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A REVIEW ON BIOMEDICAL APPLICATIONS OF CHITOSAN-BASED BIOMATERIALS

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ABSTRACT

Chitin/chitosan and their derivatives have attracted considerable interest as a potential source for biomaterials such as hydrogels due to their safety and biological activities, such as, antimicrobial, antitumor and stimulation of wound healing, etc. In particular, some kinds of covalently cross-linked (chemical) chitosan hydrogel such as chemically cross-linked chitosan hydrogel, photocrosslinked chitosan hydrogel (PCH) and ionic crosslinked (physical) chitosan hydrogels such as ionic/temperature sensitive chitosan hydrogel and polyelectrolyte complexes (PECs) composing positive or negative charge have been developed. These have been used in several applications including drug delivery carriers, hemostats, wound dressings, submucosal fluid cushion, tissue adhesive and scaffolds of tissue engineering which we originally evaluated. In this review, we described on chitosan hydrogels with particular attention on medical applications of PCH, hydrocolloids and PECs in fields of Biomedical Research.

KEYWARDS: Cross-linked Chitosan Hydrogel, Polyelectrolyte Complexes, Drug Delivery Carriers, Hematostats, Tissue Adhesive, Wound Dressing.

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INTRODUCTION

Chitin/chitosan can be produced economically from the shells of crustaceans, a waste product of the seafood industry that would otherwise pollute coastal areas. Chitosan comprises co-polymers of *N*-acetyl-glucosamine and *N*-glucosamine units linked by β-(1→4) glycosidic bonds, and can be obtained by alkaline deacetylation of chitin[1,2]. Chitosan is nontoxic and biocompatible with living tissue[3,4]. The production of chitin from shells mainly involves the removal of proteins and the dissolution of calcium carbonate in the shells. The resulting chitin is deacetylated to yield chitosan[1,2]. The term “chitosan” is used to describe polymers comprising less than 50% *N*-acetylglucosamine units[2-4]. The degree of deacetylation (DDAc) affects the solubility, hydrophobicity and electrostatic properties of chitosan, with the latter affecting the polymer’s ability to interact with polyanions through the protonated amino groups. Chitosan can be hydrolyzed by lysozyme and is thus a biodegradable polymer. Chitosan and its degradation products are nontoxic, nonimunogenic and noncarcinogenic[3-6]. Furthermore, chitin/chitosan and their derivatives have attracted considerable interest due to their biological activities, including antimicrobial[7], hypocholesterolemic functions[8], antitumor[9,10] and their stimulation of wound healing[11,12]. The present review is exclusively concerned with chemical chitosan hydrogels formed by addition of a crosslinker[13], namely covalently crosslinked such as photocrosslinked chitosan hydrogels (PCH) formed by addition of a photocrosslinker[14-16]. A second review will describe physically cross-linked chitosan hydrogel such as temperature sensitive chitosan hydrogel[17,18], polyelectrolyte complexes (PECs)[19,20] and hydrocolloid[21,22] formed by direct interaction between polymeric chains without the addition of cross linkers. An entangled chitosan hydrogels which are formed by solubilization of chitosan in an acidic aqueous medium will not be discussed further in this review, as they are limited by their lack of mechanical strength and their tendency to dissolve. In the present review articles, we focus the potential medical applications of photocrosslinked chitosan hydrogel (PCH)[14-16] and chitosan-based biomaterials such as hydrocolloid sheets composed of alginate chitin/chitosan, fucoidan hydrocolloid sheet (ACF-HS)[21,22] and polyelectrolyte complexes (PECs) composing chitosan and protein/gene[19] which we had originally evaluated, as drug delivery carriers, tissue adhesives, submucosal fluid cushion, wound dressing, hematostats, scaffolds for tissue engineering and protein/gene delivery carriers.

Chitosan-based hydrogels

Chitosan hydrogel was defined as macromolecular networks swollen in water or biological fluids. Based on the definition given here, chitosan hydrogels are often divided into two classes, namely chemical hydrogels and physical hydrogels[13,23,24]. Chemical hydrogels are formed by irreversible covalent links, as in covalently crosslinked chitosan hydrogels. On the other hand, physical hydrogels are formed by various reversible links. These can be ionic interactions as in ionically cross-linked and PECs, or

secondary interactions such as alginate/chitosan/fucoidan complexed hydrocolloid sheets (ACF-HS)[21,22]. The present review is exclusively concerned with chitosan hydrogels formed by addition of a crosslinker, namely covalently or ionically crosslinked hydrogel. In cross-linked hydrogels, polymeric chains are interconnected by crosslinkers, leading to the formation of a 3D network. Crosslinkers are molecules of molecular weight (MW) much smaller than the MW of the polymeric chains. The properties of cross-linked hydrogels depend mainly on their crosslinking density, namely the ratio of moles of crosslinking agent to the moles of polymer repeating units[23,24]. Figure 1A shows simplified scheme for temperature sensitive chitosan hydrogel which show sol-gel transition at body temperature due to a conformational change. Since chitosan lucks intrinsic thermosensitive properties, other temperature sensitive materials need to be introduced into the chitosan to make it applicable as a temperature sensitive chitosan hydrogel. For example, temperature sensitive hydrogels composed of chitosan and β-glycerophosphate (GP)[13,18] or polyethylene glycol (PEG)[13] has been prepared and investigated their sol-gel transition in response to thermal and pH changes. Those hydrogels has also been evaluated as carriers for cells and drug-delivery[25,26]. Preparation of a hydrogel containing a covalently cross-linked chitosan hydrogel requires crosslinkers which are molecules with at least two reactive functional groups that allow the formation of bridges between chitosan chains (Figure 1B). Those crosslinkers should have at least two reactive functional groups that allow the formation of bridges between polymeric chains such as glutaraldehyde as dialdehydes[13]. However, even if hydrogels are purified before administration, the presence of free unreacted dialdehydes in hydrogels could not be completely excluded and may induce toxic effects. Figure 1C shows simplified scheme for PCH which form hydrogels under short exposure to visible or ultraviolet (UV) light in the presence of light sensitive compounds (photocrosslinkers)[14-16]

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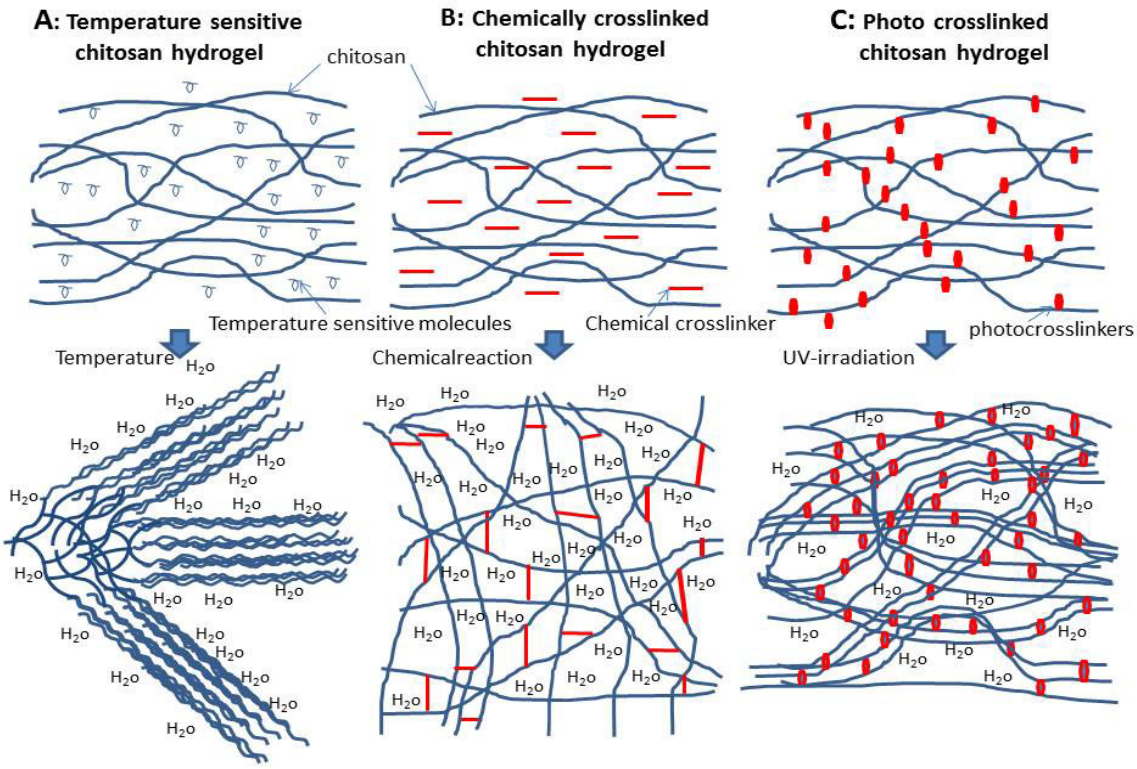

Figure 1

Simplified scheme of gelling mechanism.

A: Thermal gelation due to a change in temperature the polymer molecules rearrange from random coil to helix, then the helices assemble in dusters join together. B: Chemical crossling gelation due to chemical reaction between crosslinkers and polymers. C: Photocrosslinking due to radical reaction between photocrosslinkers and polymers. We previously described a photocrosslinkable chitosan derivative (Az-CH-LA) that contains both lactose moieties (lactobionic acid) and photoreactive azide groups (p-azidebenzoic acid) as photocrosslinker[14-16]. The chitosan used in this study had a molecular weight of 300－500 kDa with 80% deacetylation. Lactose moieties have been introduced through condensation reactions of chitosan with amino groups. Moreover, chitosan containing 2% lactobionic acid exhibited high aqueous solubility, even at neutral pH. Furthermore, application of ultraviolet light (UV) irradiation with a 250-W lamp (major peak, 340 nm; Usio Electrics Co., Ltd., Tokyo, Japan) to Az-CH-LA produced an insoluble_PCH just like soft rubber within 30 seconds and firmly adhered two pieces of ham to each other[14-16]. The Az-CH-LA solution can be injected into body and the hydrogels are then formed by applying UV light externally through skin. Basic molecules such as chitosan and protamine complexed with acidic molecules such as alginate, heparin and fucoidan form complexes through ionic interactions as PECs[13,24,26]. Reported studies indicate that polyanions and polycations can bind to proteins below and above their isoelectric points, respectively. These interactions can result in nanoparticles, hydrogels, soluble complexes and/or the formation of

amorphous precipitates (Figure 2). Main aspects studied by different authors are compositions of PECs obtained under various experimental conditions, such as the strength and position of ionic sites, charge density and rigidity of polymer chains as well as chemical properties such as solubility, pH, temperature and concentration[13,26]. Electrostatic interactions are also important because of their similarity to biological interactions. Interactions between proteins and nucleic acids, for example, play a role in the transcription process[27]. DNA/chitosan PECs[28], chitosan/chondroitin sulfate PECs and chitosan/hyaluronate PECs function as gene[29] and drug carriers[29]. Moreover, PECs that are insoluble also have potential applications as membranes, microcapsules, micro/nanoparticles and scaffolds for tissue engineering[29].

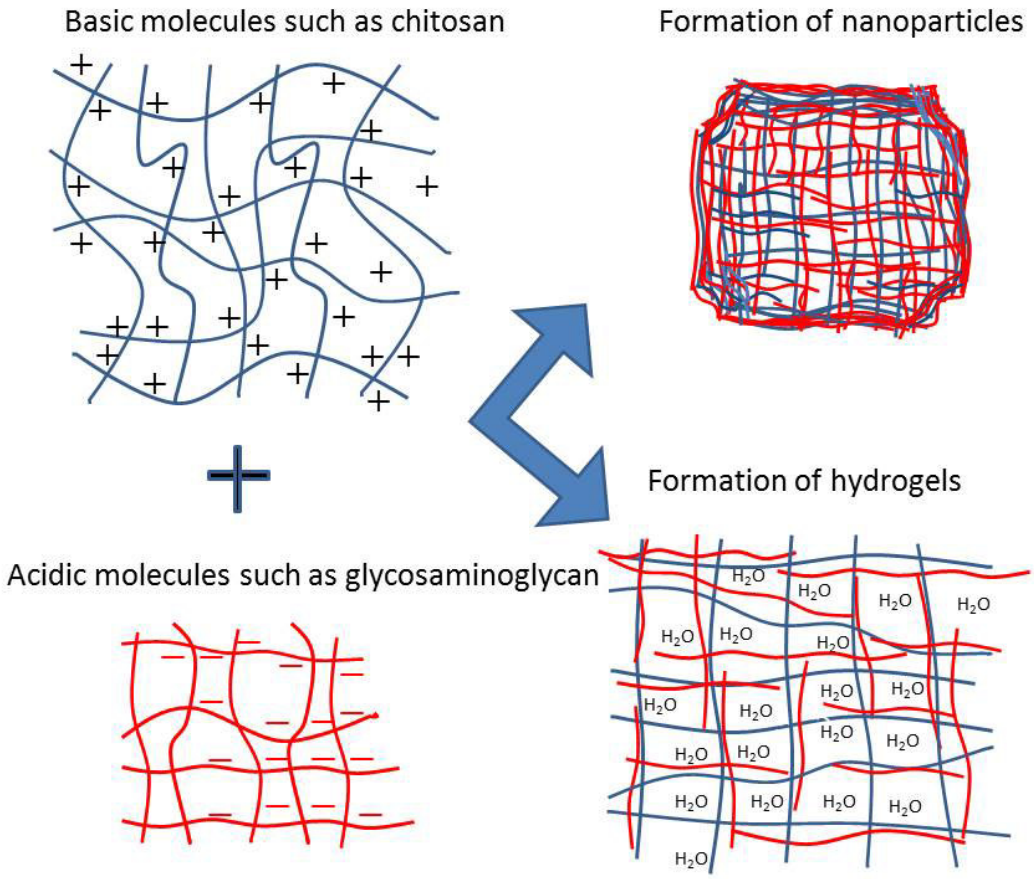

Figure 2

Formations of PECs such as nanoparticles and hydrogels.

Biological adhesives, hemostats and submucosal fluid cushion

Biological adhesive are used for tissue adhesive, hemostasis and sealing of the leakage of air and body fluids during surgical procedure. Although most bleeding in surgical procedures can be controlled by appropriate sutures, hemostasis is uncontrollable under certain conditions, such as coagulopathy, medication of anticoagulants, inflammation, infection and severe adhesion[14,15]. In addition, intractable air leakage in lung surgery has often been found, especially in emphysematous lung

disease[15]. In many cases of such uncontrollable bleedings and intractable air leakages, a number of adhesives have been utilized in hemostasis and air sealing, i.e. chemically crosslinkable gelatins[30], cyanoacrylate polymers[31,32] and fibrin glues[33,34]. Requirements for such adhesives are locally non-irritating, systematically nontoxic, appropriately flexible and biodegradable. However, cytotoxicity and severe tissue irritability have been found when using resorcinol, formaldehyde or carbodiimides for the crosslink-reaction of gelatins[30] or due to the formation of formaldehyde by degradation of cyanoacrylate[31,32]. Fibrin glue, which contains fibrinogen, thrombin, factor XIII and a protease inhibitor, utilizes the blood coagulation system for sealing tissues and currently is the most widely used surgical adhesive[33,34]. However, fibrin glue has a disadvantage in its industrial production, since human blood is used as its source. On the other hand, curable chitosan-poly (ethylene glycol)-tyramine hydrogels[35], catechol-functionalized chitosan/pluronic hydrogels[36] and PCH[14,15] were reported as chitosan-based hydrogels for tissue adhesive. The binding and sealing strengths of the PCH prepared from 2 w% Az-CH-LA solution was superior to that of fibrin glue (Beriplast P)[14,15]. A tracheal tube was inserted into the dead pig and connected to a mechanical ventilator. The lung was then punctured with a needle (1.2 mm in diameter) about 10 mm deep. One drop (about 30 µL) of 30 mg/mL of Az-CH-LA solution was applied to the puncture site and irradiated with UV light at a distance of 2 cm for 30 seconds. Subsequently, ventilation was started through a linear pulsed-air volume increase. The pressure at which air leakage reoccurred was measured and termed the “bursting pressure” of the PCH (millimeter of mercury)[14,15]. Beriplast P was also examined as a control and was measured for the bursting pressure occurred 5 minutes after application of the fibrin glue. On the other hand, one end of small intestine, trachea and thoracic aorta removed from the dead pigs was ligated with suture material, and the other side was intubated with a small catheter held in place by ligature. The catheter was connected to a syringe and a manometer. The issues were punctured with the needle, and about 30 µL of the Az-CH-LA solution was applied to the hole and irradiated with UV light for 30 seconds. The tissues were placed under water, and they were inflated until leakage bubbles could be detected in the water. The pressure required to produce this air leakage was measured as the bursting pressure. Similar experiments have been performed with the fibrin glue, with the bursting pressure measurements starting 5 minutes after application[14,15]. Results of above experiments were shown in Table 1[15]. The bursting pressures of PCH were more than that of the fibrin glue on lung, small intestine, trachea and thoracic aorta. These results suggest that the sealing strength of PCH may be sufficient to stop arterial bleeding and air leakage from the lung or trachea in surgical applications.

Table 1

Air-sealing strength of chitosan hydrogel and fibrin glue[15]

Sealing strength (mmHg) Organs (TC)	Chitosan hydrogel (PCH)	Fibrin glue
Lung	51 ± 11 (n = 4)	12 ± 2 (n = 4)
Small intestine	65 ± 5 (n = 6)	48 ± 7 (n = 6)
Trachea	77 ± 29 (n = 6)	44 ± 16 (n = 6)
Thoracic aorta	225 ± 25 (n = 6)	65 ± 15 (n = 6)

We examined the hemostatic efficacy of photocrosslinkable chitosan hydrogel-mixed photocrosslinked chitosan sponges (PCM-S) (Figure 3) after hepatic injury of rats[37]. The left lobe of the liver was penetrated with a dermal punch to produce a penetrating wound in heparinized rats. Treated rats either had PCM-S applied into the wound and then were immediately UV-irradiated, or they had TachoComb® (TC) inserted into the wound[38]. The study demonstrated that PCM-S effectively controlled hemorrhage after liver trauma in the heparinized rats. All heparinized rats in PCM-S-treated groups achieved complete hemostasis within 5 minutes and all survived. In contrast, the control heparinized rats could not stop the bleeding for more than 3 hours and all rats were died within 6 hours. TC had an intermediate effectiveness, with bleeding lasts longer than 20 minutes, resulting in three deaths of three of the eight study rats during the first 24 hours. No adverse events related to the use of the hemostatic agents (PCM-S and TC) were detected through two months in both non-heparinized and heparinized rats[37]. Furthermore, A novel emergency hemostatic kit was developed for severe hemorrhage using PCH[39].

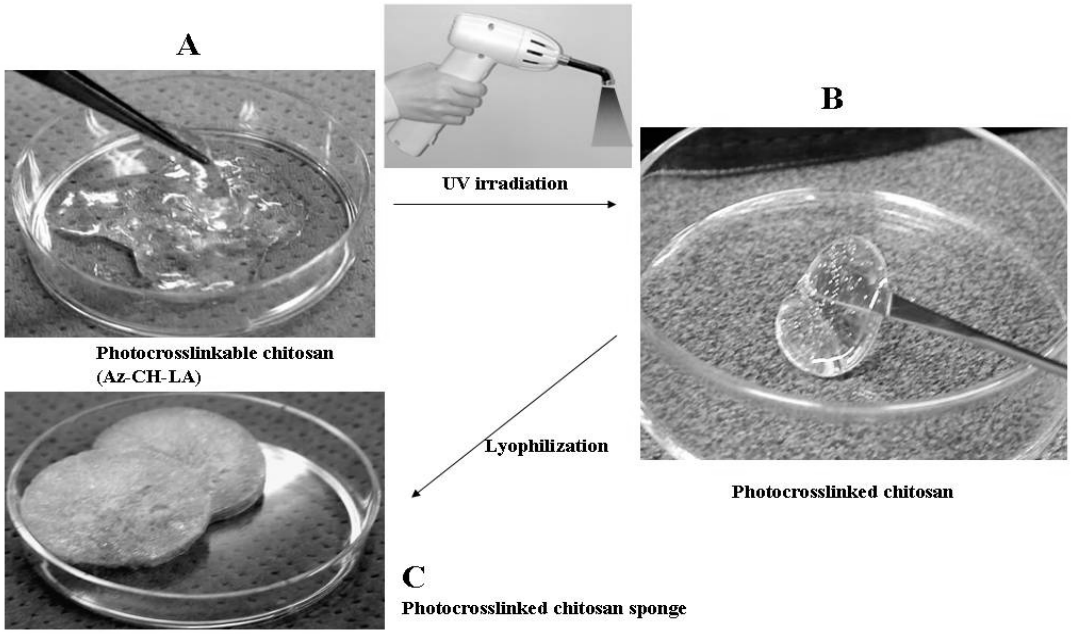

Figure 3
Formation of photocrosslinkable chitosan (A)-mixed photocrosslinked chitosan sponge (C) (PCM-S). Az-CH-LA is converted to photocrosslinked chitosan hydrogel (B) with UV irradiation.

Formation of a submucosal fluid cushion (SFC) has become integral to endoscopic endoscopic submucosal dissection (ESD) as well as endoscopic mucosal resection EMR of large superficial lesions of the gastrointestinal tract[40]. We also investigated the use of PCH as SFC[41-43]. A disadvantage of PCH-assisted ESD is to require UV irradiation using an expensive UV fiber for ESD, which may be associated with minor inflammation in residual tissues[42,43]. Furthermore, it cannot be ruled out that PCH-assisted ESD may have an association with carcinogenesis. Because homogenous UV irradiation using a simple UV lamp and fiber is technically difficult, further studies are necessary to determine the requirement and safety of UV irradiation[42,43]. Since those biomaterials as SFC were hard to inject because of their high viscosity, an application of a targeted high-pressure water jet may be required to ameliorate the endoscopic treatment of mucosal lesion[44]. The application of an ideal injectable hydrogel as SFC among those hydrogel described in this review could contribute to ameliorate the endoscopic treatment which previously could not be resected endoscopically due to their size, extent or location.

Chitosan in regenerative medicine

Regenerative medicine, one of the hottest fields in present and future life science, finally aims at the restoration or replacement of lost or damaged organ or body part with transplantation of new tissues in combination with supportive scaffolds and biomolecules. Recently, functional biomaterial research has been directed toward the development of improved scaffolds for tissue engineering[3,4,23], wound dressing[12,16,45] and drug delivery carrier[5,6]. In this regard, increasing attention has been given to chitosan and its derivatives. Chitosan and its derivatives are undisputed

biomolecules of great potential by their polyelectrolyte properties, including the presence of reactive functional groups, gel-foaming ability, high adsorption capacity, biodegradability, bacteriostatic, fungistatic and even anti-tumor influence[4,7,10]. Several requirements have been identified as crucial for the production of tissue engineering scaffolds: (1) the scaffold should be made from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the scaffold, (2) possess interconnecting pores of appropriate scale to favor tissue integration and vascularization, (3) have appropriate surface chemistry to favor cellular attachment, differentiation and proliferation, (4) possess adequate mechanical properties to match the intended site of implantation and handling, (5) should not induce any adverse response and (6) be easily fabricated into a variety of shapes and size[46,47]. The versatility of chitosan and its derivatives offer a wide range of applications since they are biodegradable and nontoxic, and can be formulated in a variety of forms including powders, gels, membranes, sponges and films for their applications. They can also provide controlled release of growth factors and extracellular matrix components. However, unfortunately chitosan alone cannot meet the long-term mechanical, geometrical, functional and cell adherent requirements[46,47]. To improve the adherent ability for seeding cells, the chitosan allow for wide range of molecules to be modified. The incorporation of collagen or biologically active RGD-containing protein peptides to chitosan as a chitosan-collagen scaffold can enhance its cell attachment ability[46,47]. Table 2 summarized on applications and benefits of chitosan in regenerative medicine including wound healing, tissue engineering and drug delivery.

Table 2
Application and benefits of chitosan in regenerative medicine.

Applications	Benefits
Wound healing	· Chemoattract macrophages and neutrophils to initiate the healing process. · Stimulate vascularization and granulation tissue formations. · Carry cytokines to accelerate the wound healing. · Preventions of scar formation and retraction.
Tissue engineering	· Intrinsic antimicrobial activity and controlled releasing of exogenous antimicrobial agents. · Non-toxic and biodegradable. · Easy to develop various forms. · Retain and controlled release cytokines, extracellular matrix, and antimicrobial agents. · Promote attachment, proliferation, and viability of various cells including mesenchymal stem cells. · Modification with various biomaterials to improve the adherent ability.
Drug delivery	· Non-toxic, biodegradable, and biocompatible with high cationic charge potential. · Produce micro (nano) particles with polyelectrolyte complex methods. · Protect DNA or functional protein from degradation by enzymes. · Retain and controlled release active agents. · Yield high transfection efficiency.

There are many other synthetic materials which can react biocompatibly with the body. Among these materials, polylactide (PLA), polyglycolide (PGA) and polylactide-co-glicolide (PLGA) etc. have received much attention because of their biodegradability and biocompatibility. Conjugation of chitosan with those synthetic materials is expected to become a key and potential technology to develop desirable scaffold materials for the tissue regenerations[48]. Stem cells with self-renewal potential and multilineage differentiation capacity have been in tissue engineering[49]. Bone marrow- or adipose tissue-derived mesenchymal stem cells have been extensively studied and have shown promising application implication[50]. Furthermore, recent studies show that chitosan has good characteristics for the attachment, proliferation and viability of mesenchymal stem cells[50]. With these promising features, they are considered as an interesting biomaterial for use in cell transplantation and tissue regeneration, and the technology for chitosan has been used to create various tissue analogs including cartilage[51], bone[52], skin[53], myocardium[54] and peripheral nerve[55] in the past decades.

Chitosan in wound healing

Chitosan possesses the characteristics favorable for promoting rapid dermal regeneration and accelerated wound healing. It is observed that chitosan has a stimulatory effect on macrophages and that it was found to act as chemoattractant for neutrophils both *in vitro* and *in vivo*, an early event essential in wound healing[53]. These cells kill microorganisms, remove dead cells and stimulate the other immune system cells, which improve overall healing by reducing the opportunity for infection[56]. The application of chitosan and its derivatives as a wound dressing has been widely studied. In a comparative study of insoluble chitin powder, insoluble chitosan powder and water-soluble chitin/chitosan (WSC) solution, WSC solution was found to have the highest tensile strength with the fastest healing rate[57]. It is likely that the superior biodegradability and hydrophilicity of WSC solution can enhance its compatibility with wounded tissues and increase its activity as a wound-healing accelerator[57]. To improve the healing process, chitosan has been combined with a variety of functional molecules such as growth factors, extracellular matrix components and antibacterial agents. The other advantages include healing of wounded meniscal tissues, and of decubitus ulcers, depression of capsule formation around prostheses, limitation of scar formation and retraction during healing[57]. We have previously reported that the application of PCH into open wounds induces a significant wound contraction, thereby accelerating the wound closure and healing process, as shown in a normal mouse[45] or rat[12] model for wound repair. In addition, the PCH showed the ability to control release of various growth factors, to serv as a novel carrier and to induce neovascularization *in vivo*. FGF-2 interacted with PCH and the FGF-2 molecules incorporated into the PCH were gradually released upon *in vivo* biodegradation of the hydrogel itself[53]. We also evaluated the effect of FGF-2-incorporated PCH on the wound healing process using healing impaired diabetic *db/db* mice (Figure 4)[59]. Our main conclusions were that FGF-2-incorporated PCH show a substantial effect to induce vascularization and granulation tissue formation and improve wound healing in the *db/db* mice[59].

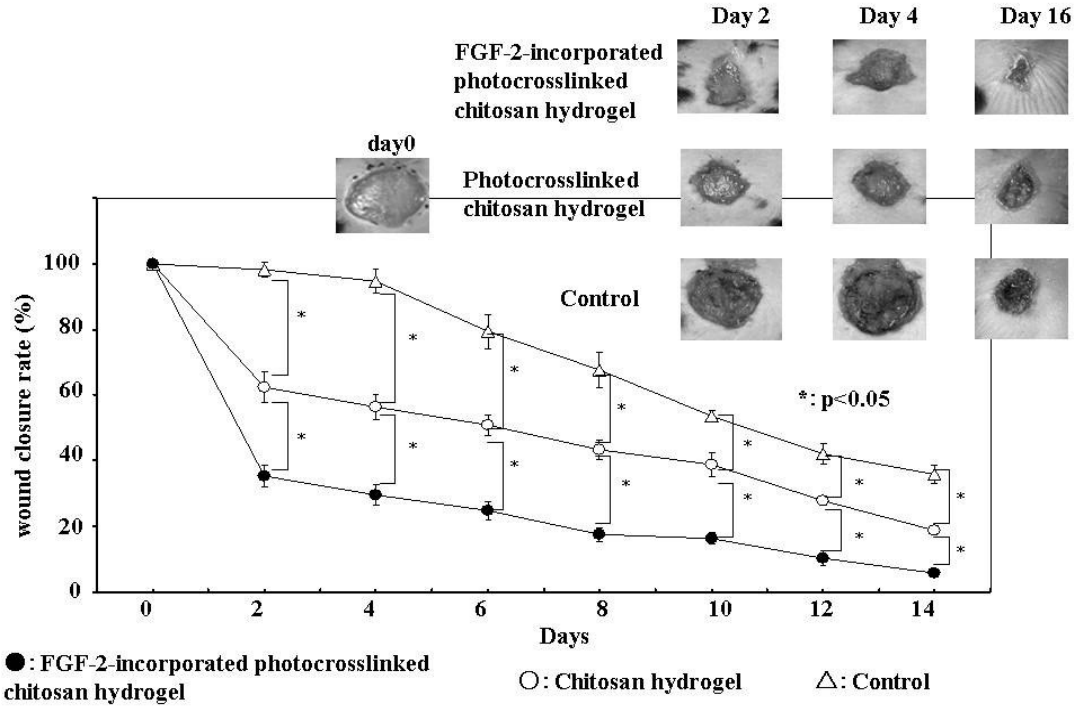

Figure 4
Enhanced wound-healing in FGF-2-incorporated PCH-treated db/db mice.

To create a moist environment for wound healing, alginate/chitosan/fucoidan complexed hydrocolloids (ACF-HS) has been developed as a functional wound dressing[21,22]. ACF-HS gradually adsorbed fluid without any maceration and the fluid adsorbance *in vitro* reached constant during 18 hours. Round full-thickness skin defects were made on the back of *db/db* mice to prepare healing-impaired wounds[22]. Application of ACF-HS could be expected that it effectively interact with and protect wound in rats, providing a good moist healing environment with exudate. Besides those, the wound dressing could have other properties like ease of application and removal, and proper adherence. After applying ACF-HS to the wounds, the mice were later killed and histological sections of the wound were prepared. The histological examinations have demonstrated significantly advanced granulation tissue and capillary formations in the wounds treated with ACF-HS on day 4, day 9 and day 14, in comparison with that in commercially available hydrocolloid wound dressing (ABSOCURE-surgical; Nitto Medical Corp., Osaka, Japan) as a positive control and non-treatment (negative control)[21,22].

Chitosan-based peptide/protein/gene delivery systems

The design of appropriate carriers for an administration of hydrophilic macromolecular drug such as proteins and peptides has been a major goal of pharmaceutical research. Protein and peptide drugs are important to treat diseases with increasing prevalence in the population such as osteoporosis and diabetes. On the other hand, vaccination

(antigenic peptides) is also important and is still a dire problem[5]. In general, drug delivery materials can support via various routes, like nasal, ocular, oral, parenteral and transdermal. Particularly, protein and peptide delivery by mucosal or oral routes would be highly desirable from a clinical and industrial perspective, and could lead to substantial advances in the development and application of proteins and peptides[20,60,61]. A series of nanocarrier systems in which proteins and peptides are associated with a chitosan based nanostructure can be formed as colloidal PECs[1,5,6]. PECs composed of chitosan or its derivatives and proteins were produced by mixing a protein solution with chitosan solution. Hence, PEC comprised of chitosan derivatives and insulin was synthesized via electrostatic interactions[62]. A second approach toward protein/nanoparticles associations was to make the colloidal PECs first and then to adsorb the proteins, as reported by various authors[63]. Since Mumper et al.[64] pioneered to apply chitosan to gene delivery systems, a lot of efforts have been made to explore the potential of chitosan and its derivatives as a non-viral vector[65-67]. DNA/chitosan complexes are prepared in acidic or neutral aqueous solution where chitosan is highly or partially ionized, respectively[65-67]. In addition to solution pH, the DDAc and molecular weight of chitosan influence the physicochemical and biological properties of chitosans and the transfection efficiencies of DNA/chitosan complexes[65-67]. The use of chitosan with more than 80% of DDAc might accelerate chitosan degradation and DNA release, since highly acetylated chitosan (less than 20% of DDAc) release DNA very slowly. Lavertu et al.[66] studied several combinations of various molecular weight and DDAc value of chitosan, and they selected two combinations of high transfection efficiency using a chitosan of 10 kDa and DDAc of 8 and 20%. The coupling between the DDAc and the molecular weight of chitosan suggests that an optimal binding strength of chitosan to DNA is required for maximum transgene expression, namely, it should be strong enough to condense and protect DNA, but weak enough to permit intracellular disassembly.

CONCLUSION

Chitin, a natural polymer of *N*-acetylglucosamine, is the second-most abundant polysaccharide in the nature after cellulose, and is derived in the exoskeletons of crustacean or shrimp, the cuticles of insects and cell walls of fungi. Chitosan comprising *N*-acetylglucosamine and glucosamine can be obtained by alkaline deacetylation of chitin and is found to be nontoxic and biocompatible with living tissue. Since chitosan can be hydrolyzed by lysozyme, it is one of the biodegradable polymers, and chitosan and the degraded products are nontoxic, nonimunogenic and noncarcinogenic. Chitosan hydrogel has attracted considerable interest due to their biological activities, that is, antimicrobiral, antitumor, hypocholesrolemic functions and stimulatory effect on wound healing. Furthermore, there are enough scientific evidences for the potentiality of chitosan hydrogels in many medical applications such as drug delivery carriers, tissue adhesives, wound dressing, hematostats, scaffolds for tissue engineering and protein/gene delivery carriers.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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