of surgical tissue defects caused by oncotomy or reducing hernia. Conventional filling agents, such as artificial mammae, artificial eyes, artificial noses and so on are utilized only to maintain the shape in the face of a particular soft tissue defect in human medicine, and there is little biological filling agent buried in a wound cavity to promote wound healing. For these reasons, further investigations have been carried out on the material, nature, shape and fitness of the wound dressing and filling agents. On the other hand, Malette (Malette, 1986) showed the effects of chitosan for wound healing in dogs. They concluded that chitosan treated wounds did not display classic healing, but simply regenerated the normal tissue elements leaving no visible scars. These reports agree with the results when using chitosan-cotton for various types of infected wounds in large animals (Minami *et al.*, 1991, 1992).

WOUND HEALING

Objectives expected for these biomaterials of chitin and chitosan would be: (1) to accelerate wound healing, (2) to be useful in unhygienic circumstances, (3) to decrease treatment frequency, (4) to give comfortable and painless wound surface protection, (5) to simplify operations, and (6) to avoid the use of antibiotics. Concerning item (6), many problems in antibiotic therapy in food animal practice have been pointed out, namely bacteria drug-resistance, drug allergies, antibiotic residues in food, death of useful bacteria in the digestive tract and of protozoa in the lumen and so on. The availability of biodegradable and non-toxic materials capable of activating host defenses to prevent infection and to accelerate the healing of the wound is desired. Applications of these biomaterials to various clinical cases of many animals are summarized in *Table 1* and *Table 2*.

Table 1. Applications of chitin biomaterials

Form	Species	Applicable Clinical Case	Reference
Cotton	Dog	Abscess, Bite Wound, Contused Wound, Fracture	Okamoto *et al.*, 1992, 1993
	Cat	Abscess, Bite Wound, Contused Wound	Okamoto *et al.*, 1992, 1993
	Human	Decubitus ulcer	Ueyama *et al.*, 1994
Sponge	Cow	Decubitus ulcer	Wada *et al.*, 1990
		Abscess, Arthritis, Contused Wound, Surgical Dead Space (Umbilical Hernia)	Minami *et al.*, 1992; Okamoto *et al.*, 1993
	Dog	Abscess, Bite Wound, Contused Wound, Surgical Dead Space (Tumor Resection, Inguinal Hernia, Fracture), Lacerated Wound, Alveolitis	Okamoto *et al.*, 1992, 1993
	Cat	Abscess, Bite Wound, Surgical Dead Space(Tumor Resection, Inguinal Hernia, Fracture)	Okamoto *et al.*, 1992; 1993
	Rabbit	Abscess	Fukumoto *et al.*,1994
	Monkey	Bite Wound	Fukumoto *et al.*,1994
	Human	Maxillosinusectomy(Chitin sponge coated gauze)	Nagashima *et al.*, 1991
		Skin and soft-tissue defect	Maeda *et al.*, 1992
Composite with NWF			
	Cow	Fetlock Deformity, Capsuloplasty, Herniorrhaphy, Tendoplasty, Redressement of entropion	Okamoto *et al.*, 1992; Minami *et al.*, 1992
	Ring-tailed Lemur	Skin Defect of Tail	Fukumoto *et al.*, 1994
	Dog	Perineocele, Skin Defect, Prothsesis of Subcutaneous Tissue	Minami *et al.*, 1994
	Cat	Skin Defect	Saito, 1995
Powder	Dog	Contused Wound	Okamoto *et al.*, 1993
	Cat	Bite Wound	Okamoto *et al.*, 1993
	Human	Surgical wound, Slow healing surgical incision, Perineal wound, Excision of a keratosis, Ulcer, Trauma, Amputation	Balassa *et al.*, 1978
Film	Human	Dermatomed wound, Fresh burn Artificial Skin	Kishimoto *et al.*, 1985; Maeda *et al.*, 1986; Yasuse *et al.*, 1992; Oura *et al.*, 1992; Yamamoto *et al.*, 1990; Oshima *et al.*, 1986a,b

In the studies on chitin and chitosan biomaterials, various wounds have been found to require different characteristics of these biomaterials. This is particularly the case in veterinary rather than human surgical treatments, due to the unfavorable circumstances and the negative reaction of animals to treatment. Furthermore, the wound

faces are subjected to constant movement and are stimulated by the applied biomaterials. It is therefore obvious that the wound contacting biomaterials can result in further injury and prevent a smooth healing process. It is essential that biomaterials for animals be flexible, adhesive, and soft to the wound.

The effects of chitin agents on various animals are summarized in *Table 1*. In dogs, cats, and cows, the wound healing effects of these chitin agents were almost the same. Chitin-sponge was applied to tumor, hernia, hematoma, patella luxation, traumatic teeth injury and castration as filling agent of surgical tissue defect, and to trauma, abscess as wound dressing or tissue defect filling agent. In 90% of cases, good healing developed. When chitin-sponge was buried in surgical tissue defect due to oncotomy, recurrence of the tumor was not recognized for 3–24 months. In one case, recurrence of the tumor developed after one month post-operatively. The tumor was placed subcutaneously on the buried chitin-sponge and so was easily removed (Okamoto *et al.*, 1993).

Chitin-NWF was applied to trauma as a tissue defect filling agent, to abscesses as a wound dressing agent, to tumor, hernia and hematoma as filling agent of surgical tissue defect, and umbilical hernia as prosthesis of suture site of hernia ring. In 88% of cases, good healing developed and recurrence of the hernia did not develop in all cases. However, in two cases of trauma and one case of hernia which developed post-infection, wound healing was achieved by removal of the chitin-NWF (Okamoto *et al.*, 1993). Chitin-NWF was also found to be beneficial as prosthesis. Synthetic materials of high tensile strength have been used for the repair of abdominal wall defects in large animals (Johnson, 1969; Mansberger, Kang and Beebe, 1973; Tulleners and Fretz, 1983; Usher, 1979). Polypropylene meshes have been described most frequently for it (Johnson, 1969; Usher, 1979). However, operative method using these materials are complicated. The operative method was simpler using chitin-NWF than other materials.

The chitin-sponge and chitin-NWF were found to be efficient as filling agents for surgical tissue defects. Conventional filling agents are used only in plastic therapy. As substitute materials for maintaining shape, synthetic products such as silicone, vinyl chloride, and styrene foam are usually used. These materials are only buried in an organism to physically regulate shape and they do not promote wound healing. In many surgical tissue defects, it is desirable for the material buried to be biodegradable and to be replaced by normal tissues.

Chitin-cotton was applied to trauma and abscesses as a tissue defect filling or wound dressing agent. In 90% cases, good healing developed (Okamoto *et al.*, 1993).

Chitin-flake was applied to trauma as a tissue defect filling or a wound dressing agent. In 89% of cases, good healing developed (Okamoto *et al.*, 1993).

In these cases of injuries including traumas and abscesses, formation of healthy granulating tissue was observed within one week after treatment. Skin defects subsequently re-epithelialized without scar formation and any functional disturbances. In 6 out of the 44 cases of trauma, and in 7 out of the 55 cases of abscesses, granulating tissue did not develop. In these cases, general conditions were serious and contaminations of the wounds were severe (Okamoto *et al.*, 1993).

Chitin had similar effects on wound healing for various animals mentioned above. In addition, when these agents were applied to various types of trauma and abscessesas a filling or wound dressing agent, in spite of the different shapes of chitin agents,

Table 2. Applications of chitosan biomaterials

Form	Species	Applicable Clinical Case	References
Cotton	Dog	Abscess, Bite Wound, Lacerated Wound, Contused Wound, Gangrenous Mastitis, Surgical Infection	Minami *et al.*, 1993
	Cat	Abscess, Bite Wound, Lacerated Wound, Contused Wound, Surgical Dead Space	Minami *et al.*, 1993
	Rabbit	Abscess, Bite Wound	Ohtake, 1994
	Cow	Abscess, Septic Pododermatitis, Interdigital Phlegmon and Hyperplasia, Arthlitis, Peri-arthritis,Lacerated, Wound, Contused Wound, Omphalitis, Mastitis	Minami *et al.*, 1992, 1993
	Horse	Canker, Decubitus Ulser, Lacerated Wound, Contused Wound, Sitfast, Thrush	Minami *et al.*, 1992
	Primates: (Ring-tailed Lemur, Olive Baboon, Guereza, Bolivian Squirrel-monkey, Japanese macaque)	Bite Wound, Lacerated, Wound, Contused Wound, Abscess	Fukumoto *et al.*, 1994
	Carnivore: (Masked Palm Civet, Raccoon Dog, Red Fox, Cheetah, Lion, Common Raccoon, Coati, Caspian Seal)	Bite Wound, Lacerated, Wound, Contused Wound, Abscess	Fukumoto *et al.*, 1994
	Hyracoidea: (Cape Hyrax)	Lacerated Wound	Fukumoto *et al.*, 1994
	Artiodactyla: (Reeves's Muntjac, Goat, Giraffe, Barbary Sheep, Fallow Deer, bactrian Camel)	Lacerated Wound, Contused Wound, Decubitus Ulser, Abscess	Fukumoto *et al.*, 1994
	Rodentia: (Capybara)	Bite Wound	Fukumoto *et al.*, 1994
	Marsupialia: (Red Kangaroo, Common Wombat)	Contused Wound	Fukumoto *et al.*, 1994
	Edentata: (Giant Anteater)	Abscess	Fukumoto *et al.*, 1994
	Squamata: (Indian Python)	Abscess	Fukumoto *et al.*, 1994
Composite with NWF			
	Dog	Contused Wound	Murae *et al.*, 1994
	Cat	Skin Defect	Saito, 1995
Fine powder			
	Cow	Mastitis	Minami *et al.*, 1992
	Dog	Abscess, Lacerated Wound	Minami *et al.*, 1993
	Cat	Abscess, Bite Wound, Lacerated Wound, Contused Wound, Pyothorax, Mammary Tumor, Stomatitis,	Minami *et al.*, 1993
	Masked Palm Civet	Bite Wound	Fukumoto *et al.*, 1994
	Turtle	Vesiculopustular Dermatitis	Fukumoto *et al.*, 1994
Stick	Cow	Injuries of Teat Canal	Tanioka *et al.*, 1992
Hollow yarn			
	Cat	Ranula	Tanioka *et al.*, 1992
	Dog	Ranula	Minami *et al.*, unpublished data
Sponge	Rabbit	Experimental Surgical Wound	Muzzarelli *et al.*, 1993
Film	Rabbit	Experimental Surgical Wound, Cornea Injury	Muzzarelli *et al.*, 1993

Form	Species	Applicable Clinical Case	References
Chitosan solution in acetic water			
	Dog	Experimental Surgical Wound, Kidney, Ureter, Penile Foreskin	Bartone *et al.*, 1988
	Human	Burn	Allan *et al.*, 1984
	Monkey	Dermatitis	Rawls, 1984; Allan *et al.*, 1984
Lauroylated chitosan			
	Cow	Teat-sealant	Carolan *et al.*, 1992
5-methyl pyrolidinone chitosan(MPC)(water soluble)			
	Human	Injury, Meniscus injury, Coating for Prosthetic Materials	Muzzarelli *et al.*, 1993
5-methyl pyrolidinone chitosan(MPC)(water soluble)			
		Bone	Muzzarelli *et al.*, 1993
5-methyl pyrolidinone chitosan(MPC)(gel)			
	Human	Decubitus Ulcer	Muzzarelli *et al.*, 1993
	Decubitus Ulcer(Tegasorb)		Neill *et al.*, 1989
N-carboxybutyl chitosan			
	Rabbit	Dermo-Epidermal Explant	Biagini *et al.*, 1992

similar effects such as healthy granulating tissue formation were induced. On the other hand, the paper or film type remedies made of chitin did not accelerate wound healing because they did not maintain contact with the wounds. However, the present materials kept in contact with the wounds. These results suggest that the shape of the chitin agent affects the healing of wounds, and that keeping chitin in contact with the wounds is important.

The wide assortment of chitosan biomaterial applications for wound healing is illustrated in *Table 2*. In bovine practice, septic pododermatitis, abscess, interdigital phlegmon and interdigital hyperplasia were treated with Chitopack C as wound dressing and/or wound filling agent (Minami *et al.*, 1993). Various dirty wounds such as bite wounds and contused and lacerated wounds by wild animals were treated with Chitopack C for bovine treatment. The amount used of Chitopack C in small animal practice was 0.1–0.3g/head. In the application of Chitopack C to purulent bovine diseases, 90% of them were cured without antibiotic therapy (Minami *et al.*, 1993). In septic pododermatitis, good granulation was not formed in quite severe cases, such as those animals that had received inappropriate long term conventional treatment; in other cases, both legs had been seriously affected at the same time, so that a normal gait was hardly possible and a decubitus ulcer had subsequently developed. In other cases, good granulating formation and epidermization without scar were observed following the disappearance of purulence in the wounds. The frequency of convalescent treatments was less than 5 times in all cases. Contracted basketball-size abscesses in cow were healed with the application of only an initial Chitopack C filling to wound cavity.

In comparison to conventional therapy with irrigation and antibiotic administration to wound cavities, the novel Chitopack C method permitted a substantial decrease in treatment frequency (Minami *et al.*, 1993). In the Chitopack C therapy of various infectious wounds, it was always observed that there was no recurrence of purulence and the treatment frequency was reduced (Minami *et al.*, 1993).

In bovine practice, acute type or chronic type mastitis were treated with direct injection of 0.006% chitosan (DAC-80) physical saline suspension (chitosan suspension; 1.3–3 mg as chitosan/mammary quarter twice a day) through the streak canal

(Minami *et al.*, 1993). The acute type cases were completely cured with 2–10 administrations of medication, and 86% of the chronic type cases were cured with 2–30 administrations of medication. It was indeed surprising that these vicious, chronic mastitis cases that had not been responsive to antibiotics, clearly returned to a normal state with a short administration of chitosan medication (Minami *et al.*, 1993).

In companion animal practice, purulent skin and subcutaneous traumas were treated with direct injection of 0.006% chitosan physical saline suspension (60–300 μg as chitosan) to wound cavity or wound surface. Septic wounds and a pyothorax were completely cured by a single or several shots of chitosan (DAC-80) suspension after aspiration of saniopurulent hydrothorax. Based on the above results, chitosan (DAC-80) suspension seems to be more efficient for these purulent diseases than chitosan (DAC-80) cotton (Minami *et al.*, 1993).

WOUND DRESSING

Chitosan had a strong effect on wound healing in the bovine and equine clinical cases (Minami *et al.*, 1992). Especially in the treatment of vicious large abscesses, ulceration of soles in cow and canker in horse, with one or several applications of chitosan-cotton, these complicated diseases were immediately cured without complications.

Trauma

In 84% cases with trauma in dog, sufficient preferable responses were obtained by chitin flake (Okamoto *et al.*,1992). The palatal areas of 2 healthy monkeys were designed as the experimental sites, from which split thickness gingival flaps of relatively uniform thickness were removed. They received a chitin membrane or lyophilized dura mater as a dressing (Ogata *et al.*, 1991). It was concluded that the chitin might protect the denuded palatal sites, promoting keratin production, and thereby facilitating rapid and effective regeneration of the oral mucosa. Chitosan cotton was used as a wound dressing for trauma of skin (Minami *et al.*, 1992).

In the clinical cases, there were no obvious differences between the chitin and chitosan agents when these agents were applied to trauma and abscesses in dog. Furthermore, these agents were also obviously effective in cats. It is likely that similar responses were induced by these agents in the cat as well as in the dog. Another interesting point was that similar effects were induced by different shapes of chitin agents when these agents were applied to trauma and abscesses as fillings and wound dressing agents. These results suggest that the shape of the chitin agent does not affect the healing of wounds.

Burn

Beschitin W (Kifune, 1987) (Unitika Co., Ltd., Kyoto, Japan), which is made of non-woven fabric of polymeric N-acetyl-D-glucosamine (chitin), and acetate chitosan (Allan *et al.*, 1984) elicit analgesic effects on burn injuries in human beings (Kishimoto *et al.*, 1987; Oura *et al.*,1992).

Development of an ideal artificial skin that is well-fit, painfree and devoid of post-operative scar formation has been anticipated. Beschitin W (Unitika Co., Ltd., Kyoto,

Japan), is an artificial skin that has been developed for these purposes (Kifune, 1987; Kishimoto *et al.*, 1987; Oura and Minagawa, 1992). Over 1000 clinical cases of its use have been reported (Kifune and Tsuratani, 1991).

The effects of chitosan-cotton are worth noting in the field of veterinary medicine. Allan *et al.* (Allan *et al.*, 1984) showed the beneficial effect of chitosan which enabled burned rats to more successfully resist the adverse effects of trauma and infection.

In chitosan, accelerating effects of wound healing to small animals such as rat and dog were well documented in a burn therapy of rat (Allan *et al.*, 1984) and the aqueous solution of chitosan acetate doesn't sting, as acid solutions usually do. In fact, it feels 'quite pleasant.' The resultant film is permeable to oxygen and has a strong ability to absorb water, both useful properties in a burn covering. It also was slowly degraded by the enzyme lysozyme, which was present in wound areas, raising the possibility that the film might not need to be periodically removed from the wound.

Dermatitis

Chitosan also can be used effectively as a topical treatment for certain types of infections (Rawls, 1984). B. K. Ghosh has used it in acetic acid solution to treat monkeys suffering from dermatitis caused by mite infestations. The dermatitis cleared up in two days, and new hair growth started in 10 days. Unlike conventional topical treatment, treatment with chitosan appears to clear up the condition permanently. It works on athlete's foot infections, too.

Purulent diseases

Ulceration: Chitosan cotton was used as a wound dressing for ulceration of the sole of cow (Minami *et al.*, 1992). On the other hand, chitosan-cotton was used as a treatment for subcutaneous infections including cutaneous erosion and ulceration, and for the acceleration of granulated tissue formation (Minami *et al.*, 1992).

Horn erosion and trauma of the skin were cured in all cases with single chitosan cotton fitting to the wound (Minami *et al.*, 1992). Ulceration of the sole was also cured by granulated tissue formation and regeneration of the sole in all cases within a month.

Septic Pododermatitis, Interdigital Phlegmon, Interdigital Hyperplasia: In the application of Chitopack C to the purulent diseases (Minami *et al.*, 1993), 88% were cured without antibiotic therapy. In septic pododermatitis, good granulation was not formed in quite severe cases, such as animals that had received inappropriate long-term conventional treatments. In other cases, both legs had been seriously affected at the same time, so that a normal gait was hardly possible and a decubitus ulcer had subsequently developed. In other cases, good granulating formation and epidermization without scar were observed following the disappearance of purulence in the wounds. The frequency of convalescent treatments were under five times in all cases.

Canker

Chitosan cotton was used as a wound dressing for a canker of horse (Minami *et al.*,

1992). Canker was treated with radical surgical debridement and with chitosan cotton packing in the debrided area. Rapid granulated tissue formation was observed and the horse was clinically normal within a month after surgery, and tolerated pulling a heavy weighted sightseeing coach.

BIOLOGICAL FILLING AGENT

Abscess

Chitosan cotton was used as a filling agent for subcutaneous abscess (Minami *et al.*, 1992, 1993). Subcutaneous abscesses were cured in all cases with single chitosan cotton packing to the wound. In the large abscess, the dead space packed with chitosan-cotton was gradually contracted by granulated tissue formation and was cured within a month. No recurrence or suppuration of the wounds were observed in any of the cases.

Purulent skin and subcutaneous trauma

In clinical medicine, it has been reported that subcutaneous implants of chitin cottons and chitin suture materials were effective for the healing of closed wound (Kifune, 1987). When chitin was experimentally implanted in dogs, the rapid formation of granulated tissue and angiogenesis, and the earlier healing of wounds in a veterinary clinic using some chitin remedies were also observed (Minami *et al.*, 1992).

In companion animal practice, purulent skin and subcutaneous traumas were treated with direct injection of 0.006% chitosan physical saline suspension (60–300 μg as chitosan) to wound cavity or wound surface (Minami *et al.*, 1993).

Tumors

When chitin-sponge was buried in surgical tissue defect due to oncotomy, these operative wounds healed without complications like scar formation or functional disturbances, and recurrence of the tumor was not recognized for 3–24 months. In one case, recurrence of the tumor developed after one month post-operatively. The tumor was placed subcutaneously on the buried chitin-sponge and so was easily removed (Okamoto *et al.*, 1993). It was suggested that chitin suppressed recurrence of tumor. N-Acetylchitohexaose, consisting solely of N-acetyl-D-glucosamine (Tokoro *et al.*, 1988), DAC-30 and DAC-70 (Nishimura *et al.*, 1984) inhibited growth of Meth-A in mice, but unmodified chitin (Nishimura *et al.*, 1984) and chitohexaose (Tokoro *et al.*, 1988) did not. In the future, long-term observation and further investigation are necessary in respect to effects of chitin for recurrence of the tumor. At the site of the removal of the tumor, chitosan-cotton was also applied as biological filling agents (Okamoto *et al.*, 1992).

Mastitis

Mastitis is conventionally treated with the injection of a remedy which consists mainly

of antibiotics through the streak canal into the gland cistern or the direct injection into the udder artery. It is well known that complete healing of mastitis cannot be expected even in the treatment of the acute type. A low percentage of acute mastitis turns into a vicious chronic type, which is less responsive to any antibiotic therapies.

In bovine practice, acute or chronic type mastitises were treated with direct injection of 0.006% chitosan powder physical saline suspension(1.3–3.0 mg as chitosan/mammary quarter twice a day) through the streak canal. One hundred per cent of acute type mastisises were completely cured with 2–10 administrations of medications, and 86% of chronic type cases were cured with 2–30 administrations of medications (Minami *et al.*, 1993). In the lactating state of colostrum, periods for disappearance of mastitis symptoms were much longer than for other states. Jensen and Eberhart (Jensen *et al.*, 1981) have pointed out that cell contents in secretions decreased dramatically in the pre- and early post-partus period. This is one of the reasons why the mastitis will occur mostly in these periods. In treatment it is therefore important how to increase the bactericidal cellular contents in the secretion. Infusion of chitosan into the mammary glands through the streak canal may induce these reactions in the udder, because of chitosan's chemotacticity for PMN cells and the enhanced CL response of PMN cells.

Bone wound

Chitosan accelerating effects of wound healing to small animals such as rat and dog were applied for bone wound repairs in dogs (Malette, 1986; Borah *et al.*, 1992). While bone repair can be sustained by physiological processes that carry it to completion without the aid of biomaterials or foreign substances, it leads to the formation of callous tissue, which, because of its irregular calcification, is quite unlike the fibrous and orderly organized tissue obtained after inserting chitosan (Schenk *et al.*, 1970; Draenert and Draenert, 1980; Volpin *et al.*, 1988).

Hemorrhagic pneumonia

Subcutaneous administration of various amount of DAC-80 (10–200 mg/kg) was tested in dogs (Minami *et al.*, 1994). Anorexia was observed in all dogs with administration of DAC-80 more than 50 mg/kg, and mortality was brought about by the more than 150 mg/kg DAC-80 administration. In characteristic hematological findings, leukocytosis was observed and an increase of serum LDH2 and LDH3 isoenzymes. From the findings of autopsy, any dying dogs showed severe hemorrhagic pneumonias. Chitosan causes a lethal pneumonia to dogs. The DAC-80-related pneumonia featured the infiltration of polymorphonuclear cells and severe hemorrhage into the interstitial spaces and alveoli. These findings closely resembled those of adult respiratory distress syndrome (ARDS) (Murray, 1977) and murine lung affect cobra venom factor injection as a model of ARDS (Mulligan *et al.*, 1993). On the other hand, IL-8 causes neutrophil infiltration in a rabbit model of lung reperfusion injury (Sekido *et al.*, 1993) and IL-8 levels is also increase in the BALF of ARDS (Miller, 1992).

ARTIFICIAL ORGAN

Capsuloplasty

Chitin-NWF and chitin-sponge were used for suppurative arthritis of the carpal joints of calves as intra-articular tissue substitutes after curettage and irrigation. Capsuloplasty for the excised necrotic capsule was performed by fixing chitin-NWF using interrupted sutures (silk, 5 USP) (Minami *et al.*, 1992). Flexor deformities of the fetlock joint were completely cured in all cases without complications. Arthritis was also cured and the purulent discharge from the joint had disappeared. The facts of suppurative arthritis disappearing and the complete functional recovery of the affected foreleg indicated that strong anti-inflammatory effects existed in chitin because of the success in capsuloplasty by chitin-NWF without infection.

Artificial vessel

Chitosan accelerating effects of wound healing to small animals such as rat and dog was applied for a hemostatic agent for vascular grafts (Malette, 1986; Fradet *et al.*, 1986; Dutkiewicz *et al.*, 1992).

Application for hernia

In the treatment of umbilical hernia of calf, the chitin-NWF was buried in the subcutaneous portion as it had a sheet form, as a prosthesis for the stitches of the hernia ring, and the peripheral portion of the sheet was fixed by an interrupted suture. Umbilical hernias were completely cured without complications (Minami *et al.*, 1992). We have also applied chitin-NWF for the reduction of a perineal hernia in a dog, utilizing the effect of rapid organization of the subcutaneous fat promoted by chitin (Okamoto *et al.*, 1993). With this chitin-NWF technique, the operating time was far shorter (< 20 min) in comparison with conventional techniques, and dyschezia disappeared on the first post-operative day and no recurrence was observed (Minami *et al.*, 1994). On the surgical reduction of the umbilical hernia in calves and foals, Fretz et al. (Fretz *et al.*, 1983) reviewed retrospectively and reported that post-operative complaints were more numerous and serious in calves (37%) than in foals (11%). In this technique, no postoperative complaints were observed. It seemed that recurrence of the hernia did not develop because of the organization of the chitin-NWF.

In the cases of surgical tissue defect due to oncotomy, herniorrhaphy or another operations, the chitin-sponge and chitin-NWF were used as filling agent. In the case of an intrapelvic tissue defect due to the reduction of perineal hernia, the chitin-NWF was wound up into a roll form so as to fit with the wound cavity along the rectum.

Artificial tendon

Chitin-NWF was used for calves with flexure deformity of the fetlock joint (Minami *et al.*, 1992). As in the case of the fetlock joint deformity, chitin-NWF was used as a

tendon substitute in the elongation technique. When the superficial and deep digital flexor tendons were cut off and the fetlock joint was elongated to the normal position, the cut parts were separated proportionally to the degree of contraction of the two flexor tendons. Flexor deformities of the fetlock joint were completely cured in all cases without complications. Good connections between NWF and the dissected flexor tendons and enough angiogenesis in the NWF were observed.

Artificial kidney

The flow-rate of water through N-acetylchitosan membranes was $10.0–23.6 \times 10^{-3}$ml/cm2min, under a pressure of 3 kg/cm^2, and was unaffected by membrane thickness in the range 12–60 μm. The increase of chain length in the N-acyl groups caused a slight decrease of the flow-rate. Low-molecular weight compounds such as D-glucose, maltose, urea, calcium chloride, sodium chloride, cyclohepta-amylose and maltodextrin (M.W. 2900) passed though N-acetylchitosan membranes, whilst high-molecular weight compounds, such as cytochrome c (M.W. 13000) did not pass through. A variety of N-acyl chitosan membranes are available today. They seem to offer advantages over Cuprophane, the cellulosic product which is commonly used for artificial kidneys, in terms of more efficient ultrafiltration of middle molecular size compounds (M.W. 1000–2000). The manufacture of Cuprophane membranes is also complicated by the need of removing copper from the product (Muzzarelli, 1983).

Contact lens

An application for chitin that may be closer to commercialization is as a contact lens material, Allan *et al.* (1984) suggest. Because chitin is highly permeable to oxygen, chitin lenses could be worn for long periods. Polymers suitable for making both hard and soft contact lenses can be made from chitin, although they would need to be treated to induce slight crosslinking to prevent the lysozymes naturally present in tears from slowly dissolving them.

Remedy for streak canal injury : chitosan stick

Unsuitable suckles with proper procedure or machine operation often miss, and cause either arctation of the streak canal orifice, teat sphincter injury, or hyperplasia of the streak canal inner walls, teat cistern etc. These teat disorders also occur with tread trauma inflicted by the animal's own feet, and/or with iatrogenic injuries of the streak canal, etc. For the treatment of streak canal injuries, twisting plugs firmed with a binder are available, which are used with antibiotic salves before insertion. However, even when these biomaterials are used, there is little amelioration in suckle condition, and orifice ectasia or dissection of teat tips are required, which frequently result in teat infection, subsequently in mammary gland infections, and in most cases in mastitis. Chitosan stick was made to solve these problems (Tanioka *et al.*, 1993).

Blocking to *E. coli* invasion was evaluated (Tanioka *et al.*, 1993). A stick equipped with a 5 cm string was inserted in a glass tube of 8 mm in diameter, then immersed in physiological saline to swell well. After removing the excess of physiological saline in the glass tube, it was vertically set in 1 ml of physiological saline containing 1×10^6

of *E. coli* for 5 min at 37°C. The stick taken out of the apparatus was cut into 1 cm in length. The piece was put into 2 ml physiological saline and stirred well, then the 0.1 ml solution was sown on an agar medium and cultured at 37°C for 24 h, followed by counting the number of colony.

As shown in *Table 3*, chitosan stick reveals excellent blocking to bacterial invasion (E.coli)compared with commercial materials. Based on these results, this chitosan stick is expected to provide a good remedy for the treatment and prophylaxis of streak canal injuries.

Table 3. Inhibition of *E. Coli* invasion by chitosan stick

	Number of Colonies			
Distance from Stick Bottom (cm)/ Stick	0~1	1~2	2~3	3~4
Polyester	57	19	4	2
Chitosan stick	77	27	1	0
Commercial A	62	23	57	33
Commercial B	715	271	73	39

Catheter

Catheters were implanted for 30 min in carotid and femoral arteries. ^{125}I-Fibrinogen was injected prior to implantation; the radioactivity of the entire catheter and extruded thrombus was determined by γ-ray spectrometry. The tridodecyl methylammonium chloride-heparin surface performs poorly in comparison to the chitosan-heparin cyanoborohydride surface. Chitosan-heparin coated polymers display excellent thromboresistance properties. The lifetime of the thromboresistance can be extended by covalently binding the heparin to chitosan with the aid of sodium cyanoborohydride. This surface treatment is useful for biomedical applications requiring blood compatibility for periods as long as four days (Muzzarelli, 1983).

DRUG DELIVERY

In recent years, much attention has been paid to the drug delivery systems using biodegradable polymers. It is important that the concentration of an administrated reagent in blood is maintained at a suitable level as long as possible for the treatment and prophylaxis of disease.

Fine powder

Fine powder of chitosan gel was prepared by cross-linking chitosan with glutaraldehyde as an intelligent material for drug delivery (Ouchi and Ohya, 1993).

Kneaded mixture

The dissolution of spironolactone from kneaded mixtures with chitosan and low-molecular wt. gelatin was studied (Acarturk, Sencan and Celebi, 1993). It was found

that chitosan and low-molecular weight gelatin significantly increased the dissolution of spironolactone compared with the drug alone.

Tablet

Tian-Rui *et al.* reported the dissolution properties of water-soluble drugs from directly compressed tablets using chitosan with a high degree of polymerization, chitosan with a low degree of polymerization, 60% deacetylated chitin or hydroxypropylchitosan, in considering a development of per-oral sustained release tablets (Tian-Rui *et al.*, 1988). There is a commercial long acting injectable solution 'Teramycine/LA' (Pfizer Co., Ltd) which contains oxytetracycline (OTC) and is used in the treatment of infectious disease of cow or pig, however, this needs repeating injections at 3–5 day intervals and gives sharp pain at the injection. Release of oxytetracycline (OTC) from tablets covered with various chitin derivatives such as carboxymethylchitin (CM-chitin), dihydroxypropylchitin (DHP-chitin), and partially deacetylated chitin (DAC), was studied (Shigemasa, Sashiwa and Saimoto, 1993). Each tablet covered with chitin derivatives did not immediately disintegrate and gave the sustained release of OTC for a long period (more than 7 days). The release of OTC·HCl were suppressed more by the tablet covered with CM-chitin < DHP-chitin < DAC-67 in this order.

Film

Most of the water-soluble chemicals except Coomassie Brilliant Blue with a strong acidic group and pullulan with a high molecular weight (5.8×10^4–38.0×10^4), were rapidly released from the films within 1h (Yomota, Komuro and Kimura, 1990). The two chemicals described above were released only in the presence of lysozyme, and their release rates were controlled by the degradation rate of the films.

Chitin derivatives

A biodegradable (lysozyme-susceptible) and water-soluble 6-O-carboxymethyl chitin (CM-chitin) was applied to delivery systems (DDS) as a carrier for sustained release of drugs. Hydrolysis (by lysozyme and peptidase) of peptides combined with CM-chitin was investigated as a model system for DDS. Incorporation of spacer drug moiety to CM-chitin molecule induced enhancement of the lysozyme susceptibility; kcat/Km value of CM-chitin-drug conjugate for the lysozyme-catalyzed hydrolysis became 45 times higher that that of CM-chitin (Miura and Tokura, 1993).

MECHANISM OF WOUND HEALING

Macroscopic findings

The macroscopic findings are summarized in *Table 4* (Okamoto *et al.*, 1993). Undulation at the chitin/NWF-implanted site on post-implantation days (PIDs) 2, 4, and 8 decreased gradually and lasted until PID 18. In the chitin group, slight rubor was seen on PID 2, although neither high temperature nor dolor was indicated at any of the implanted sites or neighboring skin tissues during the experimental period. In the

Table 4. Macroscopic findings of the implanted Site[a]

	Undulation		Rubor		Dolor		High Temp.	
PID[b]	NWF[c]	C/NWF[d]	NWF	C/NWF	NWF	C/NWF	NWF	C/NWF
2	+	+	+	+	+	–	+	–
4	+	++	+	–	+	–	+	–
8	+	++	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–

[a] Each sign was classified into following three categories;
–: not found, +: slight, ++: moderate.
[b] PID: post-implantation day.
[c] NWF: polyester non-woven fabric.
[d] C/NWF: a composite of chitin and NWF.

control group, rubor, high temperature and dolor were observed on PIDs 2 and 4, although these signs were not apparent on PIDs 8 and 18.

A large amount of slightly viscous exudate was observed around the chitin/NWF implants on PIDs 2, 4, and 8 when the implants were isolated. Moreover, small quantities of viscous exudates pooled around the implanted NWF on PIDs 4 and 8 were also observed. No exudate was observed in both groups on PID 18 (Okamoto *et al.*, 1993). No microbial agent grew on agar of any of the exudates in both groups.

The surrounding tissues of the implants were normal on four days (PID 4) after the subcutaneous implantation of a sponge-like chitin in dog (Okamoto *et al.*, 1995). The implants turned into gel on PID 4, subsequently disappeared, and were replaced with reddish granulation tissues by PID 14. During this time, many polykaryocytes were observed in the granulation tissues. On PID 28, however, histological findings showed normal structure.

In the macroscopic observation of subcutaneously implanted chitosan cotton in bovine cases, there were no clinical symptoms systematically and locally in the implanted site and animals during the experimental periods (Minami *et al.*, 1993).

Mesenchymal cell

Young stromal connective tissue organization in the presence of chitosan only, without dura mater as a support, creates a three-dimensional network where each compartment is well-represented (Muzzarelli *et al.*, 1988). These elements are generally identified as mesenchymal cells; many of them have undifferentiated aspects (Muzzarelli *et al.*, 1988). Some mesenchymal cells show an endothelial attitude, and grossly tend to encircle circulating red cells (Muzzarelli *et al.*, 1988).

Anti-inflammatory

Disappearance of suppurative arthritis and complete recovery of the affected foreleg function in capsuloplasty of cow by chitin-NWF without infection indicated that strong anti-inflammatory effects existed in chitin.

The accumulation of inflammatory cells was observed at the site in contact with the chitin-sponge on four days after the subcutaneous implantation of a sponge-like chitin in dog (PID 4) (Okamoto *et al.*, 1995). On PID 28, however, inflammatory cells were not observed. Chitin could accelerate the first phase of wound healing where inflam-

mation is accompanied with infiltration of mononuclear (MN) and polymorphonuclear (PMN) cells without any uncomfortable side effects such as high temperature and dolor.

The observations of enhanced local antiphlogistic activities by administration of chitosan could be readily explained by the data of the activation of inflammatory cells and the clinical data from large animal practice. These antiphlogistic reactions in the clinical cases were more evident from the results of the application of the chitosan suspension to small animals (Minami *et al.*, 1993).

Polymorphonuclear (PMN) cell

Analyses of the effects of polymeric N-acetyl-D-glucosamine (chitin), which was obtained from squid pen, on histiogenic activation in dogs were carried out with subcutaneous implants (5×5 cm^2) of polyester non-woven fabric (NWF) supplemented with chitin (chitin group) and NWF (control group) (Okamoto *et al.*, 1993). PMN cells with a fibrin net at the aperture of polyester fiber were observed in both groups on PID 2. Numbers of mononuclear (MN) and polymorphonuclear (PMN) cells concentrated around the implants on post-implantation day 2 (PID 2) were larger in the chitin than in the control group.

Seven days after subcutaneous implantations of chitosan in cow, numerous PMN cells gathered around chitosan fibers and in the neighboring area. After 14 days, many of the migrated PMN cells disappeared. These histological findings are highly suggestive of the fact that the chitosan fibers constitute a stimulant for the migration of PMN and mononuclear (MN) cells (Minami *et al.*, 1992, 1993).

Transmission electron microscopy analysis identifies the presence of many leukocytes in the specimens after 14 day post-implantation, showing poor healing processes (i.e. fibroblast proliferation and collagen deposition) that characterize the tissue repair at this time in an animal model (Muzzarelli *et al.*, 1988).

Migration: This assay was performed by the blind well chamber method (Blind well chamber 200–187, Neuro Probe Inc., U.S.A.), using blood collected from healthy cows and healthy mongrel dogs (Minami *et al.*, 1993). Furthermore, the effects of normal canine serum, which was separated from healthy mongrel dog blood, on PMN cell migration was assayed with the same blind well chamber method.

Bovine PMN cells were found to migrate to the chitin and chitosan suspensions through a 5μm pore size polycarbonate in the Blind well chamber (Usami *et al.*, 1994). This migration was remarkable in the more finely granulated chitin and chitosan suspensions. Bovine and canine PMN cells were found to accumulate at subcutaneously implanted chitin and chitosan agents (Minami *et al.*, 1993; Okamoto *et al.*, 1993). Intraperitoneal administration of chitin to mice was reported to increase the number of peritoneal exudate cells within 3 hr (Suzuki *et al.*, 1986). Thus the evidence seems to be in favor of a good in vitro model of the enhanced cell accumulation at chitin and chitosan-administered regions at an early stage of wound healing.

. Chitin and chitosan, water-insoluble polysaccharides, are not known to induce the bioactivity in vitro. As to the mechanism of bovine PMN cells migration to chitin and chitosan suspensions, it is thought that finely granulated chitin and chitosan them-

selves might directly or indirectly enhance the migratory activity of bovine PMN cells. It has been reported that lipopolysaccharide (LPS) and zymosan could activate PMN cells (Creamer *et al.*, 1991; Olson, 1990; Thomsen and Jensen, 1991). Glucan, the major polysaccharide of zymosan, was shown to enhance the generation of reactive oxygen species from PMN cells (Williams *et al.*, 1986). A water-soluble oligosaccharide, N-acetylchitohexose, was shown to attract mouse PMN cells (Suzuki *et al.*, 1986). Finely granulated chitin and chitosan could also enhance bovine PMN cells activity (Usami *et al.*, 1994). These results should be important in assessing the acceleration of wound healing with chitin agents.

The results of PMN cell migration to chitosan are shown in Table 5 (Minami *et al.*, 1993). In order to clarify the effects of chitosan on canine PMN cell migration, the checkerboard assay (Zigmond and Hirsch, 1973) was performed. Based on Boyden's interpretation (Boyden, 1962), these results reveal that chitosan is a chemotactic substance. There is no chemotactic agents in the supernatant as summarized in *Table 6* which confirms that chitosan is really a chemotactic substance for canine PMN cell. Furthermore, PMN cell migration was enhanced by the supplementation of serum (*Table 7*). It is evident that an arithmetic effect on PMN cell migration was obtained with the supplementation of serum into chitosan suspensions (Minami *et al.*, 1993).

Table 5. Effects of chitosan on bovine PMN cell migration

Agent[a]	Mean particle size (μm)	Number of migrated cells Mean ± S.D.(cells/mm²)
Control[b]	–	2 ± 1
Flonac C	50.0	6 ± 2
Chitofine P-1	3.5	53 ± 7
Chitofine P-2	0.9	84 ± 10

[a] 1 mg/ml in 200μl HBSS in the lower chamber.
[b] 200μl HBSS.

Table 6. Effects of supernatant of chitofine S on canine PMN cell migration

Agent	Migrated cells/mm²
HBSS	63 ±9
Chitofine S	179 ±21
Supernatant(200 G, 5min)	73 ±25
Supernatant(700 G, 5min)	52 ±16
Supernatant(3000 G, 5min)	62 ±17

Table 7. Effects of serum on canine PMN cell migration

Agent	Migrated cells/mm²
HBSS	63 ± 9
Serum	209 ± 81
Chitofine S	205 ± 52
Serum + Chitofine S	404 ± 79

Chemiluminescence (CL) response: The method of CL measurement by a Biolumat LB 9501(Berthold Co., Germany) was employed as follows. PMN cells from circulating blood in mongrel dogs were prepared with the density gradient centrifugation method. In order to investigate the relationship between CL response and complements in the serum, two types of serum were prepared. One was normal canine serum, the other was the serum inactivated with the incubation at 56°C for 30 min (Minami *et al.*, 1993).

The canine PMN cell CL responses are summarized in *Table 8* (Minami *et al.*, 1995). In the CL responses with canine serum, CL of PMN cells for chitin and chitosan was 30% and about half that for zymosan respectively. The CL response to chitin and chitosan was markedly enhanced by serum. Under serum-free conditions and with decomplementized serum, canine PMN cells did not respond to either chitin and chitosan. These results clearly indicated that canine PMN cells interacted with these preparations by opsonization with serum complement. Ross, Cain and Lachmann (1985) demonstrated that the interaction of zymosan and neutrophils was independent of C3 opsonization. In addition, Williams *et al.* (1986) reported that unopsonized zymosan, of which major carbohydrate component was a glucan, was phagocytosed by human PMN cells under serum-free conditions. The result for zymosan in *Table 8* (Minami *et al.*, 1993) agree well with the results of Williams *et al.* Our findings on the CL response of canine PMN cells to chitin and chitosan under serum-free or decomplementized conditions suggest that there are no receptors interacting with N-acetyl-D-glucosamine and D-glucosamine on the surface of canine PMN cells. After the application of chitin or chitosan to wounds, we frequently observed a moderate amount of exudate on the wound surface (Minami *et al.*, 1993). This may indicate a signal of a good response for a wounded body, because the chitin or chitosan particles opsonized by the exudate enhance the phagocytic activity of PMN cell. From these results, it is clear that chitin and chitosan act as stimulants for PMN and macrophages, inducing the migration of inflammatory cells into the wound cavity and then inducing active biodebridement by these cells.

Table 8. Effects of chitosan on canine PMN CL response

Agent	Peak count[1]		
	Normal serum[2]	Decomplementized serum[3]	Serum free[4]
Zymosan	537.4 ± 22.3	215.8 ± 5.3	122.4 ± 12.9
Chitosan[5]	269.8 ± 27.3	1.4 ± 0.7	1.5 ± 0.3
Chitin[6]	156.0 ± 12.0	2.0 ± 2.0	2.0 ± 0

[1] Peak emission count per each 1000PMN cells(Rlu/sec).
[2] 10% canine normal serum.
[3] 10% canine decomplemented serum (56°C, 30 min).
[4] Suspended in HBSS without serum.
[5] Chitosan fine powder.
[6] Chitin fine powder.

Macrophage

The effects of chitin on the activation of peritoneal macrophages were described in detail by K. Nishimura *et al.* (Nishimura *et al.*, 1984, 1986a,b,c, 1987). Peritoneal macrophages obtained from some laboratory animals are known to be activated by

chemically modified chitin, especially those with 30%, 70%, and 80% deacetylation, although such is not the case with non-deacetylated chitin. On the histological appearance of nonwoven fabric of polyester (NWF) implanted site (control) and chitin/NWF composite implanted site, numbers of mononuclear (MN) cell concentrated around the implants on post-implantation day 2 (PID 2) were larger in the chitin than in the control group and polykaryocytes were attached to many polyester fibers of the implant before day 8, but not in the case of NWF implantation (Okamoto *et al.*, 1993). This suggested that chitin might also activate MN cells in dog and induced early macrophages migration into the wound cavity.

Chitin derivatives and chitose oligomer (MW. 2,000) at a concentration of 1,000 μg/ml activated macrophage cell line (clone A2) which damaged L929 cells (Tanigawa *et al.*, 1992).

Chitosan activates macrophages for tumoricidal activity and for the production of Interleukin-1. Moreover, chitosan shows immunopotentiating activity, which is desirable for drug carriers to be administered to tumor bearing hosts, whose immunities are depressed (Nishimura *et al.*, 1986). Chitosan has an in vivo stimulatory effect on both macrophage nitric oxide (NO) production and chemotaxis (Peluso *et al.*, 1994). The macrophage NO secretion is attributed to the N-acetylglucosamine unit of the chitosan molecule rather than to the glucosamine residue (28 and 15 μM NO resp.). Moreover, the immune stimulatory effect of chitosan was very specific since other glycosaminoglycans, such as N-acetyl-D-mannosamine and N-acetyl-D-galactosamine, had no effects on NO production. In vivo experiments strengthen this hypothesis. Macrophages migrate in response to a positive gradient of chitosan, thus indicating a true chemotactic effect. N-Acetylgalactosamine or N-acetylmannosamine do not elicit a true chemotactic response whereas this is evident for N-acetylglucosamine at concentrations much below those necessary for glucosamine. The checker-board analysis suggests that N-acetylglucosamine and glucosamine induce a true chemotactic response, whereas random migration is present when different concentrations of the other substituted hexosamines are placed in both the lower and upper wells of the chemotaxis chamber (Peluso *et al.*, 1994).

Polykaryocyte

Polykaryocytes appeared slightly in the granulation tissues on seven days after the subcutaneous implantation of a sponge-like chitin in dog (PID 7), and the number of cells showed a peak on PID 14 (Okamoto *et al.*, 1995). On PID 28, however, polykaryocytes were not observed. This suggests that chitin induces polykaryocytes, which are thought to be derived from macrophages and have higher level functions than macrophages (Chambers, 1978). There may be a close relationship between the disappearance of granulation tissue and the appearance of polykaryocytes. On the histological appearance of nonwoven fabric of polyester (NWF) (control) and chitin/NWF composite implanted sites, polykaryocytes were attached to many polyester fibers of the implanted chitin/NWF before day 8, but not in the case of NWF implantation (control) (Okamoto *et al.*, 1993).

Fourteen days after subcutaneous implantations of chitosan in cow, polykaryocyte derived from macrophages attached themselves to chitosan fibers (Minami *et al.*, 1993).

Endothelial cell

When the wound healing was observed histologically at the site of lesion administered with chitin, there was increased vascularization in the lesion (Nishimura *et al.*, 1984). Then, in order to know in vitro whether this chitin promotes the new vascularization or not, the cell attachment to chitin- and chitosan-coated dishes and the proliferation of the cells (Shigemasa *et al.*, 1991) was tested using the endothelial cell line, CPAE (Dainippon Seiyaku Inc., Laboratory Products) derived from the main stem pulmonary artery of a young cow.

CPAE cells were well spread out on the control dish and the chitin-coated dish, but were not on the chitosan-coated dish. Mean adherent viable cell numbers and the standard error of each dish are shown in *Table 9* (Minami *et al.*, 1992). There was no significant differences between the cell count of the chitin-coated dish and of the control, but the cell count of chitosan-coated dish was significantly lower than the others ($P < 0.01$).

Table 9. Viable CPAE cells cultivated on chitin- and chitosan-coated dishes

Dish type	Adherent viable cell number[a] ($\times 10^4$)	Number of tested dishes
Chitin-coated	31 ± 3	4
Chitosan-coated	14 ± 2	4
Control	35 ± 6	4

[a] Number counted after 48 h cultivation at 37°C in CO_2-incubator.

Cytokines

Recently, a study of cytokines has made it clear that granulation was accelerated by IL-1, TNF-α or FGF and suppressed by IL-4 or TNF-γ (Sato *et al.*, 1990; Chensue *et al.*, 1989). These cytokines were released by macrophages, lymphocytes, fibroblasts and so on. Not only IL-1 and TNF-α are produced from macrophages (Chang, Gilman and Lewis, 1986), but these substances are also known to activate fibroblasts (Hatake, 1991). It is well known that peritoneal macrophages obtained from some laboratory animals, such as mouse, rat and rabbit, were activated with chitin derivatives, especially with DAC-70 (Nishimura *et al.*, 1984, 1986), DAC-80 (Nishimura *et al.*, 1984), and DAC-30 (Nishimura *et al.*, 1986).

The amount of IL-1 in the exudate taken from around the areas where chitin-NWF was implanted in dog increased two-fold in comparison with that of control medium alone (Okamoto *et al.*, 1992). However, this response was much lower in comparison with one unit of mouse γIL-1α. Anyway, murine thymocytes sufficiently responded to the exudates of dog. It is considered that these exudates contained IL-1. These results would suggest that IL-1 is effective for healing wounds in dog and also to human and laboratory animals such as mouse and rat.

Chitin agents may attain excellent advantages from the fact that various cells showing a biophylaxis function are allowed to migrate for the purpose of fighting the bacteria and treating the necrosing tissue in the wound.

Angiogenesis

On the histological appearance in chitin/NWF composite implanted sites at 4 days after implantation (PID 4), the newly formed granulation tissue around the chitin/NWF composite actively invaded the composite with new blood vessels, but not in the case of NWF implantation (control) (Okamoto *et al.*, 1993; Minami *et al.*, 1992). In the chitin group, the implant was organized gradually and its organization was completed on PID 18, when obvious angiogenesis toward the NWF was observed. The histiogenic tissues surrounding the implant site of the chitin group remained pink in color when the chitin/NWF was removed, whereas the histiogenic tissues were pale in color in the control group during the experimental period. Obvious angiogenesis toward the NWF was not observed macroscopically. The mechanisms of progressive angiogenesis in the implanted chitin-NWF were partially recognized with good attachment of the CPAE cells to the chitin-coated dish (Minami *et al.*, 1992).

Fourteen days after subcutaneous implantations of chitosan and chitin in a cow, a connective tissue accompanying angiogenesis was reconstructed around the fibers (Minami *et al.*, 1992, 1993).

Fibroblast

FPF assay (Wahl *et al.*, 1979) for the exudate recovered from around the areas where chitin-NWF was implanted in dog showed that the fibroblast proliferation increased four- to six-fold in comparison with that of control (Okamoto *et al.*, 1992). The assay value of the samples taken 2 days after implantation was slightly higher than that of the samples taken at 4 days. The fibroblasts cultivated with the exudate derived from chitin-NWF were active.

Slight proliferation of fibroblasts was observed at the site in contact with the chitin-sponge on four days after the subcutaneous implantation of a sponge-like chitin in dog (PID 4) (Okamoto *et al.*, 1995). From the results, it is assumed that FPF is an extremely FGF-like substance. These results would suggest that FPF is effective for healing wounds in dog and also in human and laboratory animals such as mouse and rat.

Seven days after subcutaneous implantations of chitosan and chitin in cow, a mild fibroblast activation was observed in the neighboring area around the fibers (Minami *et al.*, 1992, 1993).

Granulation

Granulating tissue can generally be divided into two types, healthy and unhealthy granulating tissues (Hataya *et al.*, 1992). It is well known that healthy granulating tissue develops only in the absence of foreign bodies such as bacteria, debris, and so forth (Clark and Denver, 1985). Formation of healthy granulating tissue which is closely related to angiogenesis is a very important factor in wound healing (Clark and Denver, 1985; Hataya *et al.*, 1992). Granulation tissues with neovasculature were observed slightly or moderately at the site in contact with the chitin-sponge on 7 and 14 days after the subcutaneous implantation of a sponge-like chitin in a dog (Okamoto *et al.*, 1995). After 28 days, however, granulation tissues formed disappeared. On the

histological appearance of chitin/NWF composite-implanted sites at 4 days after implantation (PID 4), formation of granulation tissue around the implanted composite was already definitely observed, whereas such a phenomenon was not manifested around the implanted nonwoven fabric of polyester (NWF) (control). Mitoses were observed frequently in the granulating tissue around the chitin/NWF on PIDs 4 and 8. The newly formed granulation tissue around the chitin/NWF composite actively invaded the composite with new blood vessels, and polykaryocytes were attached to many polyester fibers of the implant before day 8, but not in the case of NWF implantation (Okamoto *et al.*, 1993). It was clearly shown that NWF was rapidly organized by the chitin-induced granulation in subcutaneous tissue. As chitin was also found to promote the formation of granulating tissue with angiogenesis, chitin could have induced healthy granulating tissue within one week after treatment. In the case that granulating tissue did not develop, general conditions were serious and contamination of the wounds were severe (Okamoto *et al.*, 1993).

The effects of chitin on the mechanism of the formation of granulating tissue are unclear. Mitoses in granulation tissues around the chitin/NWF implant on PIDs 2 and 4 were accompanied with simultaneous increases in MN and PMN cells around the chitin/NWF implant. A recent study on monokines has clarified that the granulating tissue of mice is accelerated by IL-1 (Sato *et al.*, 1990) or TNF-α (Chensue *et al.*, 1989). Not only IL-1 and TNF-α are produced from macrophages (Chang, Gilman and Lewis, 1986), but these substances are also known to activate fibroblasts (Hatake, 1991).

Chitosan may be used to inhibit fibroplasia in wound healing, and to promote tissue growth and differentiation in tissue culture. Chitosan provides a non-protein matrix for three-dimensional tissue growth (Malette, 1986).

On the other hand, chitosan-cotton was used as a treatment for subcutaneous infections including cutaneous erosion and ulcerations, and for the acceleration of granulated tissue formation (Minami *et al.*, 1992). In the treatment of purulent digit disease with chitosan (Minami *et al.*, 1993), granulating formation was also observed with minimum convalescence periods without antibiotic administration to the soiled body surfaces. These effects agree well with those data obtained from equine canker (hypertrophic moist infectious pododermatitis) treatment (Minami *et al.*, 1991).

Chitosan was superior to chitin in its effect on the acceleration of granulation tissue formation.

Collagen

Scar formation which depends on both continued synthesis and catabolism of collagen is a serious problem in a wound healing process (Clark and Denver, 1985). Cartilage which is known as a wound healing accelerator increased the density of collagen in wounds where it was topically administered (Paulette and Prudden, 1959, Allen and Prudden, 1966). On the other hand, it is known that minimum scar formation remaines in the wound after treatment with chitin administration. Within one week after wounding, therefore, collagen-hydroxyproline did not increase in a rat model. The amount of collagen-hydroxyproline in the skin of the control rats was 2.30±0.69 μg/mg. Hydroxyproline in the tissues of the wound with chitin-treatment was 5.03±3.02 on day 3, 4.90±2.20 on day 5, and 3.66±0.76 on day 7. On the other hand, those

without chitin-treatment on the corresponding days were 7.38±3.29, 4.75±2.62 and 6.44±2.34. The values of hydroxyproline in the wounded tissues irrespective of chitin-treatment were significantly higher than those of the control expect for the values with chitin-treatment on day 3 and without chitin-treatment on day 5. However, the differences between the values with and without chitin-treatment were not significant except on day 7 (Yano *et al.*, 1985). Since there are, however, several reports documenting an acceleration of tensile strength with chitin (Hoffmeister *et al.*, 1964; Reynolds, Levegue and Buxton, 1960), they (Yano *et al.*, 1985) sought the mechanism of the accelerating effect of N-acetylglucosamine in other events in the process of wound healing rather than in an increase in collagen-synthesis; e.g., N-acetyl glucosamine may serve as a substrate for a reinforcement of the wounded tissues without excessive inflammatory reactions, because tensile strengths were significantly accelerated without an increase in collagen-synthesis in the study. Okamoto *et al.*, however, implanted a polyester non-woven fabric (NWF) into canine subcutaneous tissue, and observed a new formation of collagen in the implanted NWF with chitin administration but no formation of collagen in the control NWF by histological examination with Masson's trichrome stain (Okamoto *et al.*, 1993). Chitin increases collagen synthesis in the implanted NWF. Ogata *et al.* also reported an increasing collagen synthesis with chitin in the experimental created palatal mucous trauma. Type I collagen production in both the chitin and dura mater experimental groups increased more rapidly than in the group without dressing material. A detected increase in type III collagen was markedly greater in the chitin group at 3 weeks after surgery (Ogata *et al.*, 1991). Kishimoto and Tamaki (1987) confirmed that many histiocytes invaded the wound and fine collagen fibers were produced in the chitin dressing group but found little histiocyte invasion and thick collagen fibers in the non-dressed group. They suggested that histiocytes might be induced by chitin and might promote the proliferation of fibroblastic cells, which produced fine collagen in the process of burn wound healing in guinea pig skin.

In the chitin administered wound, therefore, synthesis of collagen will accelerate in the early wound healing process, but synthesized collagen will be degraded very conveniently to an appropriate amount until the final wound healing process. The degradation of wound collagen is initiated by a variety of collagenase enzymes from granulocytes (Robertson, Ryel and Taylor, 1972), macrophages (Werb and Gordon, 1975), epidermal cells, neutrophil, and fibroblasts (Clark and Denver, 1985; Donoff, McLennan and Grillo, 1971). Inflammatory accumulation at the site in contact with the chitin-sponge was also observed (Okamoto *et al.*, 1993). This suggests that excessive collagen is degraded by inflammatory cells induced by chitin.

From the result of scanning electron microscopy observation of chitosan implantation to the dura, Muzzarelli *et al.* (1988) reported that chitosan, in the relationship between chitosan and collagen production, could be considered a primer on which a normal tissue architecture is organized. Collagen fibers show a clear tendency to maintain a well-defined orientation and to form a consistent support both in the presence and absence of dura mater. The progressive deposition of collagen fibres starts from the proximity of fibroblasts that seem to guide the extracellular-oriented deposition of these fibres. In this model, the inductive role of the collagen matrix on tissue organization is further sustained by the formation of a more mature stromal tissue (Muzzarelli *et al.*, 1988).

Keratin production

A great deal of keratin protein had been produced at 5 weeks with use of the chitin dressing. It was concluded that the chitin might protect the denuded palatal sites, promoting keratin production, and thereby facilitating rapid and effective regeneration of the oral mucosa (Ogata *et al.*, 1991).

Epidermization

Though chitin had little effect on macrophages in comparison with that of DAC 70 (Nishimura *et al.*, 1984), on the human burn treatment chitin acted as an excellent wound remedy for agonizing pain and good epidermization without scars (Kifune and Tsurutani, 1991). After application of chitin sponge, the reduction of granulation and good re-epithelialization were observed. In these cases of injuries including traumas and abscesses, skin defects subsequently reepithelialized without scar formation and any functional disturbances (Okamoto *et al.*, 1993).

In the treatment of purulent digit disease with chitosan (Minami *et al.*, 1993), epidermization was also observed with minimum convalescence periods without antibiotic administration to the soiled body surfaces. These effects agree well with those data obtained from equine canker (hypertrophic moist infectious pododermatitis) treatment (Minami *et al.*, 1991).

Tensile strength

The tensile strengths of the wounds with chitin-treatment were 2.59±0.86 mV/30mm^2 on day 3, 4.67±3.30 on day 5, and 8.61±3.95 on day 7, while in the wounds without chitin-treatment, they were 1.55±0.65 on day 3, 1.57±0.77 on day 5, and 4.90±1.20 on day 7. The tensile strength of the wounds with chitin-treatment were significantly higher on days 3 and 5 than those without chitin-treatment (Yano *et al.*, 1985).

Immunological activities

Chemically modified chitins including partially deacetylated and carboxymethylated chitins were found to have potent immunological activities (Iida*et al.*, 1987; Nishimura *et al.*, 1984, 1985, 1986a,b,c, 1987).

Degradation

Allan *et al.* (1984) said that it is apparent that the effect of treatment of the burns with either low or high molecular weight chitosan is statistically different from the no treatment situation at the 95% confidence level. It is also clear that the size of the chitosan molecule plays some role with the low molecular weight being the most effective and this conclusion is valid at the 99% confidence level. If the chitosan macromolecule functions as a controlled source of low molecular weight aminosugars then the release rate would be expected to be inversely proportional to the molecular weight of the reservoir (Allan, Fox and Kong, 1978). There is, of course, evidence that D-glucosamine has a minor effect on the rate of healing of surgical incisions (Hoffmeister *et al.*, 1964). The importance of the molecular weight of

chitosan in relation to its biological behavior is also manifest in the research of Hadwiger and coworkers on the gene activation and fungistatic behavior of chitosan where heptamers showed distinctive activity (Hadwiger *et al.*, 1984).

In many surgical tissue defects, it is desirable for the material buried to be biodegradable and to be replaced by native organisms. Chitin is degraded by some enzymes such as lysozyme (Tokura*et al.*, 1983; Berger and Wiser, 1957) and chitinase (Shigemasa *et al.*, 1994; Sashiwa *et al.*, 1993). Plants and insects have chitinase (Jeuniaux, 1961), but not mammals except for goat (Lundblad *et al.*, 1974) and sheep (Lundblad *et al.*, 1979). So, chitin is thought to be degraded mainly by lysozyme in mammals (except for goat and sheep). Biodegradability is well known to be a useful characteristic for implantable materials (Kifune, 1992) and this phenomenon was clearly observed after chitin sponge implantation. Maeda*et al.* (1986) also reported that chitin did not disappear completely after 6 months when it was implanted intramuscularly in rat. An in vitro study indicated that chitin was degraded slowly by lysozyme (Tokura *et al.*, 1983). On the other hand, Muzzarelli *et al.* (1988) said that lysozyme plays an important role in the degradation of chitin in vivo; oligomers are further hydrolyzed to GlcNAc, a common amino sugar in the body, which enters the innate metabolic pathway to be incorporated into glycoproteins or to be excreted as carbon dioxide. However, little is known clearly about the process of its degradation in vivo.

There were no problems found at the site of surgical dead space after the removal of tumors and reduction of hernias in clinical cases (Okamoto *et al.*, 1992; Kishimoto and Tamaki, 1987; Malette, 1986). However, the fate of filling agents at the site of surgical dead space after removal of a tumor or in the reduction of a hernia is unclear. Regarding the chitin-sponge, it is assumed that it was attacked by enzymes, because there was no palpable foreign body at the filling site one week after the operation.

The fate of polymeric N-acetyl-D-glucosamine (chitin), which was obtained from squid pen, was determined by the subcutaneous implantation of a sponge-like chitin in dog (Okamoto *et al.*, 1995). The material was implanted at four sites, on the lumbodorsal and lumbosacral subcutaneous tissues on both sides of the midline in each dog under general anesthesia. The implants and their surrounding tissues were surgically recovered on post-implantation days (PIDs) 4, 7, 14, and 28 under general anesthesia. The implants turned into gel on PID 4, subsequently disappeared, and were replaced with reddish granulation tissues by PID 14. The macroscopic findings of the implants are summarized in *Table 10.*

Table 10. Macroscopic findings of chitin-sponge subcutaneously implanted in dog[a)]

No.	PID[b)]			
	4	7	14	28
1	–	++	+++	+++
2	+	++	++	++
3	+	++	+++	+++
4	+++	+++	+++	+++
5	+	++	+++	ND[c)]

a) Shape of chitin-sponge was classified into four categories: –: no change in shape, +: chitin-sponge turned into gel ++: slight gel remained, +++: gel was undetectable.
b) PID: Post-implantation day.
c) ND: No data.

The implanted chitin-sponge disappeared completely by PID 14. Furthermore, we have many cases where the chitin-sponge disappeared by 2 to 5 days when used as wound dressing. These phenomena suggest clearly that the degradation of chitin is promoted by contact with blood components, and that chitin is degraded by enzymes.

The diameter of the chitosan fibers at 14 days after subcutaneous implantation in bovine cases decreased to about one half compared to that at 7 days and were not degraded completely within 28 days (Minami *et al.*, 1993). Although chitosan was not degraded in the rumen of ruminants (Yoshino *et al.*, 1991), it was degraded, albeit very slowly, on subcutaneous implantation in cows. In clinical use, chitosan cotton was completely degraded within 7 days when placed on the regenerated granulation tissue of the leg of a cat injured by an automobile. Rapid degradation of chitosan has been experienced in the treatment of purulent lesions (abscesses etc) and trauma. The difference between clinical and experimental implantation is whether the inflammatory reaction has already started or not. The mechanism of chitosan degradation in animal tissue is unknown, but a wound exudate might be an important triggering factor (Minami *et al.*, 1993).

Antibacteria

The protection of the host against bacterial infection was stimulated by chitin (Iida *et al.*, 1987). Although chemically modified chitin such as 30%- and 70%-deacetylated chitin (DAC-30 and DAC-70, respectively) effectively enhanced non-specific host resistance to *E. coli* infection, unmodified chitin did not enhance such resistance and so did not show the same antibacterial function (Nishimura *et al.*, 1984). To assess chitosan's bacteriostatic properties, Allan (Rawls, 1984) tested its effectiveness against five bacterial strains and a common skin fungus. Powdered chitin, chitosan, or whole crab shell were not effective in any of the tests, but solutions of chitosan in acetic acid completely inhibited one of the bacterial strains and the fungus, and partially inhibited growth of two other bacterial strains.

As shown in *Table 11*, 80% deacetylated chitin (DAC-80) inhibited the growth of gram-positive bacteria; *B. subtilis*, *S. aureus* and *S. epidermidis*, and the gram-negative bacteria; *P. aeruginosa, E. coli, K. pneumoniae* and *P. vulgaris* at concentrations of 0.13%–0.5% (w/v) (Tanigawa *et al.*, 1992). DAC-84, DAC-88, and DAC-91 also inhibited the growth of these gram-positive and gram-negative bacteria at concentrations of 0.13%–0.5% (w/v); however, DAC-84 was not effective against *P. aeruginosa* at a concentration of 1.0%. DAC-77, at a concentration of 0.5%, inhibited the growth of *S. aureus, K. pneumoniae* and *P. vulgaris*, but it was not effective against *B. subtilis*, *P. aeruginosa*, *E. coli*, *S. epidermidis* at a concentration of 1.0%. DAC-66, at a concentration of 1.0%, was not effective against any of the bacteria tested.

The growth of almost all of the micro-organisms tested was inhibited by CO-4, as shown in *Table 12* (Tanigawa *et al.*, 1992). No bacterial growth inhibition was observed with monosaccharides such as D-glucosamine hydrochloride (GlcN HCl) and N-acetyl-D-glucosamine (GlcNAc).

To determine whether these chitins have bactericidal or bacteriostatic activity, the growth of *E. coli* was examined after incubation with DAC-80 (Tanigawa *et al.*, 1992). *E. coli* grew well when DAC-80 was removed from the medium within one

Table 11. Inhibition of bacterial growth by partially deacetylated chitins (DAC)[a]

DAC[b]	MW[c]	MIC(%)[d]						
		Bs	Sa	Se	Pa	Ec	Kp	Pv
DAC-66	190,000	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)
DAC-77	190,000	(1.0)	0.5	(1.0)	(1.0)	(1.0)	0.5	0.5
DAC-80	80,000	0.5	0.13	0.13	0.5	0.13	0.13	0.13
DAC-84	190,000	0.25	0.5	0.25	(1.0)	0.5	0.5	0.5
DAC-88	–	0.25	0.25	0.25	0.5	0.25	0.25	0.25
DAC-91	166,000	0.13	0.25	0.13	1.0	0.25	0.13	0.13

[a] Bs: *Bacillus subtilis*, Pa: *Pseudomonas aeruginosa*, Ec: *Escherichia coli*,
Sa: *Staphylococcus aureus*, Se: *Staphylococcus epidermis*,
Kp: *Klebsiella pneumoniae*, Pv: *Proteus vulgaris*.
[b] DAC-66: 66% deacetylated chitin.
[c] Molecular weight determined by GPC.
[d] Parentheses indicate ineffectiveness at a concentration of 1.0%.

Table 12. Inhibition of bacterial growth by chitose oligomers (CO)[a]

CO	MW[b]	MIC(%)[c]						
		Bs	Sa	Se	Pa	Ec	Kp	Pv
CO-1	12,000	(5.0)	(5.0)	5.0	5.0	(5.0)	(5.0)	5.0
CO-2	8,000	(5.0)	(5.0)	(5.0)	5.0	(5.0)	(5.0)	(5.0)
CO-3	4,000	(5.0)	5.0	5.0	5.0	5.0	(5.0)	2.5
CO-4	2,000	1.3	1.3	1.3	5.0	2.5	5.0	5.0
CO-5[d]	4,000	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)
GlcN HCl[e]	215	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	ND[f]	ND
GlcNAc[g]	221	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	ND	ND

[a] Bs: *Bacillus subtilis*, Pa: *Pseudomonas aeruginosa*, Ec: *Escherichia coli*, Sa: *Staphylococcus aureus*, Se: *Staphylococcus epidermis*, Kp: *Klebsiella pneumoniae*, Pv: *Proteus vulgaris*.
[b] Molecular weight determined by GPC.
[c] Parentheses mean that a concentration of 5.0%(w/v) is ineffective.
[d] N-acetyl derivative of CO-3.
[e] D-Glucosamine hydrochloride.
[f] Not done.
[g] N-acetyl-D-glucosamine.

hour, but no bacterium was observed after 48 hr exposure to DAC-80. When *E. coli* cells were incubated with DAC-80 for 3 to 24 h, the growth of the bacteria was greatly delayed, as determined by measuring absorbance at 600 nm. These results indicate that partial killing of *E. coli* had occurred.

DAC with a high degree of deacetylation were more effective than those with a low degree in inhibiting bacterial growth (Tanigawa *et al.*, 1992), suggesting that the number of amino groups in the DAC was correlated with the degree of bacterial growth. There might be an optimum ratio of deacetylation that is related to the inhibition of growth of each bacterium.

Although the molecular weights of the chitose oligomers (CO) were not directly proportional to the inhibition of bacterial growth, CO of low molecular weight (M.W. 2,000–4,000) appeared to be more effective than those of high molecular weight (M.W. 8,000–12,000) (Tanigawa *et al.*, 1992). These differences might be due to (i) the chain length of the CO, and (ii) the content of an essential component(s) for the inhibition of bacterial growth. CO-1~CO-4 inhibited bacterial growth, but an

N-acetylated CO, CO-5 with no free amino group, was inactive. These results suggest that the amino group of the CO may be essential for their inhibitory activity.

The mechanism underlying the inhibition of bacterial growth, is thought to be that the cationically charged amino group in these CO may combine, by electrostatic interaction, with anionic components, such as N-acetylmuramic acid, sialic acid and neuraminic acid, on the cell surface, and may suppress bacterial growth. However, D-glucosamine hydrochloride and chitin monomer (GlcNAc) did not show inhibitory activity (Tanigawa *et al.*, 1992). Although a strict correlation between polysaccharide chain length and inhibition of bacterial growth was not found in this study, the inhibition of growth might be caused by minute differences in the recognition of cell wall receptors for an optimum chitose oligomer chain length.

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