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Chitosan Chemistry and Pharmaceutical Perspectives

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1. Introduction

In the chitin bibliography, a major reference is the book written by Richards in 1951 "primarily for entomologists and other invertebrate zoologists" but also, in the author's hope, for chemists: the perception of the need for more chemical information is manifest in the preface of the book.[1] In fact, the chitin chemistry developed to that date was inadequate: for instance, color tests were not specific enough to provide a reliable picture of the distribution and location of chitin in living organisms.

Specific and accurate techniques for chitin identification became accessible in 1963 with the improvement of the X-ray diffraction methods by Rudall[2] and the enzymatic method of Jeuniaux.[3] The book edited by Hepburn,[4] starting with a biographical chapter honoring A. G. Richards followed by chapters on biochemical modifications of chitin by Rudall and Muzzarelli, remains a remarkable contribution.

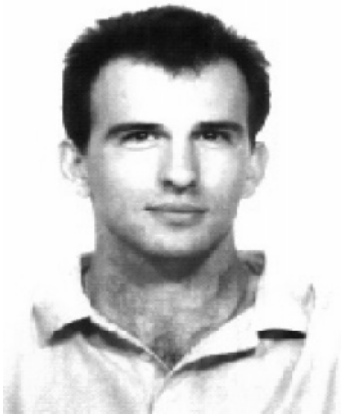
The books by Hepburn (1976)[4] and Neville (1975)[5] were immediately followed by the first book on chitin authored by Muzzarelli (1977)[6] and, same year, by the 1st International Conference on Chitin and Chitosan (proceedings published by Muzzarelli and Pariser, 1978).[7]

Prior to these events, interest in chitin was cultivated mainly by zoologists, marine entomologists, and physiologists, but in the late 1970s, chemists all over the world devoted attention to chitin. In particular, it was immediately realized that chitin was an abundant source of chitosan, the unique cationic polysaccharide (as opposed to a variety of easily accessible anionic polysaccharides), and, as such, was superior to man-made cationic derivatives of cellulose and starch. The inherent biodegradability of chitin and chitosan was interpreted as an appealing characteristic property rather than a drawback (at a time when artificial polymers such as nylon were celebrated for their inertness).

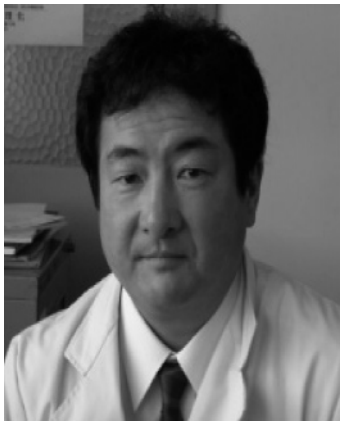
The development of chitin science in the last quarter of the century followed periods dominated by specific topics that can be roughly related to (i) technological advances (spinning, coloring, uptake of soluble species, cosmetic functional ingredients); (ii) biochemical significance (blood coagulation, wound healing, bone regeneration, immunoadjuvant activity); (iii) inhibition of biosynthesis (insecticides); (iv) chitin enzymology (isolation and characterization of chitinases, their molecular biology, biosynthesis, hydrolases with unspecific chitinolytic activity); (v) combinations of chitosan with natural and synthetic polymers (grafting, polyelectrolyte complexation; blends, coatings; (vi) use of chitosan as a dietary supplement and food preservative (anticholesterolemic dietary products, antimicrobial coatings for grains and exotic fruits). Each of these topics had a "high season" that produced a burst of publications; as a whole, a deeper knowledge on chitosan was obtained, apart from fruitful integration of interdisciplinary interests.

Majeti N. V. Ravi Kumar was born in 1972. He received his Ph.D. degree in 2000 from the Indian Institute of Technology Roorkee (formerly the University of Roorkee), India, on chitosan controlled drug-release formulations. He was associated with Professor Ramesh Gupta's Carcinogenesis and Toxicology Research Group as a postdoctoral scholar (2000–2002) at the University of Kentucky Medical Center, Lexington, KY. He was a recipient of the Alexander von Humboldt Research Fellowship (2002–2003) and worked with Professor Claus-Michael Lehr at the Department of Biopharmaceutics & Pharmaceutical Technology, Saarland University, Germany. Currently, he is Assistant Professor of Pharmaceutics at National Institute of Pharmaceutical Education and Research (NIPER), India. He has about 75 publications to his credit that include research papers, reviews, conference proceedings,and course materials. He is a member of the Royal Society of Chemistry (London), Controlled Release Society (USA), and many other scientific societies. He is on the editorial board of the *Journal of Biomedical Nanotechnology*. His current research interests are in biomaterials, drug delivery, nanoscience, and biosensitive hydrogels for protein and peptide delivery.

Riccardo Muzzarelli was born in 1937. He is Professor of Enzymology at the Institute of Biochemistry, Polytechnic University of Ancona, Italy, Doctor of Chemistry, Specialist of Radioisotope Techniques. He organized the first International Conference on Chitin and Chitosan, Boston, MA, 1977. He was Chairman and organizer of a series of Symposia at Senigallia, Italy, on Chitin Chemistry and Chitin Enzymology (1985, 1993, 1996, and 2001). He is a member of the scientific board of the international journal *Carbohydrate Polymers* (1985 to present). He is also a member of the Italian Chemical Society, Italian Society for Macromolecules, and Italian Biochemical Society. He has authored over 310 scientific articles, mainly on chitin and chitosan, and is author or editor of 14 scientific books. His famous books are *Chitin* (1977), *Chitin in Nature and Technology* (1986), *Chitin and Chitinases* (1986), and *Chitin Enzymology* (2001).



Corrado Muzzarelli was born in 1970. He is affiliated to the Institute of Biochemistry, Polytechnic University of Ancona, Italy. He has obtained his Doctor of Biological Sciences degree, with majors in marine biology, and is presently preparing to receive his Ph.D. degree in Applied Biochemistry at the University of Siena, Italy (to be completed 2004). He is a recipient of the "Braconnot" prize from the European Chitin Society, 1999. He is a member of the Italian Society for Macromolecules and Italian Biochemical Society. He is co-author of 27 publications in international journals and is co-editor of the book *Chitosan in Pharmacy and Chemistry* (2002).



Hitoshi Sashiwa was born in Osaka, Japan, in 1963. He received his Ph.D. degree from Hokkaido University (Japan) under the supervision of Professor S. Tokura in 1991. He worked at Tottori University (Japan) as Assistant Associated Professor from 1988 to 2000. He worked with Profesor R. Roy at the University of Ottawa (Canada) for 2 years (1998–2000). He worked at AIST Kansai (Japan) as a postdoctoral scholar during 2000–2004. He has been affiliated with Kaneka Co., Ltd. (Japan) since April 2004. His research interests include chemical modification of chitin and chitosan and their biomedical applications. He is a member of The Society of Polymer Science, Japan, and the Japanese Society for Chitin and Chitosan. He is the sole author of 45 publications and co-author of 30 publications.

Abraham J. Domb was born in 1953. He received his Ph.D. degree in Polymer Chemistry and B. Pharm degree in 1984 from the Hebrew University of Jerusalem, Israel. He spent 1 year as a postdoctoral fellow at Syntex Research in California and 2 years at MIT/Harvard (with Bob Langer and J. Folkman) as a research associate. From 1988 to 1992 he founded and headed the Drug Delivery and Polymer division at Nova Pharmaceuticals in Baltimore. Since 1992 he has been Professor for Medicinal Chemistry and Natural Products at the School of Pharmacy, The Hebrew University. He has published over 150 peer-reviewed articles, 42 book chapters, 2 books, and 4 special issues as well as about 70 U.S. patents (50 Issued). His research interests are in biopolymers, gene therapy, controlled drug delivery, cancer therapy, biodegradable polymers, hydrogels, coating of medical devices, nanoparticulate systems, and polymer complexes.

Today, drug delivery seems to be the topic of interest with a better understanding of the basics in chitin and chitosan chemistry, mainly chemical modifications, biodegradation, effects on various tissues, distribution to various body organs, mucoadhesion, association of chitosan with inorganic compounds, and advanced technological transformations.

The key considerations that justify this interest are that chitosan is biocompatible and does not elicit adverse reactions when in contact with human cells. Chitosan can be degraded by ubiquitous enzymes in the human body, and oligomers can activate macrophages and stimulate synthesis of hyaluronan. Moreover, they provide building blocks for the reconstruction of extracellular matrix components. On the other hand, chitosan is recognized by tumor cells, and therefore, it can bring drugs to their target selectively.

Chitosan is a safe and friendly substance for the human organism; therefore, medical and pharmaceutical applications can easily be worked out with joint efforts from specialists in various fields.

The present review intends to provide interdisciplinary insight in the scientific knowledge immediately usable to realize the potential of chitosan in the pharmaceutical field.

2. General Aspects of Chitin and Chitosan

Chitin and chitosan are polysaccharides that support numerous living organisms.[8,9] They are clearly described not only in encyclopedias, handbooks, monographs, and articles, but also in the American Standard Testing Materials (ASTM) standard guides and in the Pharmacopoeias of various countries.[10]

Most commonly, chitin means the skeletal material of invertebrates. At least 10 Gtons (1×10^{13} Kg) of chitin are constantly present in the biosphere.[11] α-Chitin occurs in the calyces of hydrozoa, the egg shells of nematodes and rotifers, the radulae of mollusks, and the cuticles of arthropods, while β-chitin is part of the shells of brachiopods and mollusks, the cuttlefish bone, the squid pen, and the pogonophora tubes. Chitin is found in exoskeletons, peritrophic membranes, and cocoons of insects. In the fungal walls, chitin varies in crystallinity, degree of covalent bonding to other wall components, and degree of acetylation.[12,13]

All these organisms synthesize chitin according to a common pathway that ends with the polymerization of *N*-acetylglucosamine from the activated pre-

cursor UDP-GlcNAc. The regulation, biochemistry, and genetics of chitin synthases are well known.

The synthetic pathway includes the action of chitin synthases that accept substrate UDP−*N*-acetylglucosamine and feed nascent chitin into the extracellular matrix. In crustacea, the Golgi apparatus is directly concerned with the synthesis and secretion of chitin.[14] In this process the nitrogen comes from glutamine. The equation for the chitin synthesis reaction is

$$\text{UDP}-\text{GlcNAc} + (\text{GlcNAc})_n \rightarrow (\text{GlcNAc})_{n+1} + \text{UDP}$$

Fungal chitin synthases are found as integral proteins of the plasma membrane and in chitosomes; a divalent cation, Mg(II), is necessary for enzyme activity, but neither primers nor a lipid intermediate are required. The enzyme is allosteric; the substrate and free GlcNAc activate the enzyme. The byproduct of the enzymatic activity, UDP, is strongly inhibitory to chitin synthase; however, it may be metabolized readily to UMP by a diphosphatase.[15]

The chitin is modified to impart the structure required by the functions of each particular tissue, via crystallization, deacetylation, cross-linking to other biopolymers, and quinone tanning. Very complex structures result, endowed with high mechanical and chemical resistance and capable of exceptional performances.

In a living organism, chitin production is finely tuned with chitin resorption to permit morphogenesis and growth: delicate equilibria therefore exist between various enzymatic systems with opposite activities.

2.1. Lipo-Chitooligomers

Rhizobia are nitrogen-fixing bacteria that promote the deformation of the root hairs of leguminous plants. The bacteria then invade the roots by means of the infection thread, a newly formed tube, and induce formation of the nodule, a specialized organ where they multiply and reduce atmospheric nitrogen to ammonia that is utilized by the plant. The symbiosis between plants and the rhizobia is specific.[16]

The lipo-chitooligomers consist of an oligosaccharide backbone of β-1,4-linked N-acetyl-D-glucosamine carrying a fatty acyl group on the nitrogen atom of the nonreducing end unit. Rhizobia produce complex mixtures of lipo-chitooligomer species: the number of units varies between 3 and 5. The fatty acyl moieties seem to reflect the composition of the fatty acyl pool present as components of the phospholipids. Special fatty acyl moieties can be present.[17,18]

The addition of lipo-chitooligomers to carrot cell line in arrested embryonic development shows that lipo-chitooligomers have a role in normal plant development, beyond legumes.[19,20] Chitin-like compounds might even play a role in vertebrate embryogenesis.[21] The frog, zebrafish, and mouse DG42 protein in vitro function as chitooligomer synthase,[22] which appears to be inhibited by polyoxin D and nikkomycin.[23]

A method to synthesize lipo-chitooligomers in vitro was based on the chemical modification of partially deacetylated chitin tetramers, i.e., on amide bond formation upon reaction of the amino group with the fatty acid anhydride in organic media at 37 °C.[24]

2.2. Chitins

In the areas of fisheries, textiles, food, and ecology, academia and industry researchers were prompted to upgrade chitin in order to exploit a renewable resource and alleviate waste problems. Today chitin and chitosan from different animal sources are commercially available.

The shells of crabs, shrimps, prawns, and lobsters coming from the peeling machines in canning factories are used for the industrial preparation of chitin. The isolation includes two steps: demineralization with HCl and deproteination with aqueous NaOH. Lipids and pigments may also be extracted. These operations are mainly empiric and vary with the differently mineralized shells, seasons, and presence of different crustaceans in the catch.

Isolated chitin is a highly ordered copolymer of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose. As a point of difference from other abundant polysaccharides, chitin contains nitrogen.[6] Chitobiose, *O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1−4)-2-amino-2-deoxy-D-glucose, is the structural unit of native chitin.[25] Bound water is also a part of the structure.

The molecular order becomes macroscopically evident when microfibrillar fragments of purified crustacean chitins are prepared in 3 M HCl at 104 °C: after removal of the acid, sonication yields colloidal suspensions that self-assemble spontaneously in a chiral nematic liquid crystalline phase and reproduce the helicoidal organization that characterize the cuticles.[26] The polymorphic forms of chitin differ in the packing and polarities of adjacent chains in successive sheets; in the β-form, all chains are aligned in a parallel manner, which is not the case in α-chitin. The molecular order of chitin depends on the physiological role and tissue characteristics. The grasping spines of *Sagitta* are made of pure α-chitin, because they should be suitably hard to hold a prey, while the centric diatom *Thalassiosira* contains pure β-chitin. According to Noishiki et al.,[27] β-chitin can be converted to α-chitin by treatment with 20% NaOH followed by washing with water.

The solubility of chitin is remarkably poorer than that of cellulose, because of the high crystallinity of chitin, supported by hydrogen bonds mainly through the acetamido group. Dimethylacetamide containing 5−9% LiCl (DMAc/LiCl) and *N*-methyl-2-pyrrolidinone/LiCl are systems where chitin can be dissolved up to 5%. The main chain of chitin is rigid at room temperature, so that mesomorphic properties may be expected at a sufficiently high concentration of polymer.[28,29]

2.3. Chitosans

Chitosan indicates a continuum of progressively deacetylated chitins. At the elemental analysis, chi-

tosans have nitrogen content higher than 7% and degree of acetylation lower than 0.40. Removal of the acetyl group is a harsh treatment usually performed with concentrated NaOH solution (either aqueous or alcoholic). Protection from oxygen, with a nitrogen purge or by addition of sodium borohydride to the alkali solution, is necessary in order to avoid undesirable reactions such as depolymerization and generation of reactive species. The excess amount of NaOH represents however an economic and ecological worry; therefore, alternatives are being sought in order to keep the NaOH to a minimum: for instance, chitin is mixed with NaOH powder (weight ratio 1:5) by extrusion at 180 °C, and highly deacetylated and soluble chitosan is obtained with just one-half of the NaOH needed for aqueous system.[30]

The presence of a prevailing number of 2-amino-2-deoxyglucose units in a chitosan permits bringing the polymer into solution by salt formation. Chitosan is a primary aliphatic amine that can be protonated by selected acids, the p*K* of the chitosan amine being 6.3. The following salts, among others, are water soluble: formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate, glycolate, and ascorbate.

Therefore, chitosan is not a cellulose-like polysaccharide considering the presence of four elements in its formula, its cationicity, and the consequent capacity to form poyelectrolyte complexes and nitrogen derivatives, according to the chemistry of the primary amino group. The film-forming ability of chitosan is another important aspect that cannot be found with cellulose. This shows that chitosan is not intractable: for instance, chitosans in water are soluble when hydrogen-bond formation is prevented by partial random re-acetylation of the amino groups, or insertion of *N*-acetylglucosamine or lactobionic acid side chains, or glycosylation at C6 via oxazoline derivatives; recent examples of chitosans bearing mono- or disaccharide side chains are available.[31,32]

Despite the alteration due to deacetylation, chitosan from crab tendon possesses a crystal structure showing an orthorhombic unit cell with dimensions $a = 0.828$, $b = 0.862$, and $c = 1.043$ nm (fiber axis). The unit cell comprises four glucosamine units; two chains pass through the unit cell with an antiparallel packing arrangement. Main hydrogen bonds are O3···O5 (intramolecular) and N2···O6 (intermolecular).[25] The crystal structures of salts and derivatives have also been determined, for instance, for chitosan ascorbate and salicylate among others. The structural unit is represented in Figure 1.

The quality of chitosan can be assessed according to various methods, some of which are recommended by the ASTM and the U.S. Pharmacopoeia (USP).[33−37]

Chitosan can be promptly depolymerized. The reaction of nitrous acid with chitosan is selective, rapid, and easily controlled: stoichiometry and products are well established. Nitrosating species attack the glucosamine but not the *N*-acetylglucosamine moieties and cleave the anydroglycosidic linkage. The rate-limiting step is nitrosation of the unprotonated amine by nitrous acid.[38] Hydrogen peroxide can also be conveniently used to depolymerize chitosan. Oligomers are prepared at ambient temperature with

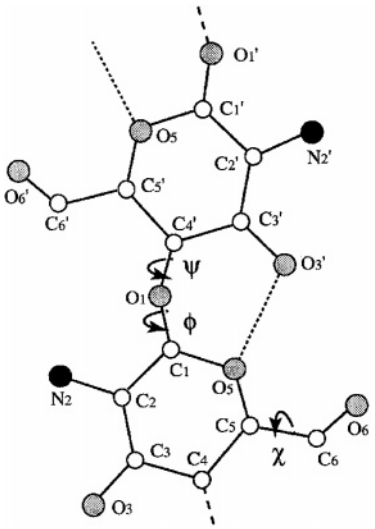


Figure 1. Chemical structure of a disaccharide segment of chitosan, showing position numbering. Two angles, psi and ø, defining the chain conformation and the angle chi defining the O6 orientation are shown. Dashed lines denote the O3−O5 hydrogen bonds. Hydrogen bonds connecting various positions of adjacent chains are omitted.

no side-product formation by fluorolysis of chitin and chitosan.[37] By enzymatic means, chitosan can be easily depolymerized by a variety of hydrolases including lysozyme, pectinase, cellulases, hemicellulases, lipases, and amylases, among others, thus showing a peculiar vulnerability to enzymes other than chitosanases.[39−44]

2.4. Chitosan Derivatives of Major Importance

Before describing the most important modified chitosans, it is fair to mention two important modified chitins. In concentrated NaOH, chitin becomes alkali chitin, which reacts with 2-chloroethanol to yield *O*-(2-hydroxyethyl) chitin, known as glycol chitin: this compound was probably the first derivative to find practical use (recommended substrate for lysozyme). Alkali chitin with sodium monochloroacetate yields the widely used water-soluble *O*-carboxymethylchitin sodium salt.[45] The latter is also particularly susceptible to lysozyme, and its oligomers are degraded by *N*-acetylglucosaminidase; thus, it is convenient for medical applications, including bone regeneration. Muramatsu et al.[46] showed that the stability of *O*-carboxymethyl chitin sponges obtained by freeze drying can be modulated by vacuum heating and γ irradiation, due to thermal crosslinking that compensates for the molecular weight decrease produced by γ-rays.

The Schiff reaction between chitosan and aldehydes or ketones yields the corresponding aldimines and ketimines, which are converted to *N*-alkyl derivatives upon hydrogenation with borohydride. Chitosan acetate salt can be converted into chitin upon heating.[47]

The following are important examples of modified chitosans that currently have niche markets or prominent places in advanced research; a detailed list of chitin and chitosan derivatives of pharmaceutical importance are presented in section 3.

2.4.1. N-Carboxymethyl Chitosan

By using glyoxylic acid, water-soluble *N*-carboxymethyl chitosan is obtained: the product is a glucan carrying pendant glycine groups.[48] *N*-Carboxymethyl chitosan from crab and shrimp chitosans is obtained in water-soluble form by proper selection of the reactant ratio, i.e., with equimolar quantities of glyoxylic acid and amino groups. The product is in part *N*-monocarboxymethylated (0.3), *N*,*N*-dicarboxymethylated (0.3), and *N*-acetylated depending on the starting chitosan (0.08−0.15).[49]

N-Carboxymethyl chitosan as a 1.0% solution at pH 4.80 is a valuable functional ingredient of cosmetic hydrating creams in view of its durable moisturizing effect on the skin.[50] The film-forming ability of *N*-carboxymethyl chitosan assists in imparting a pleasant feeling of smoothness to the skin and in protecting it from adverse environmental conditions and consequences of the use of detergents. *N*-Carboxymethyl chitosan was found to be superior to hyaluronic acid as far as hydrating effects are concerned.

2.4.2. Hydrophobic Chitosans

Hydrophobic associating water-soluble polymers are a new class of industrially important macromolecules. Hydrophobic derivatives of chitosan can be easily obtained from long-chain acyl chlorides and anhydrides. Some of these are intended to mimic the endotoxins.[51]

2.4.3. Chitosans with Methoxyphenyl Functions

The methoxyphenyl aldehydes vanillin, *o*-vanillin, syringaldehyde, and veratraldehyde react with chitosan under normal as well as reducing conditions to impart insolubility and other characteristics to chitosan. The films obtained from veratraldehyde are insoluble, biodegradable, and mechanically resistant.[52]

2.4.4. Tyrosine Glucan

These derivatives were inspired by the chemistry of the cuticle tanning in vivo. Stable and self-sustaining gels are obtained from tyrosine glucan (a modified chitosan synthesized with 4-hydroxyphenylpyruvic acid) in the presence of tyrosinase. Similar gels are obtained from 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde: all of them are hydrolyzed by lysozyme, lipase, and papain. No cross-linking is observed for chitosan derivatives of vanillin, syringaldehyde, and salicylaldehyde. With collagen + chitosan + tannin mixtures under the catalytic action of tyrosinase, partially crystalline, hard, mechanically resistant, and scarcely wettable materials are obtained upon drying. In contrast, the products obtained from albumin, pseudocollagen, and gelatin in the presence of a number of phenols and chitosan under comparable conditions are brittle. Phenoxyacetate is used in the production of penicillin and is often recycled; to remove *p*-hydroxylated derivatives of this precursor, tyrosinase is used followed by adsorption of the quinone species on chitosan.[53−55]

2.4.5. Highly Cationic Chitosans

Trimethyl chitosan was prepared from iodomethane by various authors; Curti et al.[56] reported an alternative method exploring functionalized compounds such as choline dichloride carrying the preformed trimethylammonium group that can react with chitosan to yield highly cationic chitosans (Figure 2); the other new cationic derivative is *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride as reported by Xu et al..[57] The Chitopearl products (Fuji Spinning Co., Japan) belong to this class of chitosans, where the cross-linking compound contains two quaternary nitrogens.

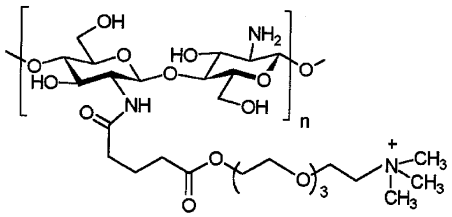


Figure 2. Highly cationic chitosan containing the triethylene glycol choline ether glutarate.

2.4.6. Polyurethane-type Chitosans

Some other types of Chitopearl spherical chitosan particles are produced from diisocyanates and suitable for chromatographic purposes and as enzyme supports.[58] Chitins of various origins in DMA-LiCl solution react with excess 1,6-diisocyanatohexane. Upon exposure to water vapor for 2 days, flexible and opaque materials are produced, whose main characteristics are insolubility in aqueous and organic solvents, remarkable crystallinity, typical infrared spectrum, high N/C ratio (0.287), and relatively high degree of substitution (0.29) but no thermoplasticity. Chitosan similarly treated under heterogeneous conditions in anhydrous pyridine yields reaction products with a lower degree of substitution (0.17). Microencapsulation of lactic acid bacteria based on the cross-linking of chitosan by 1,6-diisocyanatohexane has been performed.[59]

2.4.7. Hydroxyalkyl Chitosans

These chitosans are obtained on reacting chitosan with epoxides: depending on the epoxide conditions (pH, solvent, and temperature), the reaction may take place predominantly at the amino or alcohol group, yielding *N*-hydroxyalkyl- or *O*-hydroxyalkyl chitosans or a mixture of both, as shown in Figure 3.

3. Chemical Modification of Chitin and Chitosan

Numerous works have been published on the chemical modification of chitin and chitosan;[60−75] however, these natural polymers are still being modified to their potential, leading to various derivatives with improved properties. The following section highlights the recent studies on chemical modifications of chitin and chitosan from a pharmaceutical viewpoint.

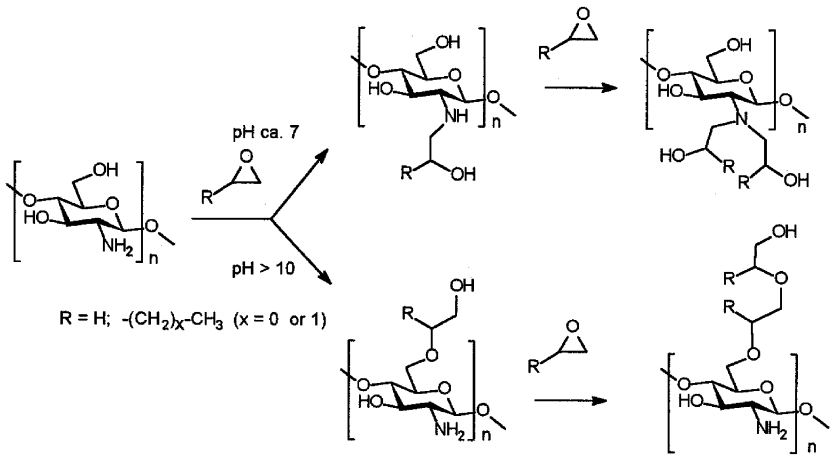


Figure 3. Reaction scheme for chitosan with epoxide. Under certain conditions, substitution degrees higher than 2 may occur.

3.1. Trimethylated, *N*-Succinylated, Thiolated, and Azidated Chitosans

Generally, cationic polymers have been used to collect and deliver DNA in vitro and in vivo. They have lead to increased transfection efficiencies.[76,77] Chitosan showed good ability to collect and deliver plasmid DNA in Cos-1 cell (monkey kidney) culture.[78] Furthermore, trimethyl chitosan chloride (TMC) (80% degree of quaternization), bearing antennary galactose residue through a 6-*O*-linked carboxymethyl (CM) group, served as DNA carrier.[79] Junginger and co-workers[80−84] reported the synthesis of TMC (**1**: Figure 4) by methyl iodide using low molecular weight (MW) chitosan (DP < 20) and evaluated their potential as gene carriers in epithelial cell line. By virtue of the strong basic property of the quaternary ammonium group, TMC is more suitable for collecting and delivering DNA than plain chitosan. Investigations were also carried out on mono-*N*-CM−chitosan (**2**), which was demonstrated to be an efficient intestinal absorption enhancer for anionic polymer such as low MW heparins in Caco-2 cell (human colon carcinoma) monolayers and in rats.[85,86]

Mucoadhesive drug delivery systems promise several advantages that arise from the localization at a given target site (I), a prolonged resistance time at the site of drug adsorption (II), and an intensified contact with the mucosa increasing the drug concentration gradient (III). Lehr et al.[87] were the first to demonstrate the mucoadhesive properties of chitosan. Recent studies suggest that polymers bearing thiol groups provide much higher adhesive properties than polymers generally considered to be mucoadhesive.[88] Kast and Bernkop-Schnürch[89] prepared chitosan−thioglycolic acid conjugate (**3**) mediated by carbodiimide. The conjugate **3** showed 10-fold increase in adhesion property compared with unmodified chitosan.

Other interesting biomedical applications using chemically modified chitosan have been reported. Ishihara and co-workers[90−92] prepared a new photo-cross-linkable chitosan bearing *p*-azidebenzoic acid and lactobionic acid (**4**). This derivative could be cross-linked by UV irradiation, resulting in a rubber-like flexible hydrogel. The hydrogel showed excellent properties such as strong tissue-adhesive, significant

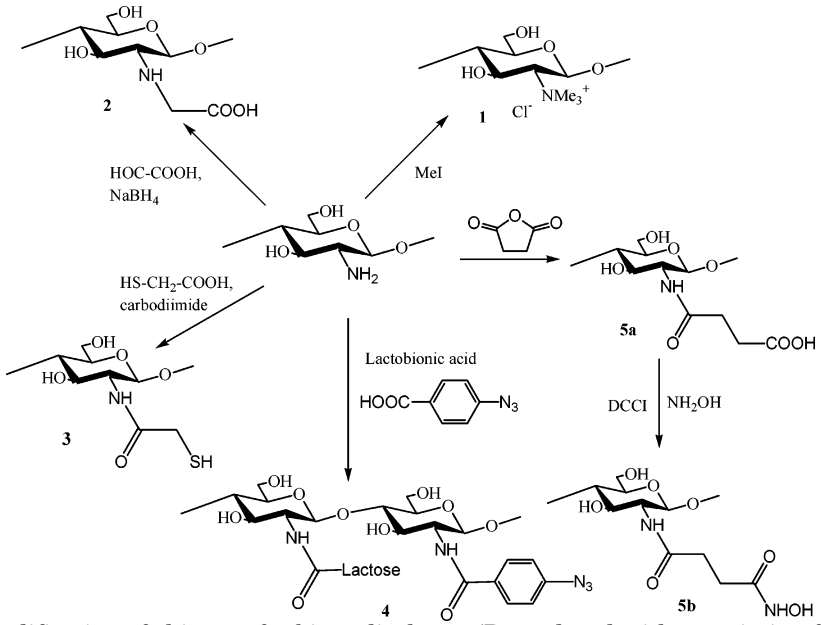


Figure 4. Chemical modification of chitosan for biomedical use. (Reproduced with permission from refs 80, 86, 89, 92, and 93. Copyright 1998, 2001, 2001, 2002, and 2001 Elsevier.)

induction of wound contraction, and acceleration of wound closure and healing, which made it suitable as a biological adhesive in surgical applications. Aiedeh et al.[93] prepared *N*-succinyl chitosan (**5a**) and its hydroxylaminated derivative (**5b**), which were crosslinked by iron(III) and investigated for drug release.

3.2. Oxychitin and Fluorinated Chitins

Sulfated chitin or CM−chitin showed biomedical aspects such as heparinoid-like anticoagulant activity,[94] immunological activity,[95] and inhibition of tumor cell invasion.[96,97] Recently, Muzzarelli et al.[98] prepared a new chitin derivative, 6-oxychitin (**6**), by regiospecific oxidation (Figure 5). The 6-oxychitin

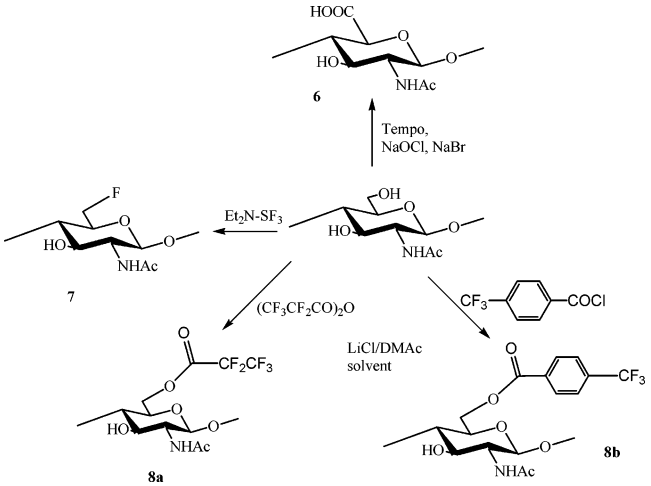


Figure 5. Chemical modification of chitin. (Reproduced with permission from ref 98. Copyright 1999 Elsevier.)

formed microcapsules with chitosan and was assayed for delivery of miconazole. Furthermore, 6-oxychitin showed hydrolytic property by lysozyme and biocompatible toward human keratinocytes.[99] The general interest in fluorinated polymers has been centered on their unique stability, low dielectric constant, low surface energy, and low water uptake. As the C−F bond can participate as a proton acceptor in hydrogen bonding, it may induce various activities via specific interaction with the biological system. Chow and Khor[100] synthesized new fluorinated chitin derivatives (**7**, **8**), and their cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using human (ATCC CCL-186) and mouse (ATCC CCL-1) fibroblast cell line. The degree of fluorination in **7** achieved 80−98% substitution of C-6 OH groups, although those of **8a** and **8b** were 41% and 5%, respectively. Since fluorinated chitin derivatives showed good cell viability of 80−100% for human fibroblast, these would be potential materials for biomedical applications.

3.3. Sugar-Modified Chitosan

Hall and Yalpani[101,102] were the first to report sugar-modified chitosan derivatives (Figure 6). They synthesized sugar-bound chitosan by reductive *N*-alkylation using sodium cyanoborohydride ($NaCNBH_3$) and unmodified sugar (**9**: method A) or sugar−aldehyde derivative (**10**: Method B), however, these sugar-bound chitosans were confined to rheological studies. Since the specific recognition of cell, virus, and bacteria by sugars has been discovered, this modification has generally been used to introduce cell-specific sugars into chitosan. Morimoto and co-workers[103−106] reported the synthesis of sugar-bound chitosans such as D- and L-fucose and their specific interaction with lectin or cells. Kato et al.[107] also prepared lactosaminated *N*-succinyl−chitosan (**12**) and its fluorescein thiocarbanyl derivative as a liver-specific drug carrier in mice through asialoglycoprotein receptor (Figure 7). Moreover, derivative **12** was a good drug carrier such as mitomycin C in liver metastasis.[108] Galactosylated chitosan (**14**: Figure 8) prepared from lactobionic acid and chitosan with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was a good candidate as a synthetic extracellular matrix for hepatocytes attachment.[109] Sponge-type complex of cationic **14** and anionic alginate also showed spheroid formation and viability of hepatocytes.[110] Furthermore, graft copolymers of **14** with poly(ethylene glycol) or poly(vinyl pyrrolidone) were useful for hepatocyte-targeting DNA carrier.[111,112]

Sialic acid is the most ubiquitous sugar present on the mammalian cell surface glycolipids and glyco-

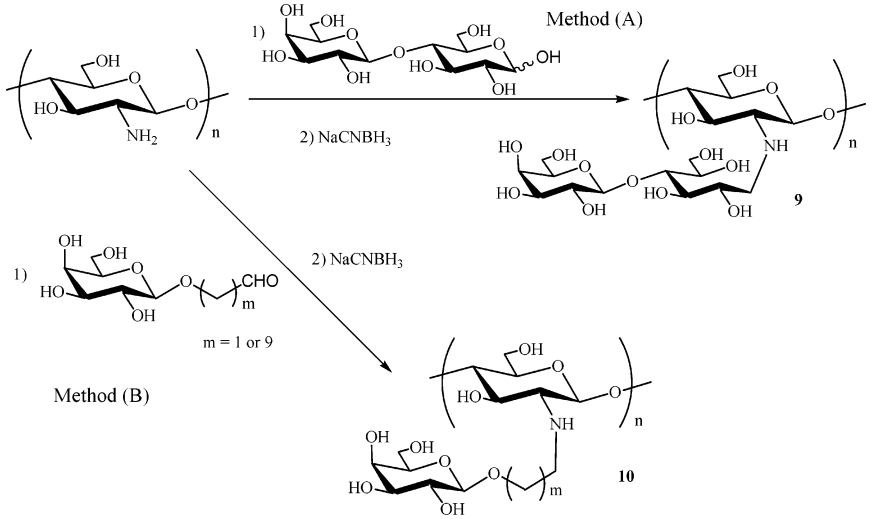


Figure 6. Strategy for the substitution of sugars to chitosan by reductive *N*-alkylation. (Reproduced with permission from ref 101. Copyright 1980 Royal Society of Chemistry.)

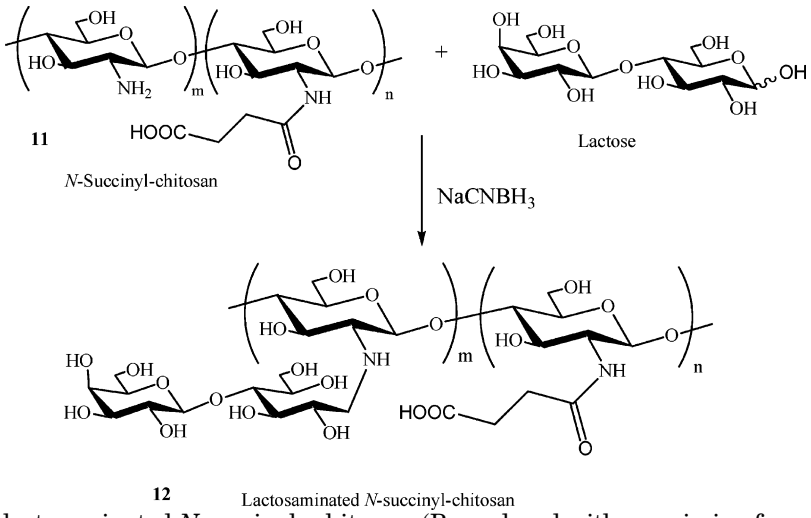


Figure 7. Synthesis of lactosaminated *N*-succinyl−chitosan. (Reproduced with permission from ref 107. Copyright 2001 Elsevier.)

proteins and is the key epitope recognized as being essential for a number of pathogenic infections. Moreover, sialic-acid-containing polymers have been shown to be potent inhibitors of hemagglutination of human erythrocytes by influenza viruses.[113−117] Sashiwa and Roy[118,119] prepared sialic-acid-bound chitosan (**16**: Figure 9) as a new family of sialic-acid-containing polymers using *p*-formylphenyl-α-sialoside (**15**) by reductive *N*-alkylation. Since derivative **16** was insoluble in water, continuous *N*-succinylation was carried out and a water-soluble derivative was obtained (**17**). The specific binding with wheat germ agglutinin lectin was shown in water-soluble derivative **17**.

Human antibodies against α-galactosyl epitope are responsible for acute rejection of xenotransplantated organs from lower animals. Artificial glycopolymers having α-galactosyl epitope are of interest from the viewpoint of medical transplantation of pig liver since they can block immune rejection. Water-soluble α-galactosyl chitosan (**18**: Figure 10), prepared following the same strategy adopted for chitosan−sialic acid conjugates, showed specific binding against α-galactosyl specific lectin (*Griffonia simplicifolia*).[120] Chitosan−sialic acid or α-glactosyl conjugates with different degrees of substitution have been evaluated.[121] These conjugates are believed to inhibit influenza viruses or can act as blocking agents for acute rejection.

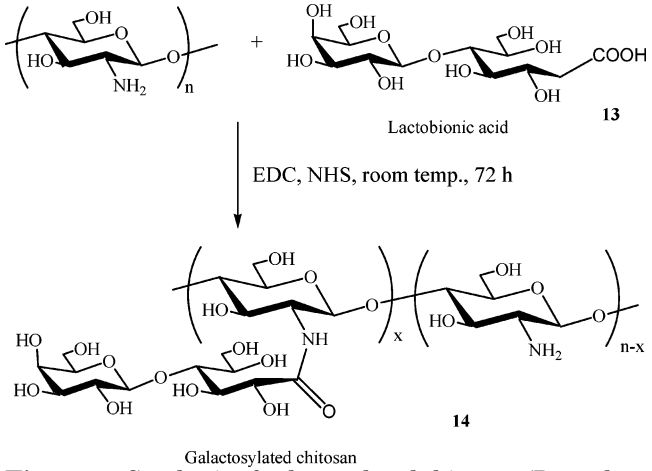


Figure 8. Synthesis of galactosylated chitosan. (Reproduced with permission from ref 109. Copyright 2003 Elsevier.)

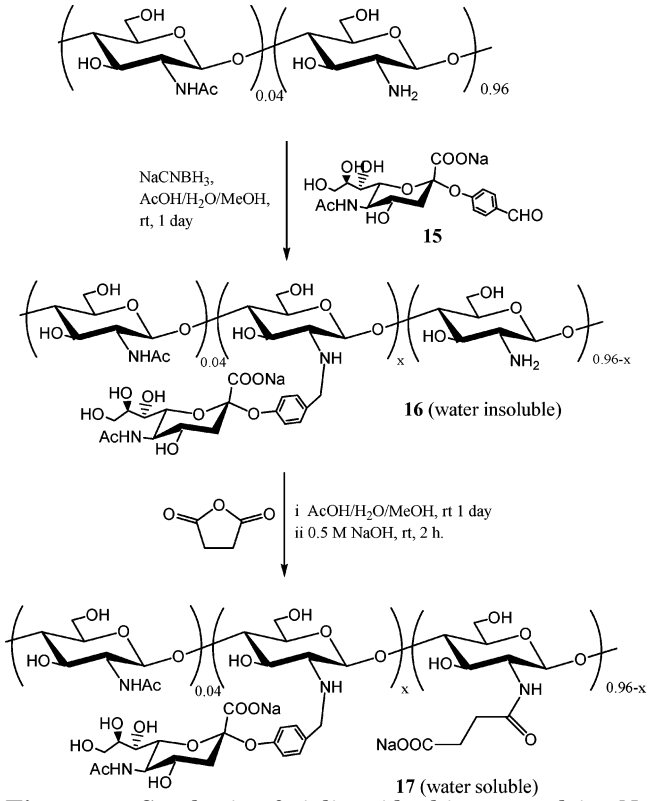


Figure 9. Synthesis of sialic-acid−chitosan and its *N*-succinylation. (Reproduced with permission from ref 119. Copyright 2000 Royal Society of Chemistry.)

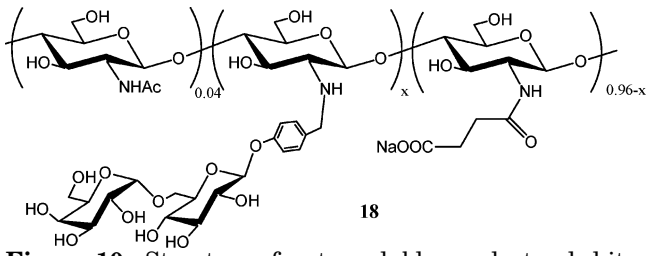


Figure 10. Structure of water-soluble α-galactosyl chitosan. (Reproduced with permission from ref 120. Copyright 2000 American Chemical Society.)

3.4. Chitosan–Dendrimer Hybrid

Dendrimers are attractive macromolecules owing to their multifunctional properties[122−124] and useful applications as viral and pathogenic cell adhesion inhibitors.[125,126] Increasing scientific efforts have gone into the design and synthesis of dendrimers.[127−129] Dendronized polymers, on the other hand, are also attractive because of their rod-like conformation and nanostructure.[130,131] Although, several investigations have been published toward the synthesis of dendronized polymers,[132,133] very few reports are available on dendronized polysaccharides, especially related to chitin and chitosan backbone.

Sashiwa et al.[134−140] established at first the synthesis of a variety of chitosan−dendrimer hybrids mainly by two procedures (Figure 11). In method A, the corresponding dendrimers bearing aldehyde and spacer are synthesized, and then these are reacted with chitosan by reductive *N*-alkylation. This procedure is advantageous because no cross-linking takes place during the reaction. However, generation of reactive dendrimer is limited owing to its steric hindrance. It is possible to generate more reactive dendrimers following method B, which uses commercial amino−dendrimers such as poly(amidoamine) (PAMAM) and poly(ethylene imine) (PEI) dendrimers. However, method B suffers from the possibilities of cross-linking. The typical example of a "tree-type" hybrid generated by method A is shown in Figure 12.[134,139] The terminology "tree-type hybrid" is based on the assumption that chitosan is a trunk, the spacer part is a main branch, dendrimer is a subbranch, and the functional sugar is a flower (or leaf). In this case, tetraethylene glycol was modified in 5 or 7 steps to synthesize the scaffold of dendrimer. PAMAM dendrimers of generation (G) from 1 to 3 bearing tetraethylene glycol spacer were prepared, attached to sialic acid by reductive *N*-alkylation, and finally attached to chitosan. The degree of substitution (DS) of dendrimer decreased with increasing generation as 0.08 (G = 1), 0.04 (G = 2), and 0.02 (G = 3) owing to the steric hindrance of dendrimer. Figure 13 shows the different types of chitosan−dendrimer hybrids.[135] Sialic acid dendron bearing a focal aldehyde end group was synthesized by a

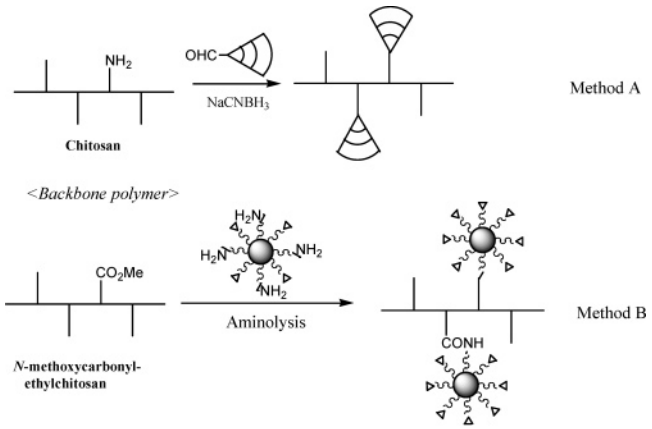


Figure 11. Synthetic strategy of chitosan−dendrimer hybrid. (Reproduced with permission from ref 136. Copyright 2001 American Chemical Society.)

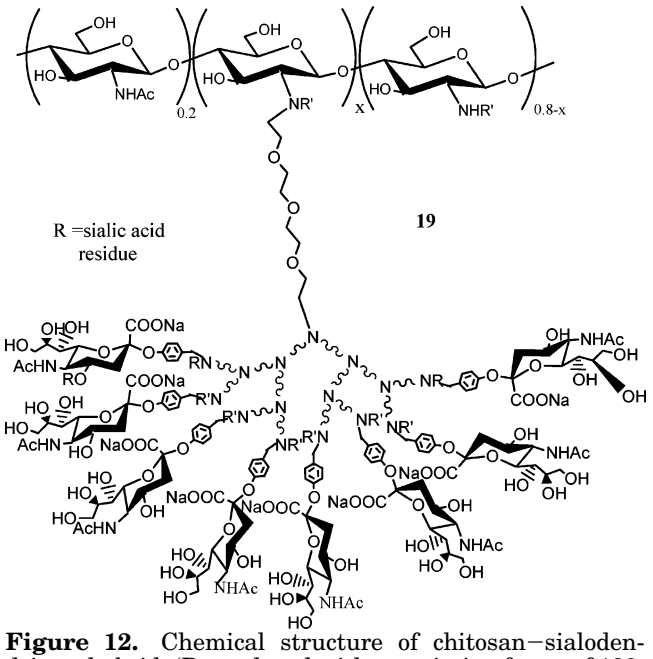


Figure 12. Chemical structure of chitosan−sialodendrimer hybrid. (Reproduced with permission from ref 139. Copyright 2002 Elsevier.)

reiterative amide bond strategy. Trivalent (G = 1) and nonavalent (G = 2) dendrons having gallic acid as the branching unit and triethylene glycol as the spacer arm were prepared and initially attached to a sialic acid *p*-phenylisothiocyanate derivative. The focal aldehyde sialodendrons were then convergently attached onto chitosan. The DS of sialodendrimer were 0.13 (G = 1) and 0.06 (G = 2). Further biological evaluation of these promising hybrids is being investigated toward the inhibition of viral pathogens including the influenza virus.

The chitosan−dendrimer hybrid prepared by method B is shown in Figure 14.[136] As the construction of hybrid was difficult from original chitosan, a derivative, *N*-methoxycarbonylethyl chitosan (**21**), was used as chitosan backbone. PAMAM dendrimers (G = 1−5) having a 1,4-diaminobutane core were attached to **21** by amidation under conditions that prevent cross-linking. The hybrids **21** could be prepared even in high generations (G = 4 or 5), although the DS of dendrimer decreased with increasing generation of dendrimer from 0.53 (G = 1) to 0.17 (G = 4) or 0.11 (G = 5). Since this hybrid was soluble in acidic water, undesired cross-linking would not occur. However, two or more intermolecular binding points were observed. In any case, sialic acid was successfully attached to the primary amine of dendrimer part with DS ranging from 0.7 to 1.4 per sugar unit, which means highly convergent synthesis of sialic acid in chitosan backbone. Given the fact that influenza virus hemagglutinins exist as several clusters of trimers (200−300/virions),[141] it is likely that the novel dendronized chitosan−sialic acid hybrids prepared by method B would present added beneficial architectures not present in previously reported sialodendrimers.[115,142−144] Preliminary biological evaluation of analogous hyperbranched sialodendrimers has already shown increased inhibitory properties.[125]

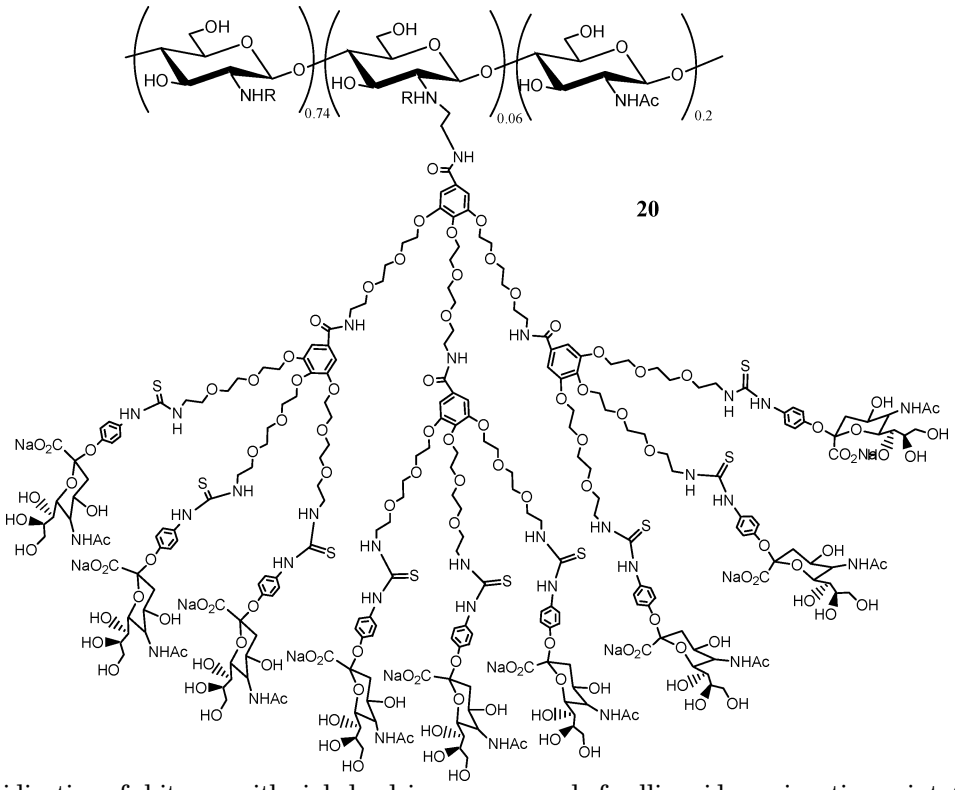


Figure 13. Hybridization of chitosan with sialodendrimer, composed of gallic acid as a junction point. (Reproduced with permission from ref 135. Copyright 2001 American Chemical Society.)

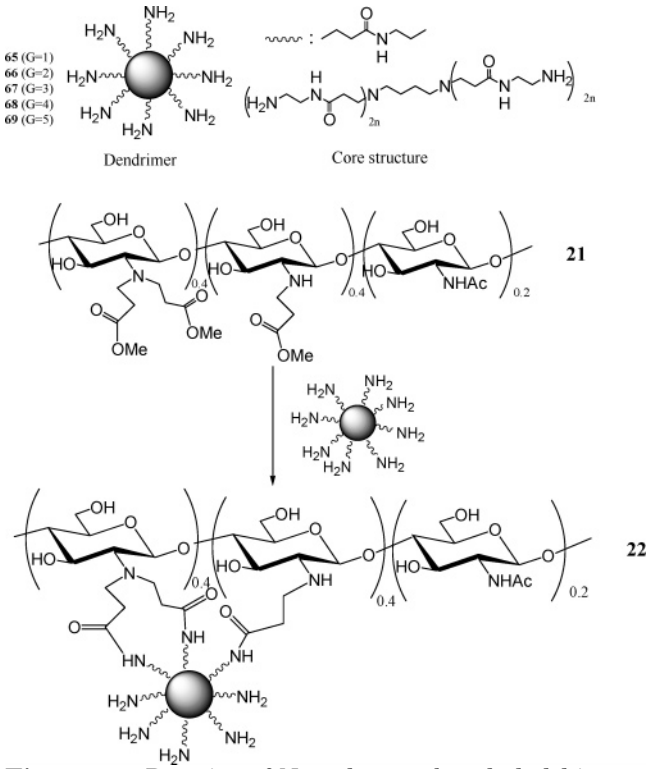


Figure 14. Reaction of *N*-methoxycarbonylethylchitosan with PAMAM dendrimer. (Reproduced with permission from ref 136. Copyright 2001 American Chemical Society.)

3.5. Cyclodextrin-Linked Chitosan

Cyclodextrins (CD) have gained prominence in recent years because the cavity, of hydrophobic nature, is capable of binding aromatic and other small organic molecules and therefore provides ideal binding sites. CD-linked chitosan is interesting for drug delivery, cosmetics, and analytical chemistry.[145] Although the functionalization of hydroxyl groups at the 6-position in CD is relatively easy, the secondary alcohols (2 and 3 positions) are most important in binding studies.[146] Sakairi and co-workers[147,148] prepared α-CD-linked chitosan (Figure 15, **23**) using 2-*O*-formylmethyl-α-CD by reductive *N*-alkylation and confirmed the host−guest complex of **23** with *p*-nitrophenol. Chen and Wang[149] obtained CD-linked

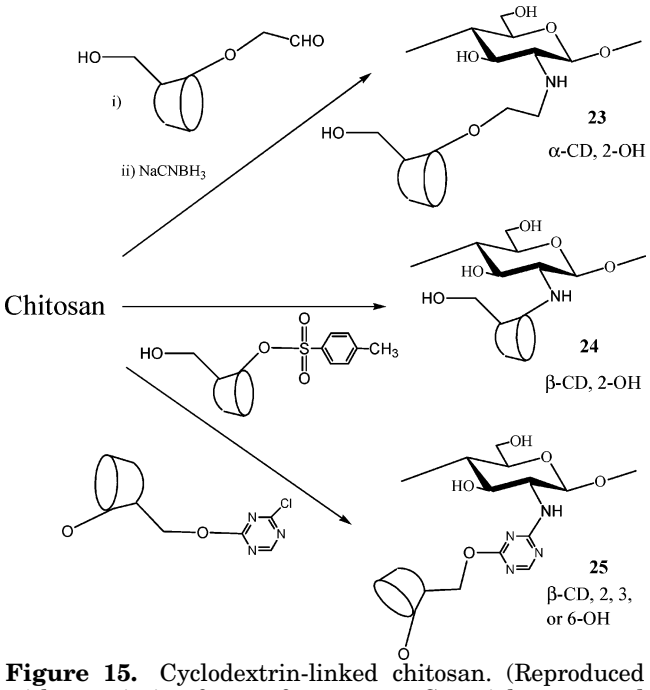


Figure 15. Cyclodextrin-linked chitosan. (Reproduced with permission from refs 148−150. Copyright 1998 and 2001 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html.))

chitosan (**24**) using tosylated β-CD and further evaluated the potential of β-CD for the release of $^{131}I_2$ in vivo. The CD-linked chitosan could also be prepared by the intermediate of its monochlorotriazinyl derivative (**25**).[150] This compound was used for decontamination of waters containing textile dyes. An insoluble cross-linked chitosan bearing β-CD was prepared using *N*-succinyl chitosan and aminated-β-CD via amide bond formation.[151] The β-CD-linked chitosan using 1,6-hexamethlene diisocyanate as spacer was also prepared by Sreenivasan.[152] This material interacts with cholesterol and might be useful as an adsorbent.

3.6. Biodegradation of Modified Chitosans

In the field of electronic equipment, such as computer and medical instruments, high-speed and high-density information processing is becoming increasingly common, and there is the danger that even low-intensity electromagnetic radiation may cause malfunction. For example, use of mobile telephones and portable computers is restricted in aircrafts and hospitals. Hence, there is a need for highly reliable electromagnetic radiation shielding materials. Biodegradability of binder between plastics and shielding materials is required for recycling of plastics, and since chitosan is biodegradable, it would be useful as a binder. Chitosan itself, however, is hydrophilic, while plastics are hydrophobic; therefore, modification of chitosan to impart solubility in organic solvents is necessary for spraying on the surface of the plastics. The hydrophobic ester group contributes solubility in organic solvents and is hydrolyzed by enzymes such as lipase (Figure 16). Sashiwa et al.[153−155] reported the synthesis of organosoluble and biodegradable acylchitosans intended for the electromagnetic shielding materials. Acylchitosans bearing long acyl chains (**27**: $n > 4$), hydrophobic pivaloyl (**28**), or benzoyl (**29**) groups showed organosoluble properties. Interestingly, *O*-acetylchitosan (**26**) showed water solubility like acetylcellulose.[155] These chitosan derivatives (**26**, **27,** and **29**) showed good biodegradation. Furthermore, these acylchitosans showed good binding properties between plastics and electromagnetic shielding materials; thus, they are useful as biodegradable binders.

Alternatively, the biodegradation has been investigated on some water-soluble chitosan derivatives prepared by Michael reaction (Figure 17).[156,157] This reaction has been developed as a new method for the chemical modification of chitosan[158] or partially deacetylated chitin,[159] and the resulting products were used as precursors for the construction of chitosan−dendrimer hybrid.[136,138] Most recently, Michael reaction of chitosan with acrylic acid was also reported using water as solvent.[156] In this case, acrylic acid acted as both proton donor to dissolve chitosan in water and reagent for Michael reaction, so that water-soluble *N*-carboxyethylchitosan (**30**) was successfully prepared. If water-soluble acryl reagents could be applied for this reaction, novel types of functional groups will be introduced by a simple procedure. Various chitosan derivatives were prepared by this reaction in water and acetic acid with various acryl reagents.[157] Biodegradation data

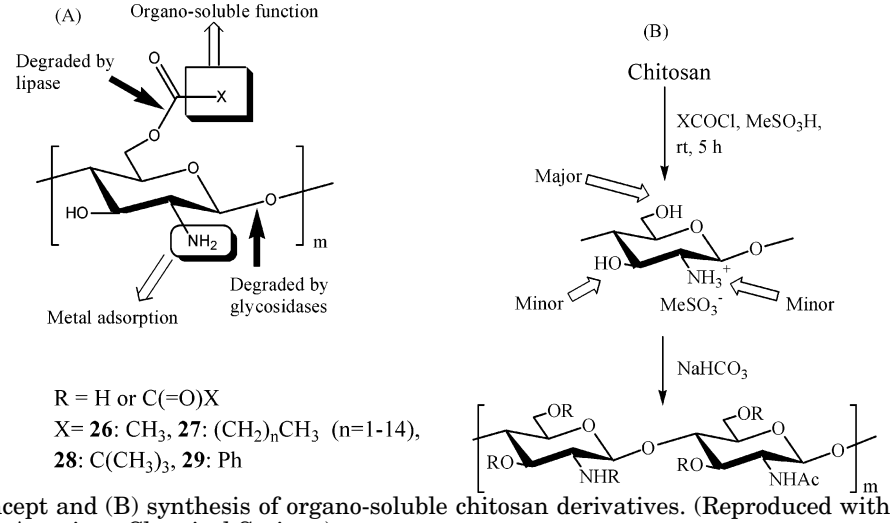


Figure 16. (A) Concept and (B) synthesis of organo-soluble chitosan derivatives. (Reproduced with permission from ref 154. Copyright 2002 American Chemical Society.)

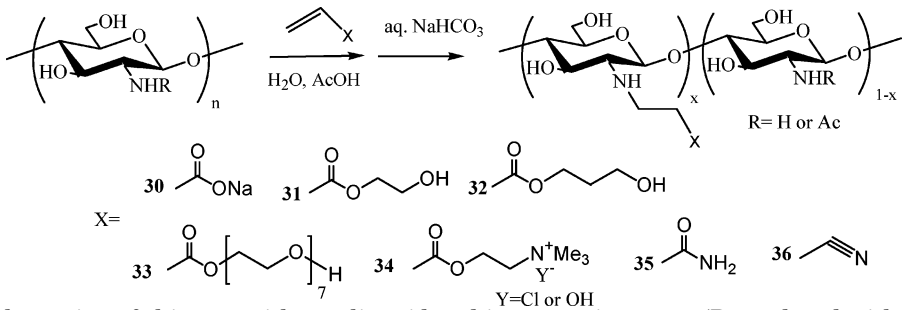


Figure 17. Michael reaction of chitosan with acrylic acid and its esters in water. (Reproduced with permission from ref 157. Copyright 2003 American Chemical Society.)

Table 1. Biodegradation of Chitosan and Its Derivatives[156,158]

sample	DS	solubility in H_2O	biodegradation,[a] %
chitosan	0.00	no	1.6
30	0.18	yes	67.3
30	0.27	yes	62.5
30	0.46	yes	51.3
31	0.44	yes	8.6
32	0.26	yes	5.0
33	0.29	no[b]	24.8
34	0.38	yes	33.6
35	0.24	no	27.8
36	0.49	no	7.7

[a] Time, 21 days. [b] Water-insoluble after lyophilization.

of these chitosan derivatives by standard activated sludge is presented in Table 1. In any case, the biodegradability was enhanced by chemical modification compared with original chitosan. Excellent biodegradability was shown in **30** with various DS, although it gradually decreased with increasing DS. Derivatives **33**, **34**, and **35** modified with poly(ethyleneglycol) (PEG), quaternary ammonium, and amido groups also showed good biodegradability. Moderate biodegradation was shown by **31** and **36** bearing hydroxyethyl and nitrile groups. These results suggest that biodegradation was very associated with the chemical structure of chitosan derivatives. Thus, carboxyl, quaternary ammonium, amido, and PEG groups were convenient for biodegradation, but hydroxyethyl and nitrile groups were not. On the other hand, biodegradation was independent of the water solubility of chitosan derivatives. Biodegradation of chitosan is quite slow by standard activated sludge. Modified chitosans are more prone to biodegradation owing to the destroyed crystalline structure of chitosan.

3.7. Crown-Ether-Bound Chitosan

Crown ethers have particular molecular structures and good complexing selectivity for metal ions. These crown-ether-bound chitosans will have a stronger complexing capacity and better selectivity for metal ions because of the synergistic effect of high molecular weight. Tang et al.[160] prepared the crown-ether-bound chitosan with Schiff's-base-type (**37a**) and its reduced form (**37b**) Figure 18. Their chemical structures were characterized by elemental analysis, IR, X-ray, and solid-state ^{13}C NMR analyses. Crown-ether-bound chitosans had not only good adsorption capacities for metal ions Pd^{2+}, Au^{3+}, and Ag^{+}, but also high selectivity for the adsorption of Pd^{2+} in the presence of Cu^{2+} and Hg^{2+}. Cross-linked types of crown-ether-bound chitosans were also reported (Figure 19).[161] These cross-linked derivatives have space net structures with embedded crown ethers, and each mesh has a certain space volume. When original chitosan reacted with 4,4′-dibromobenzo-18-crown-6-crown ether, the cross-linked product between 6-OH and NH_2 was obtained (**38**). However, this product would include heterogeneous cross-linking structure between 6-OH and 6-OH or NH_2 and NH_2. Benzylidene-protected chitosan (CTB) would produce a homogeneous cross-linking structure between 6-OH and 6-OH (**39**). These crown-ether-bound chitosans

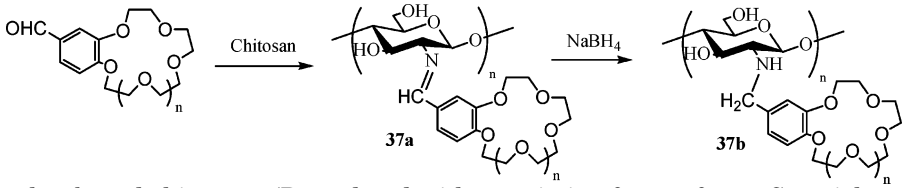


Figure 18. Crown-ether-bound chitosans. (Reproduced with permission from ref 160. Copyright 2002 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

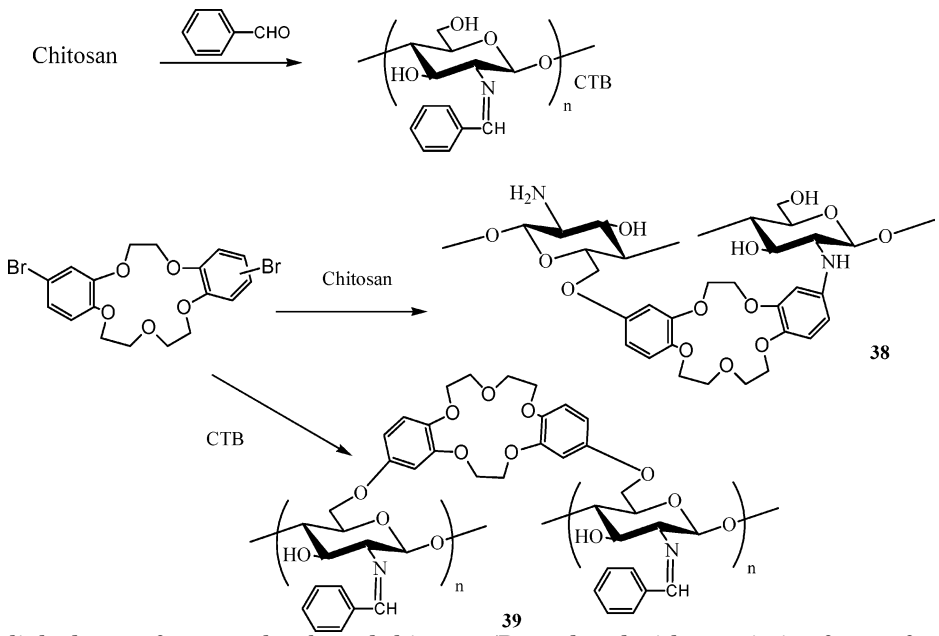


Figure 19. Cross-linked type of crown-ether-bound chitosan. (Reproduced with permission from ref 161. Copyright 2002 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

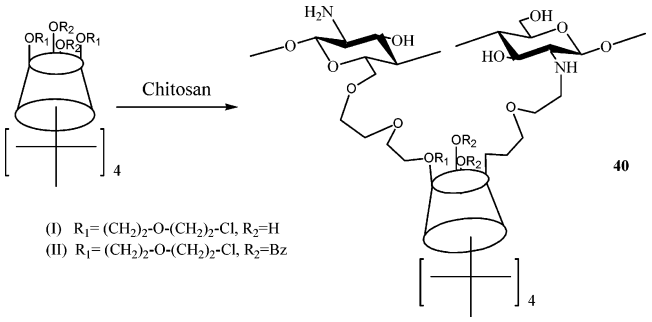


Figure 20. Calixarene-bound chitosan. (Reproduced with permission from ref 162. Copyright 2003 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

would be useful for separation and preconcentration of heavy or precious metal ions in aqueous environments.

On the other hand, calixarenes have demonstrated outstanding complex ability toward ions, organic molecules, etc, and are considered the third best host molecules, after cyclodextrins and crown ethers. Li et al.[162] reported the first synthesis of calixarene-modified chitosan (Figure 20). The adsorption properties of calixarene-modified chitosan (I and II) were greatly varied compared with that of original chitosan, especially with the adsorption capacity toward Ag^+ and Hg^{2+}, because of the presence of the calixarene moiety. These derivatives did not dissolve in general organic solvent; however, they can easily be powdered and are thus better adsorbents than simple chitosan.

3.8. Chemical Grafting of Chitosan

Graft copolymerization onto chitin and chitosan is an important study for the functionalization and practical use of them. A lot of initiators have been investigated for grafting such as ceric ion, Fenton's reagent, γ-irradiation, various radicals, and ring-opening methods.[163] An interesting feature of polyoxazoline chains is the fact that they are regarded as pseudo-peptides having good flexibility.[164] It has also been disclosed that oxazoline-grafted chitosan (Figure 21, **41**, degree of deacetylation 50%) has the capability of incorporating lipase P and catalase and increasing hydrolytic activity compared with free enzymes.[165] Furthermore, the molecular shape of water-soluble grafted chitosan **41** was evaluated by atomic force microscopy (AFM), cryo-transmission electron microscopy (cryo-TEM), and small-angle

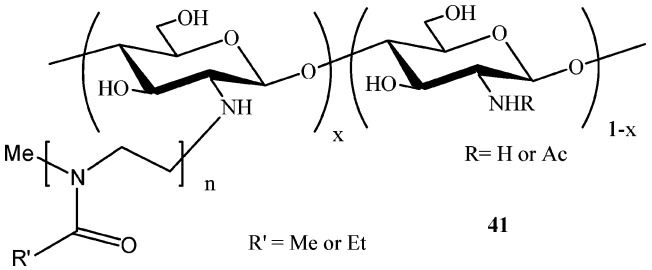


Figure 21. Oxazoline-grafted chitosan (DDA = 50%). (Reproduced with permission from ref 166. Copyright 2002 Wiley. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

neutron scattering (SANS) analysis. Grafted chitosan bearing short graft chain formed a ring structure (40−60 nm of diameter) unimolecularly, while that bearing middle chain length was monodisperse spherical (30−40 nm), whereas the longer chain aggregated intermolecularly that led to larger particles (100−400 nm).[166] These studies should be useful for the strategy to regulate molecular design and guest-binding properties of water-soluble-grafted chitosan.

Homo- and copolymers based on lactic acid have been widely used in sutures and drug-release systems owing to their biodegradability in the animal body, while pH-sensitive polymer gels have potential use in the delivery of drugs to specific regions of the gastrointestinal tract. Novel pH-sensitive physical cross-linked hydrogels were synthesized by grafting D,L-lactic acid onto amino groups in chitosan without catalysis (Figure 22, **42**).[167] pH sensitivity was due to aggregation of the hydrophobic side chains. The specific solution content of hydrogels decreased when the pH value and ionic strength were increased.

Although grafting on chitin and chitosan has been performed by high-energy irradiation or the addition of initiators such as cerium(IV) and redox system, these methods affect degradation of the polysaccharide backbone, thus giving rise to the grafted products with complicated and ambiguous structure. Kurita et al.[168] synthesized graft copolymer onto chitin by use of the mercapto group (Figure 23). Methyl methacrylate (MMA) was efficiently grafted onto mercaptochitin in DMSO, and grafting percentage reached 1300%. Although the side-chain ester groups were resistant to aqueous NaOH only, hydrolysis of ester could be achieved with a mixture of aqueous NaOH and DMSO. The grafted chitins (**43**) showed pronounced activity in terms of moisture absorption and lysozyme susceptibility when compared to unmodified chitins.

3.9. Enzymatic Modification of Chitosan

The enzymatic approach to modification of chitin and chitosan is interesting owing to its specificity and environmental impact compared with chemical modification. With respect to health and safety, enzymes offer potential by eliminating the need for (and hazards associated with) reactive reagents. Payne and co-workers[169] reported the enzymatic grafting of phenolic compounds onto chitosan to confer water solubility under basic conditions (Figure 24). Tyrosinase converts a wide range of phenolic substrates into electrophilic *o*-quinones. Using slightly acidic conditions (pH 6), chitosan could be modified under homogeneous conditions with chlorogenic acid, a natural product. The modified chitosan was dissolved in both acidic and basic conditions, although the degree of modification was low. Quinone chemistry, however, remains poorly characterized because of its complexity. It can undergo two different reactions to yield either Schiff base (**44**) or Michael-type adducts (**45**). Since it is possible for quinones to undergo either or both types of reactions with amines, as well as undergoing oligomer-forming reactions with other quinones, it is common for reactions between quinones and amines to yield a complex mixture of

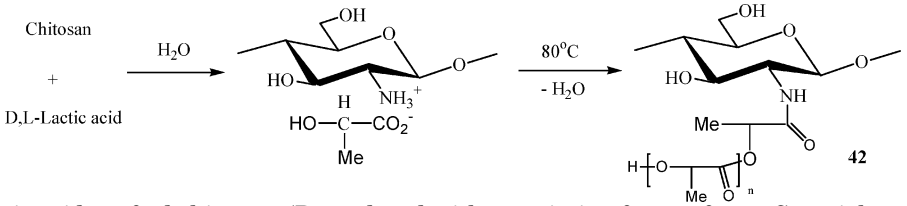


Figure 22. D,L-Lactic-acid-grafted chitosan. (Reproduced with permission from ref 167. Copyright 1999 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

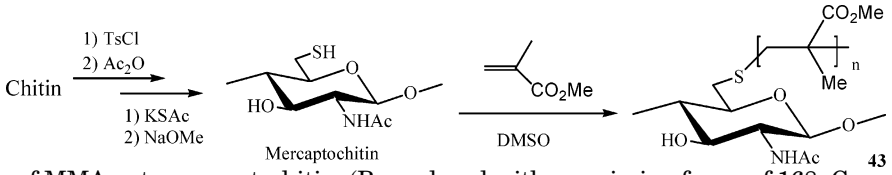


Figure 23. Grafting of MMA onto mercaptochitin. (Reproduced with permission from ref 168. Copyright 2002 American Chemical Society.)

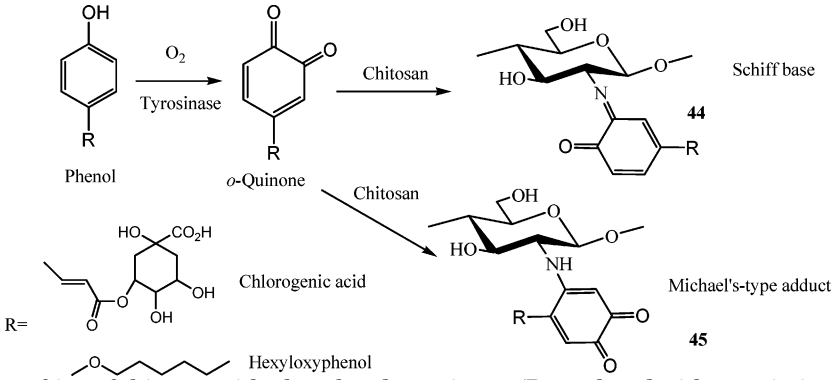


Figure 24. Enzymatic grafting of chitosan with phenol and tyrosinase. (Reproduced with permission from ref 169. Copyright 1999 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

products.[170] Furthermore, to alter the surface and rheological properties of chitosan, hexyloxyphenol was grafted onto chitosan mediated by tyrosinase.[171] On the basis of the contact angle measurements, heterogeneous modification of chitosan film yielded a hydrophobic surface owing to the substitute, while homogeneously modified chitosan offered rheological properties characteristic of associating water-soluble polymers.

Therefore, the biochemically relevant quinones studied so far are preferred materials for medical applications. For instance, menadione, a synthetic naphthoquinone derivative having the same physiological properties of vitamin K, is particularly prone to rapid reaction with chitosans and greatly modifies its spectral characteristics and increases the surface hydrophobicity of the chitosan films.[172] Research under way will provide information on the biological properties of these enzymatically modified chitosans.

3.10. Others

Colloidal systems have found numerous applications as promising delivery vehicles of drugs, proteins, antigens, and genes due to their reduced toxic side effect and improvement of therapeutic effect.[173] The micellar behavior of polymeric self-assembly system offers an advantage as one of the colloidal systems that has been widely investigated in the fields of biotechnology and pharmaceutics.[174] Precise control of the size and structure are the critical design parameters of a micellar system for drug delivery applications. To control the size of self-

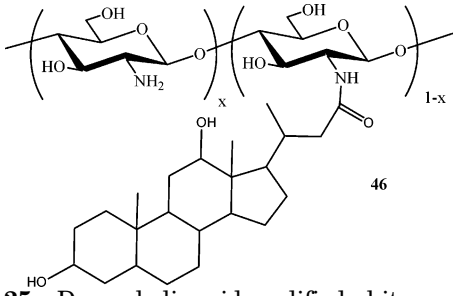


Figure 25. Deoxycholic-acid-modified chitosan. (Reproduced with permission from ref 175. Copyright 2001 American Chemical Society.)

aggregates (SA), chitosan was depolymerized with sodium nitrite and hydrophobically modified with deoxycholic acid to form SA in aqueous media (Figure 25).[175] The size of SA was 130−300 nm in diameter. Due to the chain rigidity of chitosan, the structure of SA was suggested to be cylindrical bamboo-like and might form a very poor spherical form of a bird's nest-like structure. The potential applications of SA as a gene delivery carrier were tested, and significant influence of transfection efficiency by SA was observed against Cos-1 cells (up to a factor of 10). This approach to control the size and structure of chitosan-derived SA may find a wide range of applications in gene delivery as well as general drug delivery applications. Lee et al.[176] reported the delivery of adriamycin (ADR) using SA of the deoxycholic-acid-modified chitosan (**46**). Deoxycholic acid was covalently conjugated to chitosan via EDC-mediated reaction to generate SA nanoparticles. ADR was

physically entrapped inside the SA, and slow release of ADR was achieved.

The formation of hydrogels from polymers using noncovalent cross-linking is a useful method of preparing hydrogels for drug delivery since these gels are likely to be more biocompatible as gel formation does not require the use of organic solvents or chemical reactions which may be potentially deleterious to the drug loaded. Such physically cross-linked chitosan-based gels are formed by exploiting either hydrogen-bonding or hydrophobic attractions. Uchegbu and co-workers[177] focused on the use of pendant hydrophobic groups to achieve noncovalent cross-linking. Palmitoyl glycol chitosan (GCP, Figure 26) hydrogel has been evaluated as an erodible

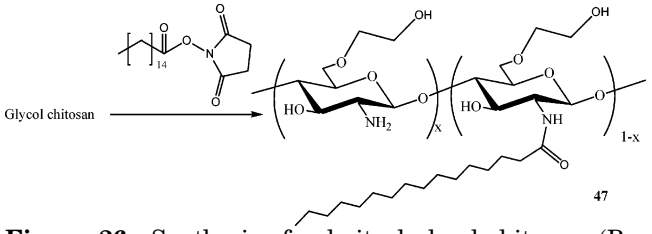


Figure 26. Synthesis of palmitoyl glycol chitosan. (Reproduced with permission from ref 178. Copyright 2002 Elsevier.)

controlled release system for the delivery of hydrophilic macromolecules.[178] Fluorescein isothiocyanate (FITC)-dextran and/or amphiphilic derivatives Gelucire 50/13 or vitamin E D-α-tocopherol poly(ethylene glycol) succinate were used as model macromolecules. Hydration and erosion were governed by the hydrophobicity of the gel and the presence of the amphiphilic additives. The controlled release of FITC-dextran was governed by the hydrophobicity of the gel. In their subsequent study, GCP hydrogel was evaluated as a deliverer of hydrophobic drugs, denbufylline, via the buccal route.[179] The buccal route has been advocated as a possible route of administration for drugs which undergo extensive hepatic first-pass metabolism or are susceptible to degradation in the gastrointestinal tract.

Glass beads have received attention as supporting material owing to their controllable and narrow size dispersion properties in addition to their mechanical strength. Sakairi and co-workers[180] reported a new hybrid that adsorbs transition-metal ions by the surface modification of nonporous beads with chitosan. Glass beads bearing aldehyde groups were produced and modified with chitosan by reductive *N*-alkylation (Figure 27). Metal ions such as Cu^{2+}, Ag^{+}, Pb^{2+}, Fe^{3+}, and Cd^{2+} were collected (over 90%) on a column of chitosan-modified glass beads. They also reported another type of chitosan-modified glass beads through a 1,3-thiazolidine linker.[181] In this case, terminal aldehyde group (**49**) produced by nitrous acid degradation of chitosan was used for the coupling with L-cysteine linker of glass beads (Figure 28). This method to prepare chitosan-modified glass beads could be applied for a variety of silica materials.

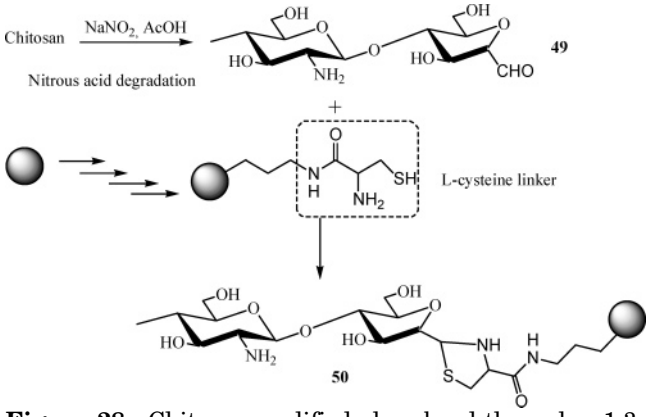


Figure 28. Chitosan-modified glass bead through a 1,3-thiazolidine linker. (Reproduced with permission from ref 181. Copyright 2003 Elsevier.)

4. Chitin and Chitosan in Different Forms

4.1. Nanoparticles

Alonso and co-workers[182] reported the preparation of nanoparticles based on ionic gelation made solely of hydrophilic polymers. The preparation process is extremely mild and involves the mixture of two aqueous phases at room temperature. One phase contains the polysaccharide chitosan (CS) and poly(ethylene oxide), and the other contains polyanion sodium tripolyphosphate (TPP). The authors claimed that the particle size (200−1000 nm) and zeta potential (between +20 and +60 mV) of nanoparticles can be modulated by varying the ratio CS/PEO−PPO. They also demonstrated that these new nanoparticles have great protein loading capacity (entrapment efficiency up to 80% of the protein) and provide a continuous release of the entrapped protein for up to 1 week.[182] Furthermore, they performed in-depth investigations to understand the physicochemical properties, surface composition, and mechanisms of protein association to chitosan and CS/PEO−PPO nanoparticles.[183] The electron micrographs showed that the particles are spherical. The CS/PEO−PPO nanoparticles exhibited a compact core surrounded by a thick fluffy coat, presumably consisting of PEO−PPO, which could not be found with the particles of chitosan alone (Figure 29).

Freeze-drying procedure for improving the shelf life of the chitosan nanoparticles using various cryoprotective agents was also investigated, and negligible differences between the freeze-dried and fresh particles were found.[184] Alonso and co-workers[185] studied two different types of chitosan in the form of hydro-

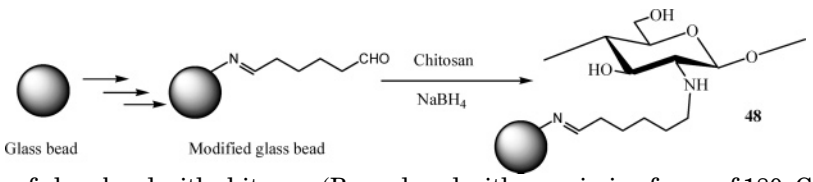


Figure 27. Modification of glass bead with chitosan. (Reproduced with permission from ref 180. Copyright 2002 Elsevier.)

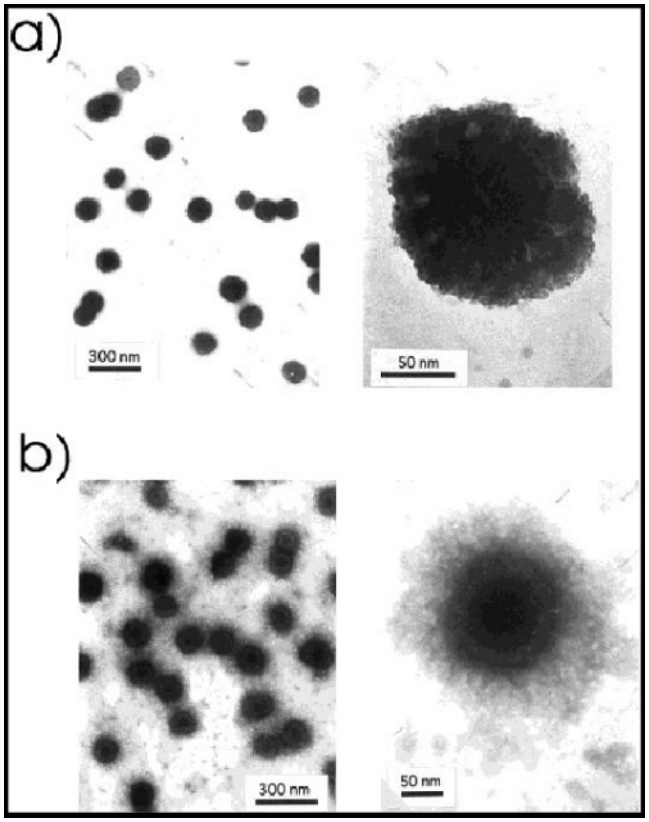


Figure 29. Electron transmission microphotography of (a) chitosan nanoparticles, (b) chitosan/PEO−PPO nanoparticles (concentration of PEO−PPO in the chitosan solution, 10 mg/mL). (Reproduced with permission from ref 182. Copyright 1997 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

chloride salt. The particles were prepared following the above-described methods, leading to a particle size of 300−400 nm with a positive surface charge and entrapment efficiency of 55% [insulin/nanoparticles (w/w): 55/100]. These particles were used to address the difficulties in the nasal absorption of insulin, discussed in the following subsections. They also explored chitosan nanoparticles for entrapment and release studies of the hydrophilic anthracycline drug, doxorubicin (DOX).[186] They approached the problem posed by hydrophilicity and cationic charge of the drug by complexing it with polyanion, dextran sulfate, leading to enhanced drug loading.[187] These nanoparticles were also used for improved delivery of the drugs to the ocular surface, and cyclosporin A (CyA) was used as a model drug.[187] These nanoparticles had a mean size of 293 nm Figure 30, a zeta potential of +37 mV, and high CyA association efficiency and loading (73% and 9%, respectively). Furthermore, in a recent review on colloidal particles as delivery systems for macromolecules, various possibilities for forming particles and their biopharmaceutical applications have been discussed.[188]

Recently, Alonso and co-workers carried out extensive investigations on the design of biodegradable nanoparticles for protein delivery.[189] As a part of this study, they prepared three types of particles of which two were related to chitosan and the third was a PEG-PLA-derived particle. They prepared sole chitosan particles as reported in their previous publications[182−187] and chitosan-coated PLGA-lecithin particles by a critically modified double emulsion method. These particles were intended for either oral or nasal administration. The preparation process of CS−PLGA particles is depicted in Scheme 1. Table 2 shows the properties of various nanoparticles used for comparison purposes in this study.[189]

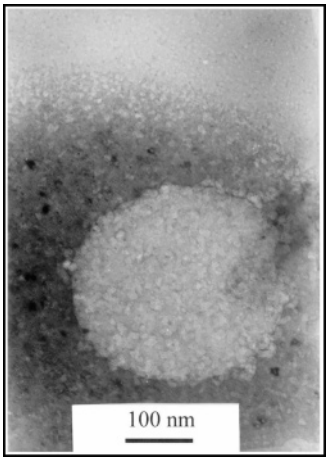
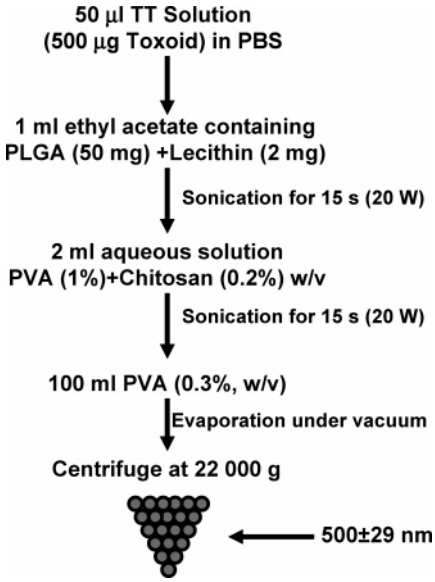


Figure 30. Transmission electron micrograph of the CyA-loaded chitosan nanoparticles. (Reproduced with permission from ref 187. Copyright 2001 Elsevier.)

Scheme 1. Flow Chart Depicting Stepwise Preparation of Chitosan−PLGA Particles



Tian and Groves[190] reported the formulation and biological activity of antineoplastic proteoglycans derived from *Mycobacterium vaccae* in chitosan nanoparticles. They prepared chitosan nanoparticles between 600 and 700 nm without the use of organic solvents. They found that the adsorption and release of bovine serum albumin seemed to be affected by the charge of the two reactants and that at high doses not all adsorbate was released. They observed an initial burst release and a further steady release for 4 h in water.[190]

Ohya et al.[191] reported PEG-grafted chitosan nanoparticles as peptide drug carriers. They observed nanoparticle formation through intermolecular hydrogen bonding in an aqueous solution. The incor-

Table 2. Particle Size, Zeta Potential, Theoretical Loading, and Encapsulation Efficiency Values of CS, PEG−PLA, and CS−PLGA Nanoparticles Containing TT and CS Nanoparticles Containing Insulin[a]

polymer	protein loaded	size (nm)	ζ potential (mV)	theoretical loading (%)	encapsulation efficiency (%)
PLA	tetanus toxoid	192 ± 12	−47.9 ± 1.5	1	36.7 ± 0.3
PEG-PLA	tetanus toxoid	196 ± 20	−23.9 ± 1.2	1	31.1 ± 0.5
CS-PLGA	tetanus toxoid	500 ± 29	+21.8 ± 1.1	1	90.0 ± 3.8
CS	tetanus toxoid	354 ± 27	+37.1 ± 5.9	10	55.1 ± 3.4
CS	insulin	337 ± 14	+36.9 ± 0.3	40	94.7 ± 2.1

[a] Reproduced with permission from ref 189. Copyright 2002 Elsevier.

poration and release of insulin was dependent on the degree of introduction of PEG chain on chitosan and observed sustained release phenomena over time.

Lee et al.[176] reported a novel and simple method for delivery of adriamycin using self-aggregates of deoxycholic-acid-modified chitosan. Deoxycholic acid was covalently conjugated to chitosan via EDC-mediated reaction, generating self-aggregated chitosan nanoparticles. The active component adriamycin was entrapped physically within the nanoparticles, and the formed self-aggregates were analyzed by photon correlation spectroscopy (PCS), fluorescence spectroscopy, and atomic force microscopy. They found self-aggregates are spherical in shape and that the initial drug concentration has an influence on the size of the particles formed. They achieved about 49.6 wt % loading efficiency with slow release phenomena over time in PBS (pH 7.2).[176] Kim et al.[175] explored these self-aggregates of deoxycholic-acid-modified chitosan (DAMC) as DNA carriers. They explained the critical aspects involved in the self-assembly formation of deoxycholic-acid-modified chitosan. Figure 31 shows the simulated structure of chitosan, DAMC, and self-aggregates formed by two and four DAMC molecules, respectively.

Yamamoto et al.[192] reported mucoadhesive liposomes coated with chitosan for drug delivery. They achieved the mucoadhesive formulation by mixing the chitosan solution with a drug-loaded liposomal suspension prepared using a thin lipid film hydration method. A linear correlation was observed between the amount of chitosan used for coating and the percentage adhesion of chitosan-coated liposomes to the intestinal sac. They observed a reduced initial burst release of carboxy fluorescein with the chitosan-coated liposomes, and a further sustained release was observed for about 24 h. They demonstrated that these nanoparticles would serve for insulin release.[192]

Yang et al.[193] investigated the formation of positively charged poly(butyl cyanoacrylate) nanoparticles stabilized by chitosan. They showed that the size of the particles is influenced by various factors such as pH, the concentration and volume of chitosan solution, and the molecular weight of chitosan. Nimodipine was used as a model drug in these studies with a resulting mean particle size of 31.6 nm.

Maitra and co-workers[194] reported a procedure to prepare ultrafine cross-linked chitosan nanoparticles in AOT/*n*-hexane reverse micellar system. They observed that the particle size is influenced by the degree of cross-linking and was found to be 30 nm when 10% of the amino groups in the polymeric chain have been cross-linked, whereas it was 110 nm when all the amino groups were cross-linked; these par-

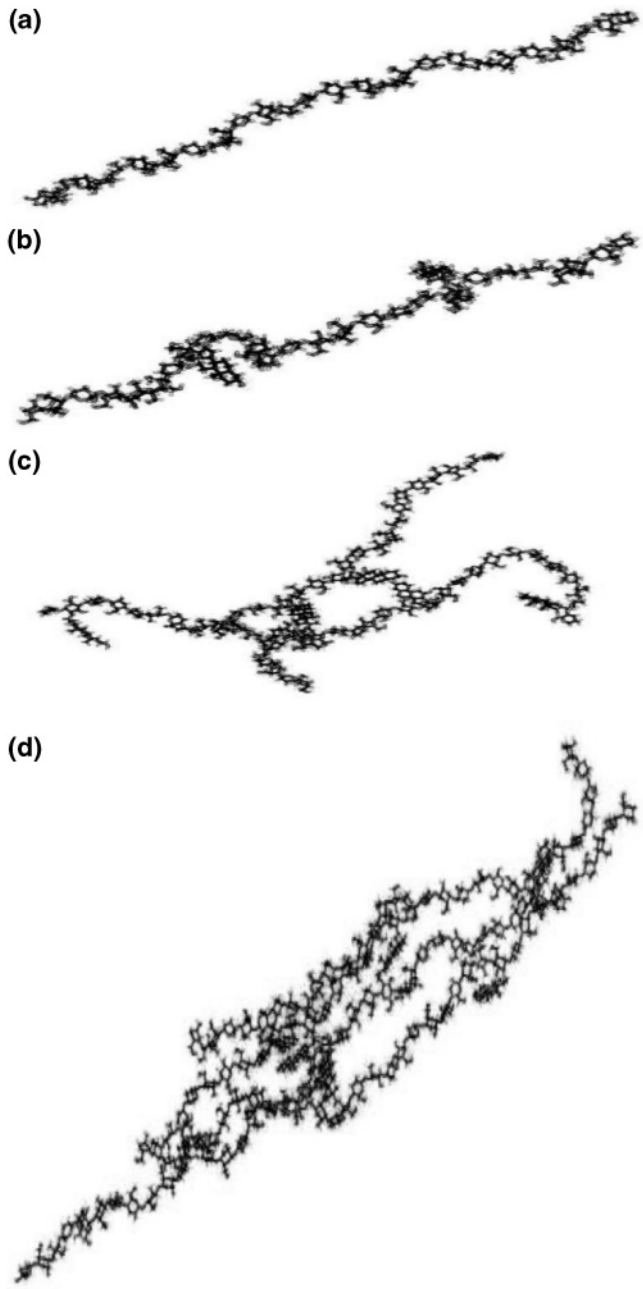


Figure 31. Simulated structure of (a) chitosan and (b) DAMC. (c and d) Simulated structure of assembled self-aggregates formed by two and four DAMC molecules, respectively. (Reproduced with permission from ref 175. Copyright 2001 American Chemical Society.)

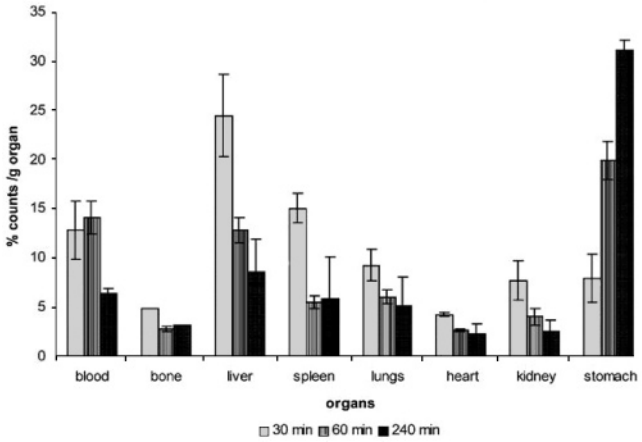


Figure 32. Organ distribution of 99mTc-radiolabeled chitosan nanoparticles, 30, 60, and 240 min postinjection. (Reproduced with permission from ref 194. Copyright 2002 Elsevier.)

ticles were thoroughly characterized. The electron micrographs reveal that the particles were spherical in shape and that lower cross-linking of the particles leads to smaller aggregates, while highly dense aggregates were formed at 100% cross-linking. The biodistribution of the particles after intravenous injection in mice showed that these particles remain in the blood for a considerable amount of time Figure 32 and distribute in the heart, liver, kidneys, bladder, and vertebral column. Other than these organs, the particles were distributed in the bone marrow (Figure 32), leaving the possibility of using these particles for bone imaging and targeting purposes.[194]

Andersson and Löfroth[195] investigated a new microemulsion based on heparin/chitosan complex suitable for oral administration. The microemulsion is based on the ingredients that are acceptable to humans. These microemulsions were studied with or without biologically active ingredients by dynamic light scattering, turbidity, diffusion-NMR, and conductivity. Appropriate mixing and modifications of these microemulsions lead to nanometer-sized heparin/chitosan complexes.[195]

Pan et al.[196] reported chitosan nanoparticles for protein delivery following a reported method[182] with slight modifications. The critical investigations include determination of the formation zone of the nanoparticles, where they used different concentrations of chitosan and TPP. On adding TPP to the chitosan solution under stirring, they observed three different systems, viz., solution, suspension, and aggregates. Varied chitosan concentrations (0.9−3.0 mg/mL) and TPP (0.3−0.8 mg/mL) lead to different particle sizes.

Nanoparticles of methotrexate (MTX) were prepared using *O*-carboxymethyl chitosan (O−CMC) as wall-forming materials and an isoelectric-critical technique under ambient condition.[197] The effects of the MTX/O−CMC ratio and amount of cross-linking agents on drug release in different media were evaluated. The changes of size and effective diameter of O−CMC nanoparticles were detected by SEM and a laser light scattering system before and after drug release. The authors claimed that these nanoparticles constitute an attractive alternative to other anticancer drugs and enzyme carriers.[197]

Kumar et al.[198] reported a emulsion−diffusion−evaporation technique to make cationic nanospheres composed of biodegradable and biocompatible copolyester poly(L-lactide acid-*co*-glycolide) (PLGA). PVA−chitosan blend was used to stabilize the PLGA nanospheres. The nanospheres have cationic surface charge and can readily bind DNA electrostatically. One of the noticeable finding from these investigations is that PVA is the most essential component required to stabilize the PLGA nanoparticles; however, the unbound PVA is hard to eliminate after several washings. On the other hand, chitosan alone could not stabilize the particles formed. Therefore, a blend of PVA−chitosan was used, leading to monodispersed cationic PLGA nanoparticles with no unbound PVA−chitosan blend Figure 33.

Since the first reports on nanoparticles for drug/vaccine delivery about 3 decades ago, a considerable amount of work has been done. It is quite understandable that nanoparticles have distinct advantages as far as pharmaceutical applications are concerned; however, they suffer from problems in scalability and in some instances reproducibility. It is very unfortunate that we are still at infancy in this area of research.

4.2. Microspheres

As a drug carrier chitosan helps overcome certain adverse characteristics of drugs such as insolubility and hydrophobicity, but the semicrystalline powder does not lend itself to direct compression. Nevertheless, chitosan powders have been evaluated in direct compression tests, and the formulations so far de-

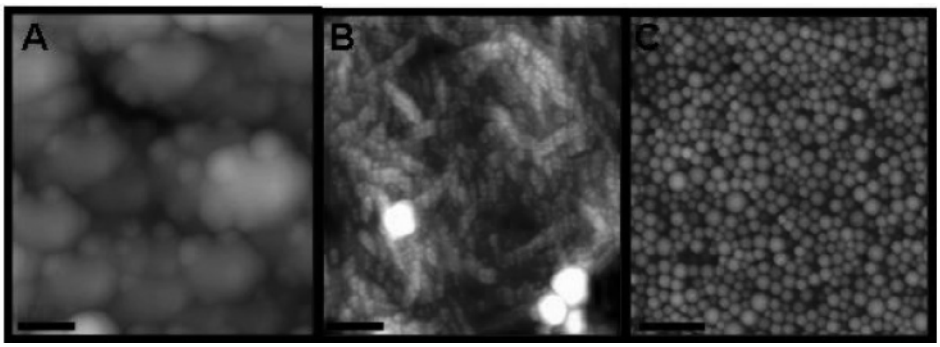


Figure 33. Atomic force microscope images of (A) nanospheres with PVA alone as stabilizer, (B) nanospheres with chitosan alone, (C) PVA−chitosan blend (bar = 150 nm). (Adapted with permission from ref 198. Copyright 2004 Elsevier.)

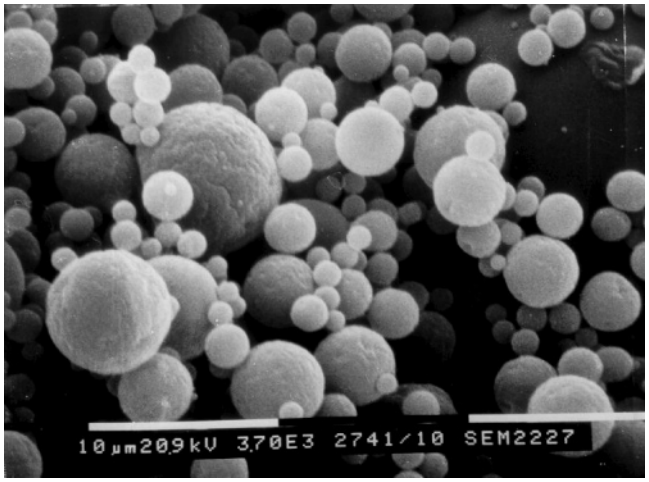


Figure 34. Methylpyrrolidinone chitosan microspheres obtained by spray drying.

veloped include excipients to facilitate compression.[199,200] For example, commercial chitosan tablets for weight control contain magnesium stearate as a binder with negative consequences on the efficacy of chitosan.

4.2.1. Preparation of Microspheres by Spray Drying

Spray drying includes four sequential stages: atomization through a spray nozzle, contact of the sprayed feed with warm air, drying of the droplets, and collection of the solid chitosan Figure 34. Chitosan solutions with drug can be fed to a spray drier at a slightly acidic pH. The size of the particles is influenced by various process parameters such as size of the nozzle, rate of feeding, and inlet air temperature. When acetone is convenient for dissolving certain drugs, aqueous solutions may contain some organic solvent, for instance, a 0.5% chitosan solution may be made with a mixture of water, glacial acetic acid, and acetone in 49.5/0.5/50 (v/v).

One point concerning exposure of the sample to hot air needs to be explained: the inlet air temperature is measured prior to flowing into the drying chamber and may be set at 160 °C or higher; however, the gradient between the wet surface and unsaturated gas actually leads to evaporation at much lower temperatures.

4.2.2. Spray-Dried Chitosans

The spray-drying technique has been applied to chitosan suspensions, chitosan salts, chitosan gelatin−ethylene oxide, and chitosan−ethylcellulose mixture. A variety of chitosan salt solutions were spray dried, leading to microspheres having diameters between 2 and 5 μm with improved binding functionality. The chitosan microsphere free-flowing powder is compressible and hence most suitable as a drug carrier.[201−209] The following are some examples.

Chitosan microspheres were prepared by spray drying using type-A gelatin and ethylene oxide−propylene oxide block copolymer as modifiers. The particle shape, size, and surface morphology of microspheres and the in vitro drug release were significantly affected by the concentration of gelatin. Betamethasone disodium phosphate (BTM)-loaded microspheres demonstrated good drug stability (<1% hydrolysis product), high entrapment efficiency (95%), and positive surface charge. Formulation factors were correlated to particulate characteristics for optimizing BTM microspheres in pulmonary delivery.[206]

By properly choosing excipient type and concentration and varying the spray-drying parameters, a high degree of control was achieved over the physical properties of the dry chitosan powders. The betamethasone release rates were influenced by the drug/polymer ratio and found to increase when the drug loading was decreased. The in vitro release of betamethasone showed a dose-dependent burst followed by a slower release phase that was proportional to the drug concentration in the range 14−44% w/w.[206]

Chitosan microspheres containing chlorhexidine diacetate, an antiseptic, were prepared by a spray drying. Chlorhexidine from the chitosan microspheres dissolved more quickly in vitro than chlorhexidine powder. The minimum inhibitory concentration, minimum bacterial concentration, and killing time showed that loading of chlorhexidine into chitosan was able to maintain or improve the antimicrobial activity of the drug, the improvement being particularly high against *Candida albicans*. It should be noted that the drug did not decompose despite its thermal lability above 70 °C. Buccal tablets were prepared by direct compression of the microspheres with mannitol alone or with sodium alginate. After their in vivo administration, determination of chlorhexidine in saliva showed the capacity of these formulations to give a prolonged release of the drug in the buccal cavity.[210]

An inclusion complex composed of progesterone and hydroxypropyl-β-cyclodextrin (HPCD) was prepared by the spray-drying and freeze-drying methods. Progesterone alone and its inclusion complex with HPCD were incorporated into chitosan by spray drying and freeze drying. Release data showed significant improvement of the dissolution rate of progesterone, and a controlled release was obtained in the presence of chitosan.[211]

Gemfibrozil, an acidic antihyperlipoproteinemic drug, was covalently linked to poly[α,β-(*N*-2-hydroxyethyl-D,L-aspartamide)] (PHEA) and poly [α,β-(*N*-3-hydroxypropyl-D,L-aspartamide)] (PHPA) by an ester linkage. Microspheres composed of different molecular weight chitosans alone or as a mixture with 2-hydroxypropylmethyl cellulose (HPMC), PHEA, or PHPA and with different polymer/drug ratios (2:1 and 3:1, w/w) were prepared by spray drying. In vitro dissolution tests showed that microspheres were always suitable to modulate gemfibrozil release and that the best conditions were achieved by microspheres composed of the low molecular weight chitosan combined with PHPA/HPMC with either a 2:1 or 3:1(w/w) polymer/drug ratio.[212]

The general chemical behavior of chitosan, however, should be considered in order to avoid certain difficulties stemming from its insolubility at pH higher than 6.5 and its reactivity under the thermal conditions of the sprayer. For example, spray drying of 1−2% chitosan in acid solution at 168 °C seems to be easy; however, the release of a drug from the

spray-dried chitosan depends on the acetic acid concentration due to the acetylation reaction occurring at the temperature to which the salt is exposed, certainly lower than 168 °C but significantly high for side reactions to occur. In fact, the degree of acetylation of chitosan increased during spray drying affected its enzymatic degradability.[208]

Chitosan can be spray dried at neutral pH if a colloidal suspension is prepared with NaOH. Nevertheless, this preparation is time consuming due to the difficulties involved in washing and removal of excess alkali and salts. Chitosan has been recently found to be soluble in alkaline media, viz., NH_4HCO_3 solutions, by forming ammonium carbamate (Chit-$NHCO_2^-NH_4^+$), i.e., a transient anionic form that keeps it soluble at pH 9.6, while reversibly masking the polycationic nature of chitosan. Because ammonium carbamates and NH_4HCO_3 decompose thermally and liberate CO_2, NH_3, and water, this alkaline system is perfectly suitable for producing chitosan microspheres by spray drying.[213]

4.2.3. Spray-Dried Polyelectrolyte Complexes

Chitosan forms polyelectrolyte complexes with polyanions, such as 6-oxychitin, heparin, carrageenan, pectin, xanthan, acacia gum, hyaluronic acid, alginic acid, poly(acrylic acid), carboxymethyl cellulose, DNA, and other macromolecules.[205,214−227] These reactions are very fast and in general lead to the immediate disordered precipitation of the insoluble coacervates upon mixing, particularly when the final pH value is neutral or higher. The polyanions are normally in the form of sodium salt and show alkaline hydrolysis that contributes to chitosan insolubilization upon mixing. Some examples are discussed below.

Continued blood anticoagulation is achieved by subcutaneous administration of low molecular weight heparin or with an orally active anticoagulant such as warfarin. An oral heparin would avoid the inconvenience of subcutaneous injections and adverse events associated with warfarin. Alginate beads with entrapped heparin were enteric coated with chitosan and cellulose acetate phthalate via carbodiimide. No significant release of heparin was observed from enteric-coated microspheres (12%) during treatment with 0.1 M HCl, pH 1.0, for 4 h, but acid-treated capsules released almost all the entrapped heparin into Tris-HCl, pH 7.4, media within 6 h. The formulation provided controlled release of heparin thanks to the polyelectrolyte complexes formed by chitosan with alginate and heparin.[228]

Microparticles containing gentamicin, an antibacterial, were prepared using chitosan, hyaluronan, and a combination of both polymers by a solvent evaporation method. These formulations were administered nasally via an insufflator. The microparticulate systems composed of chitosan and chitosan + hyaluronan considerably enhanced the bioavailability of gentamicin (31.4% and 42.9%, respectively) with hyaluronan microparticles inducing a less significant enhancement (23.3%). Previous in vitro dissolution and studies on frog palate indicated that these microparticulate formulations were all mucoadhesive. Furthermore, these particles demonstrated prolonged drug release and better bioavailability of gentamicin when compared with a gentamicin solution administered nasally in vivo. In the chitosan + hyaluronan formulation, the polymers appeared to improve the absorption of incorporated gentamicin synergically in comparison to the individual polymers.[229]

The diffusion of a model protein, bovine serum albumin, from dried chitosan-coated alginate microcapsules was studied. Factors tested included alginate and chitosan concentration, calcium concentration in the gelation medium, loading rate, chitosan molecular mass, and pH of the gelation medium as well as washing procedure.[230]

Alginate/chitosan particles were prepared by ionic gelation (Ca^{2+} and Al^{3+}). The release of sodium diclofenac was prevented at acidic pH, while it was complete in a few minutes at pH 6.4 and 7.2. The alginate/chitosan ratio and the nature of the cation controls the release rate of the drug.[231]

Chitosan gel beads were prepared in 10% amino acid solution (pH 9) and modified by forming an electrostatic complex between the amino group of chitosan and the carboxyl group of chondroitin sulfate. The in vitro release of prednisolone from the gel beads was studied by implanting into air pouches prepared subcutaneously on the dorsal surfaces of mice. No inflammatory response was observed. The in vivo release of prednisolone from the gel beads and their biodegradation were slower than in controls.[232]

From the foregoing studies it is evident that preparation of microspheres by spray drying is not an easy task. The difficulty is due to immediate coacervation of polyelectrolytes of opposite charges upon mixing, and the resulting suspensions clog the sprayer and prevent production of microspheres. As seen above, some alternative methods have also been tried to make chitosan−polyanion microparticles. One of the major hurdles in preparing chitosan−polyanion microparticles by spray-drying technique is that chitosan itself is insoluble at pH values above 6.3; for instance, it is known that phase separation occurs when hyaluronan is mixed with chitosan, particularly for stoichiometric ratios (SR = $-NH_2/-$COOH) close to 1.0.

The complex formation corresponds to charge neutralization at least partially; therefore, the degree of complexation by charge neutralization is usually provided. For the system chitosan + hyaluronan, the degree of complexation is ca. 1.0 regardless of the stoichiometric ratio value (provided SR > 1); moreover, the degree of complexation is nearly independent of the degree of acetylation of chitosan up to 0.40. For higher degrees of acetylation (randomly reacetylated chitosans) the complexation mechanism involves less cooperation. The chitosan−hyaluronan insoluble complexes are stable in acidic and alkaline media: they do not dissolve in NaOH but dissolve in 0.2 M HCl. The complex is destabilized in NaCl brines.[233]

4.2.4. Use of Chitosan Carbamate Ammonium Salt

Chitosan hydrochloride salt solution (10 g, containing 1.00 g of chitosan and a stoichiometric amount

of HCl) was poured into a saturated NH_4HCO_3 solution (prepared from 40 mL of water and 9.6 g of salt; 20 °C; final pH 9.6) and incubated at 20 °C for 5 days with no stirring to react according to the following equation

$$\text{Chit-NH}_2 + \text{NH}_4\text{HCO}_3 = \text{Chit-NHCO}_2^-\text{NH}_4^+ + \text{H}_2\text{O}$$

Additions of NH_4HCO_3 were made on the second and fourth day. The rigid and transparent hydrogel was separated by centrifugation at 12 000 rpm and kept at 4 °C (ca. 10 g); no syneresis and no microbial growth occurred over 30-day storage at 4 °C.

The chitosan carbamate ammonium salt prepared from chitosan hydrochloride was poured into a 4-fold weight of water and stirred to obtain a clear solution that was immediately submitted to spray drying to obtain amorphous chitosan-free base, as demonstrated by instrumental analysis. The expanded form of these microspheres was revealed by their bulk density (0.025 g/cm^3), which was much smaller compared to microspheres from systems containing acids other than HCl.[213]

For the preparation of spray-dried polyelectrolyte complexes, the polyanion (alginate, carboxymethyl cellulose, 6-oxychitin, carboxymethyl guar, hayluronic acid, and others) was dissolved in dilute NH_4HCO_3 solution and mixed with the chitosan carbamate solution just before spray drying. The excess NH_4HCO_3 decomposed thermally between 60 and 107 °C; on the other hand, the carbamate function released carbon dioxide under the effect of the temperature at which the spray drier was operated, thus regenerating chitosan at the very moment of the polyelectrolyte microsphere formation.

$$\text{Chit-NHCO}_2^-\text{NH}_4^+ \xrightarrow{\text{heat}} \text{Chit-NH}_2 + \text{CO}_2 + \text{NH}_3 \text{ carbamate decomposition}$$

$$\text{NH}_4\text{HCO}_3 \xrightarrow{60-107\ ^\circ\text{C}} \text{CO}_2 + \text{NH}_3 + \text{H}_2\text{O} \text{ salt decomposition}$$

In most cases the microspheres were insoluble. The polysaccharides might be partially cross-linked via amido groups formed by the carboxyl groups of the polyanion and the restored free amino group of chitosan. The susceptibility to enzymatic hydrolysis by lysozyme was poor, mainly because lysozyme, a strongly cationic protein, can be inactivated by anionic polysaccharides.[234]

Despite the chemical differences (alcohol groups in guaran, carboxyl groups in xanthan, and partially esterified carboxyl groups in pectin), these three polysaccharides on combination with chitosan in the microspheres appear to be able to bring chitosan into solution. This is particularly interesting if one considers the solubility of these three polysaccharides in water and their important applications in the food and pharmaceutical industries.

4.2.5. Preparation of Microspheres by Multiple Emulsion/Solvent Evaporation and Coacervation Methods

This multiple emulsion technique includes three steps: (1) Preparation of a primary o/w emulsion in which the oil-dispersed phase is constituted of CH_2Cl_2 and the aqueous continuous phase is a mixture of 2% v/v acetic acid solution:methanol (4/1 v/v) containing chitosan (1.6%) and Tween (1.6 w/v); (2) Multiple emulsion formation with mineral oil (oily outer phase) containing Span 20 (2% w/v); (3) Evaporation of aqueous solvents under reduced pressure. Details can be found in various publications.[215] Chemical cross-linking is an option of this method if the cross-linking agent is added just after the emulsion formation; enzymatic cross linking can also be performed.[235] Physical cross-linking may take place to a certain extent if chitosan is exposed to high temperature.

The emulsion technique is convenient when the drug is particularly sensitive to certain parameters connected to the spray drying. The emulsion technique may be associated to cross-linking or other treatments of the microspheres. The following examples are self-explanatory.

Microspheres of polyacrylamide-grafted−chitosan were used to encapsulate indomethacin, a nonsteroidal antiinflammatory drug (NSAID). The microspheres were produced by the water-in-oil emulsion technique and cross-linking with glutaraldehyde. The release of indomethacin is dependent upon the degree of cross-linking and on the amount of drug loading. This was further supported by the calculation of drug-diffusion coefficients.[236]

Microspheres of chitosan, cross-linked with glutaraldehyde, sulfuric acid, or heat treatment, have been prepared to encapsulate diclofenac sodium. Chitosan microspheres were produced in water-in-oil emulsion followed by cross-linking in the water phase. The cross-linking of chitosan took place at the free amino group in all cases and lead to the formation of imine groups or ionic bonds. Polymer crystallinity increased after cross-linking. Microspheres had smooth surfaces, with sizes in the range 40−230 μm. Loading of diclofenac sodium has been carried out by soaking the already swollen cross-linked microspheres in a saturated solution of diclofenac sodium. Up to 28−30% w/w loading was observed for the sulfuric acid cross-linked microspheres, whereas 23−29% and 15−23% of loadings were obtained for the glutaraldehyde and thermally cross-linked microspheres, respectively. The in vitro release studies were performed in 7.4 pH buffer solution. The 32% glutaraldehyde cross-linked microspheres showed the slowest release, i.e., 41% at 420 min, and the fastest release of 81% at 500 min by thermal cross-linking for 3 h.[237]

Chitosan microspheres containing 5-fluorouracil (5-FU), tegafur (FT), and doxifluridine (DFUR) were prepared by the dry-in-oil method using silicone oil with no surfactant as a dispersion medium. The fairly large chitosan microspheres had drug contents of 4−22% (w/w).[238]

Eudragit RS microspheres containing chitosan hydrochloride were prepared by solvent evaporation using acetone/liquid paraffin solvent system, and their properties were compared with Eudragit RS microspheres without chitosan. The content of pipemidic acid, an antibacterial, increased in larger microspheres as a consequence of accumulation of undissolved pipemidic acid particles in larger drop-

lets. Pipemidic acid release was faster from microspheres with chitosan.[239]

Extensive investigations on pH-sensitive chitosan microspheres were carried out.[240−245] Diclofenac sodium, thyamine hydrochloride, chlorphenramine maleate, and Isoniazid were used as model drugs in these investigations. In these studies, widely used products in medical and pharmaceutical areas, viz., glycine and poly(ethylene glycol), were employed as spacer groups to enhance the flexibility of the polymer networks and influence the swelling behavior through macromolecular interactions. The procedure is based on adding drugs to chitosan solution, and microspheres were prepared by simple coacervation. The swelling behavior, solubility, hydrolytic degradation, and drug loading capacity of the beads were investigated.[242,243] Effect of the cross-linker on microsphere properties was studied by varying the amounts of cross-linker.[243] The microspheres exhibit high pH sensitivity. The swelling ratio of the microspheres at pH 2.0 is higher than that at pH 7.4. This pH-sensitive swelling is due to the transition of bead network between the collapsed and expanded rates, which is related to ionization degree of amino groups on chitosan in different pH solutions. Chitosan−PEG system showed faster release rates, when compared to the chitosan−glycine system, due to the water diffusivity and pore forming properties of PEG; however, the drug release continued beyond 48 h in both cases. The effects of the amount of drug loaded and cross-linking agent on the delivery profiles were reported as well.[240−245] Kumar[246] reviewed various applications of chitin and chitosan with an emphasis on pharmaceutical drug delivery systems.

4.3. Hydrogels

Gel materials are utilized in a variety of technological applications and are currently investigated for advanced exploitations such as the formulation of "intelligent gels" and the synthesis of "molecularly imprinted polymers".

A typical simple example of gel formation was provided with chitosan tripolyphosphate and chitosan polyphosphate gel beads. pH-responsive swelling ability, drug-release characteristics, and morphology of the chitosan gel bead depend on polyelectrolyte complexation mechanism and molecular weight of the hydrolyzed chitosan.[247] The complexation mechanism of chitosan beads gelled in pentasodium tripolyphosphate or polyphosphoric acid solution was ionotropic cross-linking or interpolymer complex, respectively. The chitosan−polyphosphoric acid gel bead is a better polymer carrier for the sustained release of anticancer drugs in simulated intestinal and gastric juice medium than the chitosan−tripolyphosphate gel beads. Pet food based on these gels has been developed. The good performances of chitosan itself and its derivatives in the dietary food and in the pharmaceutical areas, accompanied by the more thorough understanding of the chemistry of chitosan-based gels, would certainly lead to increasing applications soon. This biopolymer, for its unique cationic nature, has gained a position among the hydrocolloids.

One of the simplest ways to prepare a chitin gel is to treat chitosan acetate salt solution with carbodiimide to restore acetamido groups. Thermally not reversible gels are obtained by *N*-acylation of chitosans: *N*-acetyl-, *N*-propionyl-, and *N*-butyryl chitosan gels are prepared using 10% aqueous acetic, propionic, and butyric acids as solvents for treatment with appropriate acyl anhydride. Both *N*- and *O*-acylation are found, but the gelation also occurs by selective *N*-acylation in the presence of organic solvents, such as methanol, formamide, and ethylene glycol. The importance of many variables has been studied by determining their effects on the gelation, such as chitosan and acyl anhydride concentrations, temperature, molecular weight of acylating compound, and extent of *N*-acylation. Probably the gelation is due to the aggregation of chitosan chains through hydrophobic associations. Applications for *N*-acylchitosan gels are reported in the literature.[248]

Chitosan gels can also be prepared by using large organic counterions. The process involves mixing of heated solutions of chitosan acetate and sodium salt of either 1-naphthol-4-sulfonic acid (NSA) or 1-naphthylamine-4-sulfonic acid, followed by cooling. The chitosan concentration required for gel formation is low, about 2−5 g/L, and similar to the concentrations used for gel formation with other polysaccharides such as the carrageenans.

Gellike properties were found in *N*-carboxymethyl chitosan: this behavior was explained in terms of association of ordered chains into a cohesive network, analogous to that in normal gels but with weaker interactions between associating chains, i.e., a weak gel.[50]

Chitosan gel beads could be prepared in amino acid solutions of pH about 9, despite the requirement for a pH above 12 for gelation in water.[249] This phenomenon was observed not only in amino acid solutions but also in solutions of compounds having amino groups. A solute concentration of more than 10% was required for preparation of gel beads at pH 9. Gelation of the chitosan beads required about 25−40 min, depending on the species of amino acid.

Novel pH-sensitive hydrogels were synthesized by grafting D,L-lactic acid onto the amino groups in chitosan without a catalyst; polyester substituents provide the basis for hydrophobic interactions that contribute to the formation of hydrogels.[167,250] The swelling mechanisms in enzyme-free simulated gastric fluid (SGF, pH 2.2) or simulated intestinal fluid (SIF, pH 7.4) at 37 °C were investigated. The crystallinity of chitosan gradually decreased after grafting, since the side chains substitute the $-NH_2$ groups of chitosan randomly along the chain and destroy the regularity of packing between chitosan chains. Water uptake of the hydrogels was investigated as a function of side-chain length and degree of substitution. The influence of pH and salt concentration on the swelling behavior of the hydrogels was determined and interpreted.

Stable and self-sustaining gels were obtained from tyrosine glucan (a modified chitosan synthesized by reaction of chitosan with 4-hydroxyphenylpyruvic acid) in the presence of tyrosinase that oxidizes the phenol to quinone, thus starting cross-linking with residual free amino groups. Gels were also obtained

with 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde.[52,235]

As an extension of these works, a mushroom tyrosinase was observed to catalyze the oxidation of phenolic moieties of the synthetic polymer poly(4-hydroxystyrene) (PHS) in water−methanol. Although oxidation was rapid, only a small number of phenolic moieties of the PHS polymer (1−2%) underwent oxidation. Enzymatically oxidized PHS was observed to undergo a subsequent nonenzymatic reaction with chitosan. Ultraviolet spectra of this chitosan film suggested that oxidized PHS was grafted onto chitosan.[251,169]

Further progress[252,253] led to internally skinned polysulfone capillary membranes coated with a viscous chitosan gel and useful as immobilization matrix for polyphenol oxidase. Bench-scale, single-capillary membrane bioreactors were used to determine the influence of the chitosan coating on product removal after substrate conversion by immobilized polyphenol oxidase during treatment of industrial phenolic effluents. The results indicate that greater efficiency was achieved in the removal of polyphenol oxidase-generated products by the chitosan membrane coating as compared with chitosan flakes.

A very popular cross-linking agent for chitosan is glutaraldehyde, as proposed by Muzzarelli et al.[254] Chitosan networks were obtained by reaction with glutaraldehyde in lactic acid solution (pH 4−5) at molar ratio amino groups/carbonyl functions about 10−20: reduction gave stable chemical gels. Arguelles-Monal et al.[255] studied the rheology of the chitosan−glutaraldehyde gel system. By reaction of chitosan with aldehydes, *N*-alkylidene- or *N*-arylidene chitosan gels are produced: the extent of modification of the amino groups is about 80%, and the minimum amount of aldehyde required for gel formation increases by increasing the aldehyde molecular weight. The gels are colorless, rigid, and infusible up to 200 °C. By using glutaraldehyde, chemical gels are produced following cross-linking interchains: the rate of gelation depends on the chitosan and glutaraldehyde concentrations, temperature, and addition of neutral electrolytes.[256]

Investigations on novel biocompatible hydrogels based exclusively on polysaccharide chains were reported, where chitosan was linked with dialdehyde obtained from scleroglucan by controlled periodate oxidation.[257] The reaction takes place at pH 1,0 and reduction of the resulting Schiff base is performed with $NaCNBH_3$. The swelling capacity of the hydrogel is remarkable, which is dependent on the highly hydrophilic character of both polysaccharides and the pH of the bathing solutions.

Semi-interpenetrating network was synthesized with chitosan, cross-linked by glyoxal, and poly(ethylene oxide).[258] Chitosan was characterized by its degree of deacetylation, determined by infrared spectroscopy, and its average viscometric molecular weight. Swelling studies were performed on the chitosan/poly(ethylene oxide) semi-interpenetrating network and the reference hydrogel (cross-linked chitosan) at pH 1.2 and 7.2. The semi-interpenetrating network displayed a high capacity to swell, adjustable by pH. Rheological studies performed in simple shearing and oscillation showed that the semi-interpenetrating network had elastic properties. Young modulus was determined by texture analysis, in uniaxial compression and indentation. Comparison between the semi-interpenetrating network and the reference gel, regarding the mechanical and swelling properties, demonstrates the interest of the addition of poly(ethylene oxide).

Poly(ethyleneglycol) dialdehyde diethyl acetals of different molecular sizes were synthesized and used to generate in situ PEG dialdehydes for the cross-linking of partially reacetylated chitosan via Schiff's reaction and hydrogenation of the aldimines. The water-soluble products obtained were thoroughly characterized. Upon freeze drying, they aggregated to yield insoluble soft and spongy biomaterials that swelled immediately upon contact with water. When exposed to papain and lipase, at physiological pH values, progressive dissolution of the biomaterials was observed, but no dissolution took place with lysozyme, collagenase, and amylase. They were found to be biocompatible toward Caco-2 cells. These cross-linked partially acetylated chitosans seem to be suitable for medical applications when prompt resorption is sought.[259]

At a high degree of protonation of the amino groups, the cationic chitosan spontaneously forms macromolecular complexes by reaction with anionic polyelectrolytes. These complexes are generally water insoluble and form hydrogels. Several reviews on polyelectrolytes have been published.[260,261] A variety of polyelectrolytes can be obtained by changing the chemical structure of component polymers, such as molecular weight, flexibility, functional group structure, charge density, hydrophilicity and hydrophobicity, stereoregularity, and compatibility, as well as changing reaction conditions, such as pH, ionic strength, polymer concentration, mixing ratio, and temperature. This, therefore, may lead to a diversity of physical and chemical properties of the complexes.

Polyelectrolytes of chitosan with other polysaccharides, proteins, DNA, and synthetic and inorganic polymers were investigated. A hydrogel with high sensitivity was prepared with chitosan (DA = 0.18) and dextran sulfate:[262] the maximum volume of the complex gel was observed in a dilute NaOH solution at pH 10.5 and was about 300 times as large as the volume at pH values below 9. This behavior, probably due to both equal and high densities of amino and sulfate groups and flexibility of anionic polymer chains, might be useful in various application fields.[263]

Microcapsules can be used for mammalian cell culture and the controlled release of drugs, vaccines, antibiotics, and hormones. To prevent the loss of encapsulated materials, the microcapsules should be coated with another polymer that forms a membrane at the surface. A most well-known system is the encapsulation of the alginate beads with poly-L-lysine. A covalently cross-linked superswelling sodium alginate gel type was also investigated for encapsulation of insulin producing cells.[264] Because poly-L-lysine has some limitations due to high cost and toxicity, systems of alginate beads coated with

chitosan at different DA or its *N*-acyl derivates have been developed.[265] The microcapsules were prepared in one step by extrusion of a solution of the drug and sodium alginate into a solution containing calcium chloride and chitosan through interpolymeric ionic interactions. The drug release during the storage of microcapsules in saline was found to be pH dependent.

Recently, calcium-induced alginate gel beads containing chitosan were prepared using nicotinic acid to investigate the release of the vitamin and the uptake of bile acids into the complex, after oral administration.[266] The possible applicability of chitosan-treated alginate beads as a controlled release system of small molecular drugs with high solubility was investigated.[267] The beads were prepared by the ionotropic gelation method, and the effect of various factors (alginate, chitosan, drug and calcium chloride concentrations, the volume of external and internal phases, and drying methods) on bead properties were also investigated. Spherical beads with 0.78−1.16 mm diameter range and 10.8−66.5% encapsulation efficiencies were produced. Higher encapsulation efficiencies and retarded drug release were obtained with chitosan-treated alginate beads. It appeared that chitosan-treated alginate beads may be used for a potential controlled release system of small molecular drugs with high solubility, instead of alginate beads.

Risbud and Bhonde[268] studied polyacrylamide−chitosan hydrogels for sustained release of antibiotics in an attempt to explore the favorable properties of both polyacrylamide and chitosan. Hydrogels were prepared by cross-linking acrylamide−chitosan mixture (8:2 v/v) with *N*,*N*′-methylene bisacrylamide and characterized further by SEM and octane contact angle measurements. Swelling, cytotoxicity, and release studies of the hydrogels were performed. Amoxicillin was used as a model drug. They found the hydrogels to be porous with a pore size of ~104 ± 7.61 μm and also observed matrix degradation after the drug was released from the hydrogels at pH 7.4. They observed no pH-dependent swelling; however, a swelling ratio of about 16.0 was found in all the buffers studied at the end of a 24 h period. About 56% of the drug was released in the 24 h period and 77% release in a 75 h period, showing sustained release nature of the matrix.[268]

Chenite and co-workers[269−272] reported novel thermosensitive chitosan gels possessing a physiological pH that can be held liquid below room temperature for encapsulating living cells and therapeutic proteins; they form monolithic gels at body temperature. The first report is all about the transformation of pH-gelling cationic polysaccharide solutions into thermally sensitive pH-dependent gel-forming aqueous solutions without any chemical modification or cross-link.[269] The gel formation was due to the addition of polysalts bearing a single anionic head, such as glycerol−, sorbitol−, fructose−, or glucose−phosphate salts (polyol− or sugar−phosphate). They found the chitosan/glycerol phosphate (C/GP) gels are well suitable for the delivery of sensitive biological materials such as proteins and living cells. The subcutaneous injections of gels formulated with a bone-inducing growth factor (BP) demonstrate that the C/GP gels can deliver active BP leading to de novo cartilage and bone formation in an ectopic site. Subcutaneous gel implants had bean-like shape and remained morphologically intact when excised at 3, 6, 12, 24, 48, and 72 h and 7 days. Studies were also performed by encapsulating various cell lines in an attempt to demonstrate the cytocompatibility of the C/GP gels and found more than 80% of the cell viability over extended period of time when cultured in vitro. In the subsequent studies the characterization of gels for the sustained delivery of drugs,[270] rheological characterization,[271] and effect of steam sterilization on thermogelling were performed.[272]

Molinaro et al.[273] evaluated the biocompatibility of the four chitosan/GP solutions in vivo and concluded that a higher degree of deacetylation of the chitin chain is desirable for superior biocompatibility. Ruel-Garièpy et al.[274] reported thermosensitive chitosan-based hydrogel-containing liposomes for the delivery of hydrophilic molecules. The so developed system is found capable of releasing macromolecules over a period of several hours to a few days in a controlled manner; however, with low molecular weight lipophilic compounds, the release is completed within 24 h. They proposed a mathematical model to characterize the release kinetics.[271] Cascone and Maltinti[275] reported hydrogels based on chitosan and dextran for the release of human growth hormone.

Cerchiara et al.[276] reported physically cross-linked chitosan hydrogels as topical vehicles for hydrophilic drugs. The drug-loaded hydrogels were prepared in a two-step synthesis, where the first process involves the preparation of physically cross-linked chitosan hydrogels and the second step involves incorporation of the drug. The in vitro release studies were carried out using porcine skin. From the detailed investigations it was found that the physically cross-linked chitosans with lauric and myristic acids increased the skin permeation of the drug with respect to the liquid solution or suspension. The increase of absorption rate was assumed to be due to interaction of the polymer with the stratum corneum.

Matrix granules containing microcrystalline chitosan were investigated as a gel-forming excipient in human volunteers with ibuprofen and furosemide as model drugs. The results of the bioavailability studies indicate that microcrystalline chitosan matrix granules allow a simple preparation of slow-release and perhaps stomach-specific dosage forms.[277]

Kim et al.[278] reported water and temperature response of semi-IPN hydrogels composed of chitosan and polyacrylonitrile cross-linked with glutaraldehyde and found that water uptake of hydrogels is temperature dependent.

4.4. Films

The choice of biomaterials suitable for forming the carrier film matrix and the barrier films were dictated by various factors, viz., (a) compatibility with the gastric environment, (b) stability during the time of drug delivery, (c) adequate mechanical properties, (d) ease of fabrication and cost, and (e) no appreciable

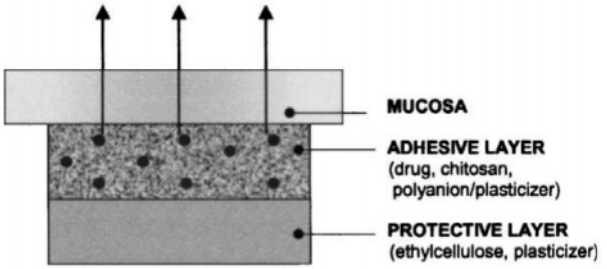


Figure 35. Schematic explanation of the structure of mucoadhesive bilayers. (Reproduced with permission from ref 281. Copyright 1998 Elsevier.)

swelling in water and softening point above 37 °C. Several polymers were found to fill all or most of the above criteria and used to prepare carrier films. Often some plasticizers and excipients were used along with the base polymer to impart a suitable degree of flexibility and facilitate diffusion of the drug from the films, respectively. Chitosan possesses good film-forming properties, and an update on chitosan films for pharmaceutical applications is presented here.

Tomihata and Ikada[279] reported in vitro and in vivo degradation profiles of chitin and chitosan films by solution casting method using specimens of varied deacetylation degrees, viz., 68.8%, 73.3%, 84.0%, 90.1%, and 100%; the thickness of the films was 150 μm. The equilibrated water content of the films decreased with increase in deacetylation, while the tensile strength of the water-swollen films increased with increasing deacetylation. The maximum water content and minimum tensile strength observed for a specimen deacetylated between 0 and 68.8 mol % may be ascribed to the lowered crystallinity by deacetylation of chitin, since both chitin and chitosan are crystalline polymers. Unlike their physical properties, in vitro and in vivo degradations of these films occurred less rapidly without passing a maximum or minimum, as their degree of deacetylation became higher. The degradation of the films was studied in vivo as subcutaneous implants. It was found that the rate of in vivo biodegradation was very high for chitin compared with that for deacetylated chitin. The films which were more than 73.3% deacetylated showed slower biodegradation. No noticeable tissue reactions were found with highly deacetylated derivatives including chitosan, although they had cationic primary amines in the molecule.

Recently, there has been a growing interest in grafting of vinyl monomers onto chitosan for biomedical and industrial applications.[163] This chemical combination of natural and synthetic polymers yields new materials which could have desirable properties including biodegradability. Singh and Ray[280] prepared chitosan films grafted by 2-hydroxyethyl methacrylate (HEMA) under ^{60}Co γ-irradiation. They observed that the tensile properties largely affected by the graft levels.

Alonso and co-workers[281] carried out design and evaluation of chitosan/ethylcellulose mucoadhesive bilayered devices for buccal drug delivery. The investigations highlight preparation of devices comprising a drug-containing mucoadhesive layer and a drug-free backing layer, Figure 35, by two different

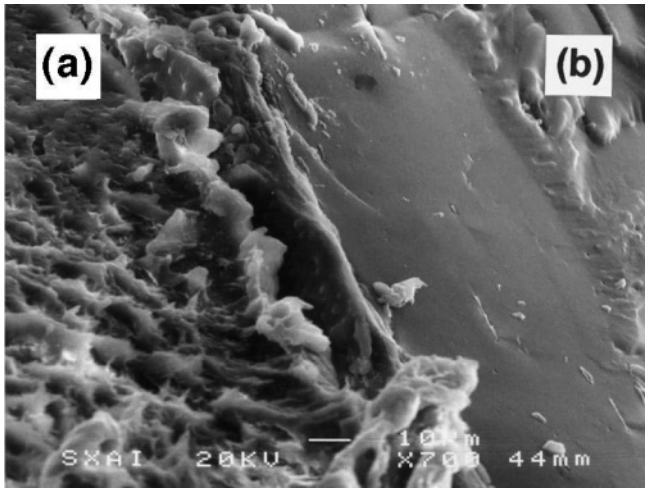


Figure 36. Scanning electron micrograph of a cross-section of a mucoadhesive bilaminated film: (a) ethylcellulose/DBP backing layer and (b) chitosan/glycerin mucoadhesive layer. (Reproduced with permission from ref 281. Copyright 1998 Elsevier.)

methods. Bilaminated films were produced by casting/solvent evaporation technique, and bilayered tablets were obtained by direct compression. The mucoadhesive layer was composed of a mixture of drug and chitosan with or without an anionic cross-linking polymer (polycarbophil, sodium alginate, gellan gum), and the backing layer was made of ethylcellulose. It was clearly evident that hydrophilic chitosan was easily laminated onto hydrophobic ethylcellulose and that a perfect binding between the mucoadhesive and the backing layers was achieved, Figure 36. The double-layered structure design was expected to provide drug delivery in a unidirectional fashion to the mucosa and avoid loss of drug due to wash out with saliva. Using nifedipine and propranolol hydrochloride as slightly and highly water-soluble model drugs, respectively, it was demonstrated that these new devices show promising potential for use in controlled delivery of drugs to the oral cavity. The drug release was due to the solubility and diffusion coefficient of the drug in the polymer film, consequently leading to solubility in the release medium. SEM observation of the chitosan films revealed the absence of pores before and after the drug-release assay. The un-cross-linked chitosan-containing devices absorbed a large quantity of water, gelled, and then eroded, allowing drug release. The bilayered films showed sustained drug release in a phosphate buffer (pH 6.4). Furthermore, tablets that displayed controlled swelling and drug release and adequate adhesivity were produced by in situ cross-linking the chitosan with polycarbophil.

Polyelectrolyte complex (PEC) of pectin, chitosan, and hydroxypropyl methylcellulose was prepared as a model for bimodal drug release.[282] The PEC formation between pectin and chitosan was investigated by examining the viscosities of supernatant solutions after removal of the precipitated complex. The PEC formation was found to be pH dependent. The amount of pectin, relative to chitosan, required for optimal PEC formation increased as the pH of the solution was reduced, and at pH less than 1.3, there was no

evidence for the formation of the PEC. Minimal swelling was observed when the pectin:chitosan weight ratio was optimal for PEC formation, suggesting formation of the PEC in situ. The permeability of the films to paracetamol as a model compound was dependent on film composition and markedly increased after exposure to pectinolytic enzymes, used to mimic conditions in the colon. These results led authors to conclude that similar formulations, applied as a film coat to tablets, could be used to achieve bimodal drug release with colonic conditions acting as a trigger for an increased rate of release.[282]

Chitosan films made with acetic acid (chitosan−AA) and lactic acid (chitosan−LA) have been tested for their potential as wound healing materials.[283] Mechanical, bioadhesive, and biological evaluation of the prepared films were carried out. The results were compared to the commercial preparation Omiderm. Chitosan−LA exhibited a lower tensile strength; however, it was more flexible and bioadhesive than chitosan−AA. Chitosan−LA and Omiderm did not cause any skin allergic reactions, in contrast, chitosan−AA showed skin irritation. Nevertheless, no sign of toxicity was encountered when the extracts of three preparations were administered parenterally.[283]

Chitosan membranes to provide an alternative means of evaluating transdermal drug delivery systems were developed and evaluated.[284] The membranes were prepared by cast-drying method. The effects of concentration of chitosan, sodium tripolyphosphate, as well as cross-linking time on flux and lag time were studied using central composite design. A mathematical model was developed to assess the permeation of the drugs through different animal skins. The investigations suggest that chitosan membrane at a particular composition simulated the permeation of diclofenac sodium through rat skin.[284]

Poly(ethylene glycol) (PEG) is water soluble and available in different molecular weights and exhibits useful properties such as protein resistance, low toxicity, and immunogenicity. Furthermore, the PEGs can abrogate the immunogenicity of proteins and preserve their biological properties. Taking advantage of the biocompatibility of chitosan as well as that of PEG, Zhang et al.[285] reported chitosan−PEG blend films for improved protein adsorption and cell growth. These extensive studies suggest that PEG enhanced the protein adsorption, cell adhesion, growth, and proliferation, but the effects were impaired by excessive PEG. The experiments also demonstrated that the optimum PEG concentration helped to maintain the natural structure of the protein adsorbed on the materials and that maintaining the natural structure benefited cell growth. However, authors found no noticeable differences on the mechanical properties and biocompatibility of the films when different molecular weights of PEG were tested, suggesting that molecular weight differences were not significant enough to affect the material properties.[285]

Several other modifications were made to improve the properties of chitosan films. One of the most recent studies described the reactions with acid chlorides and acid anhydrides.[286] Critical analysis led them to conclude that the substitution took place at the amino groups of chitosan, thus forming amide linkages, and the modification proceeded to a depth of at least 1 μm. The improvement in hydrophobicity was observed when the films carried stearoyl groups ($C_{17}H_{35}CO-$). However, modifications with succinic anhydride or phthalic anhydride did produce hydrophilic films. The improved surface hydrophobicity affected by the stearoyl groups promoted protein adsorption. In contrast, selective adsorption behavior was observed in the case of the chitosan films modified with anhydride derivatives. Lysozyme adsorption was enhanced by H-bonding and charge attraction with the hydrophilic surface, while the amount of albumin adsorbed was decreased possibly due to negative charges that gave rise to repulsion between the modified surface and albumin. Thus, authors have demonstrated that by heterogeneous chemical modification it is possible to fine tune surface properties that can influence the response to biomacromolecules.[286]

Biocompatible chitosan−gelatin composite films were prepared by varying the ratio of constituents, and their possible applicability in tissue regeneration has been evaluated in cultured PC12 cells.[287] Increased water uptake of chitosan film was observed when blended with gelatin. Composite film exhibited a lower Young's modulus and a higher percentage of elongation-at-break compared with chitosan film, especially in a wet state. The results also further indicate that the amount of adsorption of fibronectin on composite films was much higher than on chitosan film. The cells cultured on the composite film with 60 wt % gelatin differentiated more rapidly and extended longer neurites than on chitosan film. These results suggest that the soft and elastic complex of chitosan and gelatin, which has better nerve cell affinity compared to chitosan alone, is an interesting biomaterial for nerve regeneration. Chitosans and modified chitosans have been extensively investigated for various other applications as well.[246,288−293]

4.5. Fibers

4.5.1. Early Approaches to Fiber Production

The subject of fiber production from chitin and chitosan has been reviewed from various viewpoints due to inherent interest in the exploitation of chitin as a textile material.[294−299] Fiber production, however, has appeared since the beginning to be a challenging and difficult task. As it became established for cellulose, the xanthate process was developed for chitin, but the chitin fiber had poor tensile strength.[300] The unsatisfactory mechanical properties and environmental concerns with the xanthate process led to the development of halogenated and amide−LiCl solvent systems.

The halogenated solvents such as chlorinated hydrocarbons, chloroethanol with sulfuric acid, and trichloroacetic acid were developed in the early 1970s.[301] Chitin filaments were proposed as absorbable surgical sutures even though the halogenated solvents act as plasticizers.[302,303]

The trichloroacetic−chloral hydrate−dichloroethane (4:4:2) solvent system was found to dissolve

chitin, but the resulting filaments had no substantially improved mechanical properties.[304,305] Moreover, implementation of these spin systems represents a problem: trichloroacetic and dichloroacetic acids are corrosive and degrade chitin even after a short exposure, chlorohydrocarbons are environmentally unacceptable, formic acid can act as a sensitizer, and fluorinated alcohols and ketones are toxic.

A new approach was based on the *N*,*N*-dimethylacetamide−LiCl and *N*-methyl-2-pyrrolidone−LiCl solvent systems[306,307] that permitted obtaining better dry tensile strengths, although they did not provide adequate wet tenacities due to poor crystallinity and poor consolidation of the fiber. A further problem was the removal of Li from the fiber once it had formed, because the Li ion solvates the chitin amido groups. It also seems that these fibers could not offer adequate knot resistance, and these data jeopardized their further studies for surgical applications, despite the industrial efforts profused.[308]

4.5.2. Properties of O-Acylchitins

The studies on the chemical synthesis of *O*-acyl chitins were followed by studies on their biocompatibility, and hence, their potential use as materials for blood contacting surfaces has been investigated by measuring, inter alia, their critical surface tensions, clotting times, and plasma protein absorption (Table 3).[309]

The accessibility of chitin, mono-*O*-acetylchitin, and di-*O*-acetylchitin to lysozyme, as determined by the weight loss as a function of time, has been found to increase in the order chitin < mono-*O*-acetylchitin < di- *O*-acetylchitin.[310] The molecular motion and dielectric relaxation behavior of chitin and *O*-acetyl-, *O*-butyryl-, *O*-hexanoyl, and *O*-decanoylchitin have been studied.[311,312] Chitin and *O*-acetylchitin showed only one peak in the plot of the temperature dependence of the loss permittivity, whereas those derivatives having longer *O*-acyl groups showed two peaks.

The peak corresponding to the higher temperature relaxation process, β, moved to higher temperatures with increase in the size of the *O*-acyl group, excluding chitin itself, as did that for the lower temperature γ process in the three derivatives that exhibit this second relaxation process. The presence of two relaxation processes in these three samples, but only one in chitin and *O*-acetylchitin, was confirmed by NMR measurements. A comparison with the side chain relaxation in poly(*n*-alkyl methacrylate)s[313,314] indicates that the β relaxation is due to the motion of the *O*-acyl groups as a whole and hence is observed in all the samples, while the γ relaxation is due to local motion of the *O*-acyl group and hence only arises with the longer members of the series.

Table 3. Film Properties of Chitin and *O*-Acylchitins (chitin esters at 3 and 6 positions) with Relevance to Biomedical Applications (modified from ref 309)

O-acyl group	acylation degree	clotting time ratio		albumin adsorption (μg cm^{-2})
		glass	siliconized glass	
chitin	0	1.3	0.7	0.02
formyl	1.4	1.6	1.0	0.01
acetyl	2.0	3.8	1.9	0.02
propionyl	1.9	2.4	0.9	
butyryl	1.8	2.0	1.2	0.06
hexanoyl	2.0	2.5	1.1	0.05
decanoyl	1.8	2.5	1.1	0.06
dodecanoyl	1.9	2.5	1.1	0.11
benzoyl	1.9	2.6	1.0	0.04

4.5.3. Dibutyryl Chitin

Among the *O*-acyl chitins, dibutyryl chitin (DBC), an ester of chitin at 3 and 6 positions, having the prerogative of being soluble in various solvents, such as methanol, ethanol, ethylene chloride, and acetone, has attracted particular attention. Therefore, it becomes easy to manufacture woven, nonwoven, and knitted textiles useful for medical applications.

Dibutyryl chitin with a degree of *O*-substitution of ca. 1.8 was obtained using methanesulfonic acid as both catalyst and solvent;[315] the DBC filaments were manufactured as follows: dry-spun fibers were obtained from a 23% solution of DBC in acetone into air (elongation at break 34−47%); the wet spinning was performed from a 16% solution in dimethylformamide into a water coagulating bath (elongation at break 8.3%). The tensile strength was found to be small, as justified by the low crystallinity and low internal overall orientation of the filaments.[316]

A simpler and cheaper method for the synthesis of dibutyrylchitin using perchloric acid as a catalyst and butyric anhydride as acylation agent has been worked out under heterogeneous conditions on krill, shrimp, crab, and insect chitins. The preferred krill chitin had a degree of acetylation of 0.98 and an intrinsic viscosity of 13.33 dL/g (determined in DMAc + 5% LiCl solutions), which corresponded to a viscosity average molecular weight of chitin $M_v = 286.7$ KDa. The acylation mixture was prepared by pouring perchloric acid into butyric anhydride at ca. −12 °C and added to the chitin powder placed in the reactor (ca. 0 °C for 30 min, ca. 20 °C later). Weight-average molecular weight values were usually in the range 120−200 KDa. DBC fibers were spun using Pt−Au spinneret with a hole diameter of 80 μm; 14.5% solution of polymer in DMF was used as a dope. Fibers of DBC had a linear density of 34.7 tex, tenacity in standard condition of 11.9 cN/tex and in the wet state of 6.80 cN/tex, knot strength of 1.06 cN/tex, extensibility of 7.40% in the dry state and 8.04% in the wet state. In the IR spectrum of DBC there was no hydroxyl group band at ca. 3500 cm^{-1}. There were new strong bands characteristic of the fatty acid esters at 1740 and ca. 1450 cm^{-1}.[317]

As for the biological properties of DBC, the percentage of the haemolysis counted from three samples of chitin was as low as 0.086%, for dibutyryl chitin it was 0.72%, and in both cases did not exceed the value of 1% admitted for the standards. The evaluation of cytotoxicity was carried out on the reference cell series of the mouse fibroblasts 3T3/Balb, after 48 and 72 h. No agglutination, vacuolization, or cells membrane lysis was observed. The intracutaneous irritation of each extract sample was evaluated on albino New Zealand rabbits: intracutaneous reactivity of the DBC extracts showed no irritation.

The pathomorphological findings including macroscopic and microscopic assessment testified excellent biocompatibility. The healing process included the short exudative phase, which after 14 days was followed by the proliferative phase. The latter ended with the development of a connective tissue capsule, whose thickness was ca. one-fourth of the fiber diameter. A significant resorption of DBC fibers was observed during the fifth month since implantation.[318]

Because *O*-acyl chitins appear to be scarcely susceptible to lysozyme, the susceptibility of DBC to lipases has been studied in order to get insight on its biodegradability in vivo. The changes in infrared and X-ray diffraction spectra of the fibers support the slow degradation of DBC by lipases.[319]

Considering the experimental evidence available, nonwoven DBC seems suitable for incorporation into modified chitosans having recognized efficacy in wound healing, such as methylpyrrolidinone chitosan, in view of imparting mechanical strength to the dressing, while avoiding use of nondegradable fibers.[320]

4.5.4. Chitosan Fibers

Nontoxic and noncorrosive solvent systems can be used when chitosan is considered instead of chitin for the manufacture of fibers. Many articles and patents address the spinning of chitosan solutions. Although there was interest in taking advantage of the liquid crystalline nature of chitosan,[321] no great improvement of the mechanical properties was obtained. The same can be said for alkyl chitin fibers.[322]

Cross-linking agents have been proposed for improvement of chitin fibers in the wet state. Epichlorohydrin is a convenient base-catalyzed cross-linker to be used in 0.067 M NaOH (pH 10) at 40 °C. The wet strength of the fibers was considerably improved, whereas cross-linking had a negligible effect on the dry fiber properties.[323] Of course, the more extended the chemical modification, the more unpredictable the biochemical characteristics and effects in vivo. Every modified chitin or modified chitosan fiber should be studied in terms of biocompatibility, biodegradability, and overall effects on the wounded tissues.

The present trend is to coat other polysaccharide fibers with a film of chitosan or modified chitosan to impart novel characteristics to the textile. For example, a detailed study has been made on alginate fibers coated with various chitosans that actually form polyelectrolyte complexes and, under certain conditions, improve the general characteristics of the fiber while keeping the biochemical significance of chitosan.[324] Another study described wet-spun chitosan−collagen fibers, their chemical modification, and their blood compatibility.[325]

This approach to the use of chitosan seems to lead to satisfactory results, as testified by the Japanese production of socks, underwear, and similar items.

4.6. Tablets

Chitosan has been investigated as an excipient in the pharmaceutical industry to be used in direct tablet compression, as a tablet disintegrant, for the production of controlled release of solid dosage forms, or for the improvement of drug dissolution. An immediate release formulation was obtained when chitosan was used as a pharmaceutical excipient for directly compressed tablets.[326] Chitosan direct compression tablets triggered the sustained release of prednisolone,[327] a water-insoluble drug. The release rate was controlled by changing the relative amounts and degree of deacetylation of chitosan and carboxymethylcellulose. A diclofenac tablet formulation produced using both the direct compression and wet granulation methods with chitosan also displayed sustained release characteristics.[328] These studies suggest that the release rate is affected by the degree of *N*-deacetylation of chitosan. Furthermore, the report concluded that the ionic strength of the dissolution medium did not affect the dissolution of the tablets; however, the pH of the medium had a significant effect.[328] A decade later, Sabnis et al.[209] demonstrated the possible interaction of diclofenac with chitosan amino groups leading to the sustained release of the former. Further studies evaluated the bioadhesive strengths of chitosan and hyaluronate complexes and found that complexes exhibited superior bioadhesiveness in comparison to chitosan alone and were also pH dependent.[329] Chitosan and alginate tablets showed sustained release of theophylline irrespective of pH.[330]

A mucoadhesive polymer was developed from the chitosan−EDTA conjugate which inhibited enzymatic activity:[331] it could be used for overcoming enzymatic barriers for orally administered peptide drugs; however, in terms of adhesive properties, it was found to be 42% lower than plain chitosan.[332] Inhibition of the enzymatic activity with chitosan−EDTA was also observed.[333] Low insulin degradation was observed using direct compressed tablets of chitosan−EDTA, suggesting its usefulness as a potential delivery system for protein and peptide drugs via oral route.[334] Bilayered tablets obtained by direct compression and comprising a drug containing a mucoadhesive chitosan layer and a backing layer of ethyl cellulose displayed controlled delivery of nifedipine and propranolol hydrochloride.[335] Diltiazem tablet formulations were made by direct mixing of the drug with a mixture of chitosan and sodium alginate with a view to make mucoadhesive formulations for oral delivery.[336] The bioavailability of the drug varied with the route of administration and was found to be 69.6% and 30.4% when a tablet formulation of 1:4 chitosan/alginate was administered sublingually and orally to rabbits, respectively.

Phaechamud et al.[337] investigated chitosan citrate as a film former and its compatibility with water soluble anionic dyes and drug dissolution from coated tablets. The interaction of various dyes with chitosan studied using FTIR indicated that anionic dyes tend to interact with cationic polymer chitosan. Brilliant Blue and Green FS solutions (concentration 0.02−1.00% (w:w) chitosan) were made. Propranolol.HCl tablets coated with these colored film-coating solutions exhibited good appearance, and no color migration was observed. Drug dissolution from these coated

tablets was dictated by the pH of the medium, corresponding to the ability of chitosan to be protonated. These investigations suggested that color incorporation slightly retarded drug dissolution in acidic medium, where the drug dissolved from Brilliant Blue coatings was faster than from that of Green FS. This was expected due to the positive charge of Brilliant Blue and more SO_3H groups in its molecular structure, exhibiting higher water solubility. The drug dissolution from freshly prepared coated tablets, coated tablets after exposure to accelerated conditions, and storage at room temperature for 12 months was evaluated. In the later studies, the release mechanism of the chitosan-coated tablets with a view to establish the mechanistic model for drug release was investigated.[338] The conclusions for this study were made assuming that the core tablets are spherical and the drug dissolution is diffusion dependent. In order for the drug release to occur, the dissolution medium penetrates through the coated film and leaches out the drug through the film and the lag time, t_{tag}, is set for these processes. It was predicted that the coated film itself dissolves and becomes thinner with time. As it is completely dissipated, the undissolved core tablet becomes naked, allowing the drug release to proceed just as the spherical core tablet. Drug-release rate was given as a differential equation. Model adaptation was carried out to the release data of propranolol hydrochloride tablets coated with chitosan and kept in different storage conditions. The authors claimed that the goodness-of-fit of model was better than any other conventional equations tested, and estimated model parameters were informative.

Microsphere technology was adopted for the development of chitosan tablets containing chlorhexidine diacetate as a buccal formulation.[210] Tablets used in these studies were prepared by direct compression of the microparticles with mannitol alone or with sodium alginate. The microparticles were prepared by a spray-drying technique. Chlorhexidine in the chitosan microspheres dissolves more quickly in vitro than does chlorhexidine powder. The antimicrobial activity of the microparticles was investigated as minimum inhibitory concentration, minimum bacterial concentration, and killing time. Chitosan microparticles alone have an antimicrobial activity due to the polymer itself, and it was further found that the loading of chlorhexidine into chitosan is able to maintain or improve the antimicrobial activity of the drug. The improvement is particularly high against *Candida albicans*. This is important for a formulation whose potential use is against buccal infections. After their in vivo administration, determination of chlorhexidine in saliva showed the capacity of these formulations to give a prolonged release of the drug in the buccal cavity.

Indomethacin tablet formulations using various polysaccharides or synthetic polymers as binders were developed and enteric coated with Eudragit-L 100 to give protection in the stomach.[339] These tablets were evaluated for their potential as colon-specific drug delivery systems in vitro. The dissolution studies suggest that the rate is dependent on the type and concentration of the polysaccharide or synthetic polymer binder used. The results demonstrate that enteric-coated tablets containing 3% chitosan as a binder showed only 12.5% drug release in the first 5 h, which is the usual upper gastrointestinal transit time, whereas tablets prepared using guar gum as binder failed to protect drug release under similar conditions, thereby leading to the conclusion that chitosan is superior to guar gum for water-insoluble drugs used in these studies. The authors also suggested that the formulations developed using chitosan and Eudragit could serve as highly site-specific devices since drug release would be at a retarded rate until microbial degradation or polymer solubilization takes place in the colon.[339]

Tablets were compressed from a physical mixture containing salicylic acid (model drug), chitosan, and magnesium stearate.[200] Five commercial chitosans, varying in degree of deacetylation and molecular weight, were selected. Tablets were compressed at 5000, 10 000, and 15 000 psi using a Carver and a single punch tablet press. The interaction between chitosan and the drug has been studied using differential scanning calorimeter. Analysis of variance (ANOVA) indicated that the compression did not significantly affect the crushing strength or the release profile of drug from the chitosan matrix tablets. Furthermore, the ANOVA also indicated that the tablet press used during manufacture did not affect the above properties; however, the chitosans significantly affected the crushing strength as well as the release profile of drug from chitosan matrix tablets. The same group studied directly compressed tablets (200 mg) containing tetracycline, chitosan, and magnesium stearate prepared for exploring spray-dried chitosans as excipients.[204] The chitosans used were spray and thermally dried, previously *N*-deacetylated, and depolymerized. The tablets were characterized for dimensions, weight, friability, crushing strengths, disintegration, and dissolution and found that certain parameters such as the tablet weights, thickness, and diameters were not affected by the chitosan selected. However, friability of tablets containing thermally dried chitosans was generally higher (and crushing strengths were lower) than tablets containing spray-dried chitosans. It was noticed that chitosan molecular weight, degree of *N*-deacetylation, and drying method used significantly affected crushing strengths; on the other hand, disintegration times were affected only by the type of chitosan but not by the drying method used. The dissolution studies show the dependency on the chitosan type, while the drying method has no influence.

Most recently, *N*-acylation of chitosan with various fatty acid (C6−C16) chlorides with a view to increase its hydrophobicity and their application in drug delivery has been reported.[340] The studies with unmodified chitosan suggested a low degree of order and a weak tablet crushing strength. *N*-Acylation was dealt with different chain lengths in which chitosan acylated with a short chain length (C6) possessed similar properties but exhibited significant swelling; however, acylation with longer side chains

(C8−C16) resulted in a higher degree of order and crushing strength but lower swelling. Of the various possible modifications, the best mechanical characteristics and drug-release properties were found for palmitoyl chitosan (substitution degree 40−50%) tablets with 20% acetaminophen as a tracer. The high stability of these monolithic tablets appears to be due to hydrophobic interactions between side chains, as shown by a more organized structure. Drug dissolution profiles in vitro showed direct dependency on the degree of functionalization of the release rates: this suggests that palmitoyl chitosan excipients are interesting compounds for oral and subdermal pharmaceutical applications.

Junginger and co-worker's[341] carried out extensive research on modified chitosans, especially TMCs, as discussed in various sections of this review. Several of the in vitro and in vivo studies suggest TMC as a potent and safe absorption enhancer of peptide drugs; however, until now it has always been administered as a solution. The difficulties in administering the peptide drugs in solution form led to the need for a solid oral dosage form. One of their recent studies is a report on Minitablet and granule formulations as solid oral dosage forms for the delivery of peptide drugs with the *N*-trimethylchitosan chloride (TMC) as absorption enhancer. Minitablets were deemed suitable as a dosage form due to their ability, as components of multiple unit dosage forms, to disperse from each other before disintegration, effectively increasing the area in which the polymer can exert its absorption-enhancing action. One of the main focuses of this study is to see that the polymer is released from the dosage forms prior to the release of the peptide, together with achieving maximum release of both ingredients. Desmopressin (1-(3-mercaptopropionic acid)-8-D-arginine vasopressin monoacetate (DDAVP)) was used as model peptide drug. Minitablet formulations of DDAVP granules containing tetraglycerol pentastearate and TMC granules were evaluated. DDAVP granules with tetraglycerol pentastearate were specifically aimed at delaying the release of the peptide from the dosage form. Burst release of TMC was attempted with TMC granules. Release profiles for both the optimized minitablet formulation as well as the granule formulation showed that the release of DDAVP was effectively delayed from the formulation compared to the control. In comparison, more TMC was released and at a faster rate from the granule formulation than the optimized minitablet formulations. Both the optimized minitablet formulation and the granule formulation show suitable release profiles for the delivery of peptide drugs with TMC as absorption enhancer in solid oral dosage forms.[341]

5. Drug Delivery

5.1. Oral Administration

5.1.1. Drug Delivery to the Colon

Delivery of proteic drugs that are either destroyed or poorly absorbed in the upper gastrointestinal tract through colonic absorption is a mode of introducing the drugs to the systemic circulation. Peptide and proteic drugs undergo poor intestinal absorption due to their vulnerability by proteolytic enzymes in the gastrointestinal tract and poor membrane permeability. The absorption of these drugs in the colon can be enhanced because of the low activity of proteolytic enzymes there and long residence time for the drug.[342]

For the treatment of colon diseases, such as ulcerative colitis and Crohn's disease, direct drug targeting to the colon tissues reduces the dose to be administered and the incidence of adverse effects. In these instances, chitosan is safe, as demonstrated not only by the use of dietary supplements in healthy subjects but also on patients.

Although the pathogenesis of Crohn's disease remains unclear, dietary fat is thought to exacerbate intestinal inflammation. Patients therefore should be on a low-fat diet. Eleven outpatients were given tablets daily of a chitosan and ascorbic mixture (chitosan, 1.05 g/day) for 8 weeks. Patients did not interrupt their respective therapies for Crohn's disease. The chitosan and ascorbic mixture significantly increased the fat concentration in the faeces during treatment. The results indicated that oral administration of a chitosan and ascorbic acid mixture in patients with Crohn's disease is tolerable and increases fecal fat excretion.[342]

5.1.1.1. Enteric Coatings. Among the various approaches to colon-specific drug delivery (formation of a pro-drug, time-dependent delivery systems, pH-sensitive polymers) the use of synthetic polymers is increasing. In particular, the Eudragit products for enteric coatings are based on anionic polymers of methacrylic acid and methacrylates and are recognizable by codes L and S. They contain −COOH as a functional group and dissolve at pH 5.5−7. The different grades are available as aqueous dispersions, powders, and solutions in organic solvents.

For the processing of Eudragit, additional excipients are required. Plasticizers lower the minimum film-forming temperatures as well as the glass transition temperatures: the addition of a plasticizer like triethyl citrate increases the flexibility of the film coatings. To prevent the stickiness of film coatings, some glidants such as talc or glycerol monostearate are also necessary. Eudragit shows very high pigment binding capacity: the amount of pigments that can be incorporated or bound into the film coating is as much as three times the amount of Eudragit.

The Eudragit L-type polymers are also used for taste masking via salt formation, or others are applied in eroding matrix tablets, e.g., together with polymers for sustained release. These synthetic pH-sensitive polymers as well as accompanying excipients and compounds used for pro-drug preparation may accumulate in the body or originate scarcely known metabolites.

The pH of the stomach is acidic and increases in the small and large intestines. This pH variation in different segments of the gastrointestinal tract has been exploited for colon-specific delivery. Coating the drug core with pH-sensitive Eudragit has been successfully used for colon drug delivery of Asacol,

Asamax, and Salofalc. Those polymers are insoluble in acidic media, thereby providing protection to the drug core in the stomach and to some extent in the small intestine, but dissolve at a pH of 6 or more, releasing the drug in the colon. However, the pH of the gastrointestinal tract is subject to individual variations depending upon diet, disease, age, sex, and the fed/fasted state.[344,345] Due to the simplicity of the formulation of this device, many marketed preparations utilize this approach.

The mechanism by which 5-ASA was effective in treating colitis may be related to the inhibition of cyclooxygenase. Two groups of arachidonic acid metabolites may play mediating roles in colon inflammation. These are cyclooxygenase products (primarily prostaglandins) and lipoxygenase products. Musch et al.[346−348] reported that prostaglandin levels were elevated in the colonic mucosa, serum, and stools of patients. 5-ASA reduced prostaglandin production, thereby inhibiting the activity of cyclooxygenase.

5.1.1.2. Alternatives to Synthetic Polymers. On prolonged use, man-made polymers and excipients may accumulate in the body, and therefore, biodegradable polymers should be preferred. The degradation of the drug carrier in the colon is mainly due to bacterial flora. The stomach and duodenum have a microflora of less than 1000 CFU/ml: these are mainly Gram-positive bacteria. The microflora of the colon, instead, is in the range $10^{11}-10^{12}$ CFU/mL and consists of anaerobic bacteria, e.g., *Enterobacteria, Enterococci, Clostridia, Eubacteria, Bifidobacteria,* and *Bacteroides,* that ferment materials left undigested in the small intestine, such as carbohydrate polymers. In fact, for this fermentation, the microflora produces a vast number of enzymes such as β-glucuronidase, β-xylosidase, α-arabinosidase, β-galactosidase, nitroreductase, azoreductase, pectinases, deaminase, and urea dehydroxylase.[349] Because of the presence of these enzymes only in the colon, the use of bacterial degradable polymers for colon-specific drug delivery seems to be a more site-specific approach as compared to other approaches. These polymers shield the drug from the environments of the stomach and small intestine and are able to deliver the drug to the colon. On reaching the colon, they undergo assimilation by microorganisms or degradation by enzymes or breakdown of the polymer backbone leading to a subsequent reduction in their molecular weight and loss of mechanical strength. They are then unable to hold the drug any longer.[350−356]

The use of polymers susceptible to the hydrolytic bacterial enzymes seems today to be the most convenient approach to the delivery of drugs to the colon. The action of a single hydrolase is usually inadequate to explain the degradation of a carrier (for instance, a commercial β-glucosidase was found inadequate to mimic the effects of rat colonic enzymes,[357] even though it may work in vitro[358]), but one should keep in mind that most hydrolases have unspecific activities, i.e., they split the chains of polymers that are not their typical substrates. For example, chitosan is susceptible to lipase, pectinases, amylases among others, while the association chitosan + pectin is highly sensitive to pectinases.[359−361] The biodegradation of tablets containing lactose, hydroxypropyl cellulose, and prednisolone and coated with chitosan and cellulose acetate phthalate was studied in vitro: when treated with rat colonic extract the chitosan biodegradation was 40% of the original weight, compared to 10% with autoclaved extract. The formulation was found to be suitable for the delivery of prednisolone.[362]

The ability of alginates, pectins, guaran, dextran, xanthan, chitosans, and chondroitins to act as substrates for the enzymes produced by the bacterial microflora in the colon has been taken into consideration for the preparation of drug carriers further endowed with filmogenicity, biocompatibility, gel-forming capacity and other convenient qualities for the manufacture of multicomponent carriers.[339]

5.1.1.3 Advantages of Chitosan. If one considers chitosan, at first glance it would appear not to be suitable as a pH-sensitive polymer: in fact, it is soluble at acidic pH values and becomes insoluble at approximately pH 6.5. Nevertheless, an enteric coating can protect chitosan from the acidity of the stomach. When the preparation reaches the intestine, the enteric layer dissolves due to high pH and the drug-bearing chitosan core remains exposed to the bacterial enzymes, thus releasing the drug. This is certainly an advantage because in the solid form chitosan would not flocculate the bacteria while it would remain exposed to the hydrolytic activity of the bacterial enzymes. Chitosan, in fact, is digested largely by secreted rather than cell-associated bacterial enzymes.[363,364]

As soon as chitooligomers are set free by hydrolases, they become carbon sources for the growth of intestinal bacteria. *Lactobacillus lactis* utilizes the oligomers $(GlcNAc)_{1-6}$, the monomer and dimer being bifidogenic substances.[365−367]

Chitosan capsules enteric-coated with a layer of hydroxypropylmethyl cellulose phthalate have been evaluated for colon delivery of drugs.[368] In vitro studies showed that the capsules loaded with a soluble dye, 5-(6)-carboxyfluorescein, showed little release in simulated gastric juice for 2 h (transit time in stomach) and in artificial intestinal juice (next 4 h), but the presence of rat caecal contents (33%) in the dissolution fluid increased the release rate of the drug from the capsules from 20% to 100% in the next 4 h. This suggests that the flora present in the rat caecal content may have produced enzymes for the degradation of chitosan.

Studies carried out in wistar rats using insulin as the drug showed improvement in absorption of insulin using these capsules.[196] Peaks of plasma insulin concentration and marked hypoglycemic effect were seen 6−12 h after the oral administration of chitosan capsules loaded with insulin. It was also seen that insulin absorption in the large intestine increased in the presence of absorption enhancers such as sodium glycocholate. The latter was found to be more effective than aprotinin, soybean trypsin inhibitor, sodium oleate, bacitracin, and *n*-dodecyl-β-D-maltopyranoside.

Acceleration of healing with R68070 (a thromboxane synthase inhibitor) on 2,4,6-trinitrobenzene sulfonic-acid-induced ulcerative colitis in rat using chitosan capsule as a carrier was compared to a suspension of R68070 in carboxymethyl cellulose.[369] However, when the doses were increased, a marked reduction in colitis was seen with R68070 when given in chitosan capsules rather than in carboxymethyl cellulose suspension, leading to the conclusion that these capsules have a good potential for being used as carriers in colon-specific drug delivery systems. Most important, chitosan capsules loaded with 5-ASA provided a higher therapeutic effect than 5-ASA suspensions with carboxymethyl cellulose, based on myeloperoxidase activity, damage score, and colon/body weight ratio.

The release of 5-ASA and sodium diclofenac from chitosan or chitosan−HCl was studied in vitro by using matrixes prepared by compression of microspheres with drug powders (20%). The action of rat caecal microflora accelerated the drug release from the chitosan matrix, which was a reliable system for 5-ASA (t_{50} = 1.97 h) or sodium diclofenac (t_{50} = 3.58 h).[370]

Chitosan capsules manufactured by Aicello Chem. Co. Ltd. were filled with 5-ASA and then coated with hydroxypropylcellulose phthalate as an enteric coating dissolved in acetone + ethanol (1:1). Analogous preparations were made with insulin. Levels of 5-ASA in the rat large intestine 4 h after oral administration were close to 0.45 mg (i.e., 1/10 of the administered quantity) compared to ca. 0.12 mg for the same amount of 5-ASA as a CMC suspension.

In practice, the enteric-coated capsules were stable in the stomach and the small intestine. They were degraded by microorganisms in the caecal content. The delivery to the colon was specific, and the healing effect was accelerated by chitosan. Similarly, insulin absorption in the large intestine was improved with absorption enhancers such as sodium glycocholate.[346,371,372] Gelatin capsules were coated with Eudragit containing chitosan particles for the same purposes

The effect of chitosan tripolyphosphate nanoparticles (250−400 nm) on the absorption of insulin was studied by measuring the decrease of the plasma glucose concentration and the relative pharmacological availability.[373] Chitosan tripolyphosphate showed excellent association with insulin and improved the intestinal absorption of insulin to a greater extent compared to insulin−chitosan solution.

Semisynthetic derivatives of chitosan i.e., chitosan succinate and chitosan phthalate, were prepared by reacting chitosan separately with succinic and phthalic anhydrides.[374] Sodium diclofenac was dispersed in their matrixes. In vitro studies showed that these matrixes resisted dissolution under acidic conditions and showed improved dissolution under basic conditions, suggesting their suitability for colon-specific drug delivery systems.

5.1.1.4. Natural Enteric Coatings. Pectin, being soluble in water, is unable to protect its drug load during passage through the stomach and small intestine, but pectin chitosan complexes were found to be suitable for colon-specific delivery of indomethacin and paracetamol.[375]

In earlier studies the formation of chitosan pectin complexes was deemed interesting because of the efficacy in controlling the release of indomethacin and sulfamethaxazole while maintaining the biodegradability towards pectinases.[376]

An ionic complex of highly methoxylated pectin (anionic) with Eudragit-RL (cationic, insoluble pH-independent) was incorporated in swellable Eudragit-NE30D (neutral ester) and used for targeting drugs to the colon.[377] In the case of polyelectrolytic complexes of pectin and chitosan, the lowest permeability to paracetamol was found for materials having a prevalent content of nonamidated low-methoxylated pectins and highly deacetylated.[378] Indomethacin was also studied in similar carriers.[221]

Combination of pectin, chitosan, and hydroxypropylmethyl cellulose in the ratio 3:1:1 is insoluble and degraded by pectinases. These tablets were used in human volunteers to carry ^{99m}Tc and found to be able to pass through the stomach and small intestine intact, as is evident by scintigraphic images. The results led to the conclusion that degradation of these coatings takes place in the ascending and transverse colon by colonic bacteria. Figure 37. [379]

Guaran has been similarly evaluated as a compression coating: the formulation with 150 mg of guaran as a coating showed 95% release of 5-aminosalic acid in the presence of rat caecal content after 26 h.[380]

Galactomannan from locust bean should be cross-linked for these purposes, considering its solubility in water. However, combination of galactomannan and chitosan proved capable of protecting the core tablet containing mesalazine during conditions mimicking mouth to colon transit. When galactomannan and chitosan were in a ratio of 4:1, the bioavailability and dissolution profile in rat caecal content qualified the complex as a carrier for drug targeting to the colon.[381]

Chitosan−alginate beads loaded with a model protein, bovine serum albumin, were investigated to explore the temporary protection of protein against acidic and enzymatic degradation during gastric passage. During incubation in simulated gastric fluid (pH 1.2), the beads showed swelling and started to float but did not show any sign of erosion. Release studies were done in simulated gastric fluid (pH 1.2) and subsequently in simulated intestinal fluid (pH 7.5), where the beads were found to erode, burst, and release the protein. Chitosan-reinforced calcium−alginate beads showed delay in the release of albumin. The multilayer beads disintegrated very slowly. The enzymes pepsin and pancreatin did not change the characteristics of albumin-loaded chitosan−alginate beads. Alginate beads reinforced with chitosan offer an excellent perspective for controlled gastrointestinal passage of proteic drugs.[382]

Chitosan microspheres obtained by emulsification in paraffin, coconut, or sunflower oil and cross-linked to various degrees with glutaraldehyde were dispersed in poly(acrylic acid) solution for coating. Once dry, they were dispersed in alginate solution (3%) and

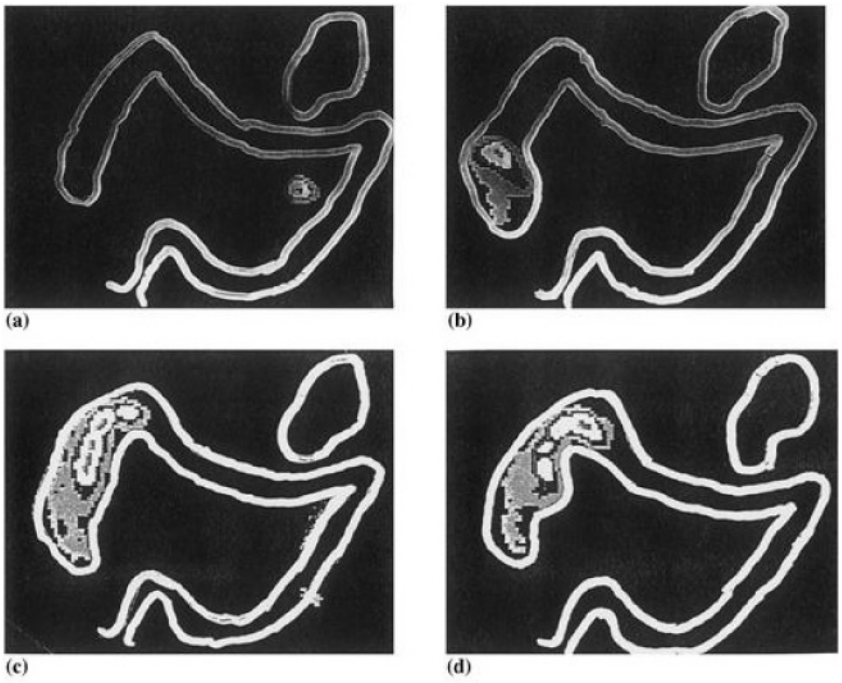


Figure 37. Scintiscan showing the tablet (intact) in the small intestine of subject 4. Time after tablet administration = 60 min. (b) Scintiscan showing the tablet having disintegrated and starting to spread in the ascending colon of subject 4. Time after tablet administration = 305 min. (c) Scintiscan showing the tablet having extensively spread in the ascending colon of subject 4. Time after tablet administration = 365 min. (d) Scintiscan showing the tablet having extensively spread toward the transverse colon of subject 4. Time after tablet administration = 405 min. (Reproduced with permission from ref 379. Copyright 1999 Elsevier.)

microcapsules were obtained by dropping the latter suspension into $CaCl_2$ (2%) solution. The mucoadhesivity was then evaluated. The mechanism involved liberation of the chitosan polyacrylate microspheres from the microcapsules in the intestine and attachment of the microspheres to the colon mucosae for prolonged drug release.[383] It is known, in fact, that chitosan strongly reacts with mucin, even in the presence of minor amounts of polyacrylate.[384]

In conclusion, it was demonstrated that chitosan capsules were stable in the stomach and small intestine. However, in rats, they were specifically degraded by microorganisms in the caecal contents when they reached the colon. Furthermore, insulin absorption from the large intestine was improved by concomitant administration with a variety of additives. 5-ASA can be delivered specifically to the colon, and the healing effect of 5-ASA was accelerated using chitosan capsules. These capsules may therefore constitute useful carriers for the colon-specific delivery of peptides, including insulin, and antiinflammatory drugs, including 5-ASA.[346]

It seems that the preparation of chitosan 5-aminosalicylate salt could be a further extension of these studies; however, the characteristic properties of this salt are unknown, while the chitosan salts of gentisic, salicylic, and acetylsalicylic acids have been characterized.[385]

5.1.2. Cholesterol and Overweight Lowering

When discussing the role of chitosan as a cholesterol lowering nutriceutical and considering possible mechanisms for its action, one should keep in mind that animal studies might not be predictive of results in humans because of the presence of chitinases in the digestive systems of many animals, as a point of difference from humans.[386] Chitosan for the oral administration to humans is generally recognized as safe.[387]

5.1.2.1. Cholesterol Lowering in Animals. Sugano et al.[388] were the first to report the cholesterol lowering ability of chitosan, and the report suggests that a diet containing 5% chitosan reduced liver cholesterol concentration by one-half or more in cholesterol-fed rats, and a similar trend was observed by other investigators as well.[389] In a 4 week study, a 4% chitosan diet reduced both liver and serum cholesterol dramatically in rats fed diets containing 1% cholesterol + 0.1% bile salts.[390] Chitosan has also been shown to reduce plasma cholesterol in cholesterol-fed broiler chickens at dietary concentrations of 1.5−3.0%.[391,392] Thus, the ability of chitosan to reduce cholesterol in animal models is well established.

The apoprotein E-deficient mouse has extreme hypercholesterolemia, atherosclerosis develops rapidly, and the lesions are histologically similar to those seen in humans.[393] Deficient mice fed diets containing 5% chitosan for 6 months had serum cholesterol only 64% of the control group.[394] The atherosclerotic lesion area in the aortic arch was reduced by one-half and in the total aorta by 42% in mice fed chitosan. These results strongly suggest that chitosan consumption has the potential to reduce the atherosclerosis risk in humans.

5.1.2.2. Cholesterol Lowering in Humans. Chitosan was first shown to reduce serum cholesterol in humans in 1993, when adult males fed chitosan-

containing biscuits for 2 weeks (3 g/day for week 1, 6 g/day for week 2) experienced a significant decrease of 6% in total cholesterol.[395] The subjects also demonstrated a 10% increase in HDL cholesterol. However, in a 28 day study in overweight subjects given a daily dose of approximately 0.6 g/day of chitosan, no reduction in total cholesterol was detected.[396] The failure to find a cholesterol reduction in this study was due to the small quantity of chitosan used. Two studies have reported serum cholesterol reductions with chitosan treatment. Obese women consuming 1.2 g of microcrystalline chitosan for 8 weeks demonstrated significant reductions in LDL, although not total serum cholesterol.[397] Eighty-four female subjects with mild to moderate hypercholesterolemia receiving 1.2 g of chitosan per day experienced a significant decrease in total serum cholesterol.[398]

The results of the animal studies and human trials provide convincing evidence that chitosan is effective in lowering total and LDL cholesterol. Chitosan appears to be active in humans at rather low doses, with as little as 1.2 g per day producing significant reductions in serum cholesterol.

5.1.2.3. Mechanism of Cholesterol Lowering. In vitro, chitosan has been reported to bind bile acids with approximately one-half or equal capacity of cholestyramine, a strong synthetic anion exchanger.[399,400] The role of the accompanying anion is important: for instance, chitosan orotate salt has enhanced capacity for bile acids.[401]

Three-fold fecal bile acid excretion in rats fed diets containing 7.5% chitosan compared to cellulose-fed animals was observed,[402] but in several studies, rats fed diets containing 5% chitosan did not have increased fecal bile acid excretion.[400,403] However, increased fecal bile acid excretion with chitosan feeding has been found in humans and rabbits.[395,404] A reduction in cholesterol absorption could have explained the cholesterol lowering effect of chitosan.[405] Thus, preponderant evidence supports a reduction in cholesterol absorption by chitosan.

Bile acid binding within the small intestine could interfere with micelle formation, which is necessary for cholesterol absorption. Nauss et al.[406] reported that, in vitro, chitosan-bound bile acid micelles in toto, with consequent assimilation of bile acids, cholesterol, monoglycerides, and fatty acids. Yet another explanation is provided by the finding that, in vitro, chitosan inhibits pancreatic lipase activity.[407]

An additional remark is that chitosan is an unspecific substrate for a number of lipases, as demonstrated in vitro;[408] thus, soluble chitosan may further depress the activity of lipases toward lipids by acting as an alternative substrate.

Chitosan upon reaction with bile acids (cholate, taurocholate, etc.) forms insoluble salts that collect lipids by hydrophobic interaction.[176,409,410] The most important aspect of this situation is that bile acids, once sequestered by chitosan, are no longer available as emulsifiers for the correct formation of the emulsion necessary for the digestion of lipids. It is known that the pancreatic lipases in order to hydrolyze triglycerides require a certain dimension of the oil droplets in the emulsion: now, when the bile acids become scarce, their capacity as emulsifiers leads to inadequate emulsions and then to scarce hydrolysis of triglycerides. Ample information on digestive lipases supports these views.[411−413] The presence of bile salts in one case activates the bile salts-dependent lipases and in the other case provide the emulsion necessary to the pancreatic lipases for enzymatic activity. Models have been proposed and discussed.[414−417] As soon as the bile salt availability decreases due to chitosan ingestion, lipases become unable to work adequately and assimilation of lipids by the organism decreases sharply.

5.1.2.4. Overweight. Studies on the effect of chitosan on human adiposity suggest that results may differ depending on whether the subjects are eating ad libitum or are on a weight-loss diet. Overweight subjects consuming 2.4 g/day of chitosan for 28 days, also consuming their normal diet, showed no change in body weight during the trial.[418] However, in three studies using subjects fed weight reduction diets of 1000 kcal per day, those given a chitosan-containing supplement lost twice as much weight over a 4-week period than those given the placebo.[419,420]

A 6-week study was conducted on obese adults who lost more body weight (−2.3 vs 0.0 kg), body fat (−1.1% vs 0.2%), and absolute fat mass (−2.0 vs 0.2 kg). The combination of glucomannan, chitosan, fenugreek *Gymnema sylvestre,* and vitamin C was found to be effective.[421]

Chitosan greatly increases fecal fat excretion when consumed in sufficient amounts.[422] Therefore, chitosan may be useful for promoting weight loss. However, chitosan can accelerate weight loss when subjects follow a low-calorie diet but will be ineffective in those consuming their normal diets.

Chitosan is also effective in lowering serum cholesterol concentration and hypertension in subjects with a restricted diet. Of course, the quality and the chemical form of chitosan should be adequate to the scope.

5.1.3. Osteoarthritis Prevention and Treatment

Osteoarthritis is treated with a variety of pharmacologic therapies including acetaminophen, nonsteroidal antiinflammatory drugs (NSAIDs), cyclooxygenase-2 inhibitors, intra-articular steroids, viscosupplements, vitamins, and capsaicin that are mostly directed at pain relief and do not address the issue of correcting the degeneration of connective tissue.[423] On the other hand, there is interest in the use of glucosamine and *N*-acetyl-D-glucosamine (NAG), building blocks of the connective tissue in joints [e.g., glycosaminoglycans (GAGs), chondroitin, and hyaluronic acid]. Glucosamine, occurring in the connective and cartilage tissues, contributes to maintaining the strength, flexibility, and elasticity of these tissues. Glucosamine is not only a substrate for the synthesis of GAGs, but also stimulates their synthesis and prevents their degradation. Additionally, these compounds have been shown to offer protection against oxidative damage.[424]

A problem with use of oral glucosamine or its derivatives has been its relatively short half-life in

blood, and thus, a sustained release form of glucosamine has been sought. The use of polymeric forms such as chitin and chitosan was explored.[425−428] The bioavailability of glucosamine was evaluated in normal healthy volunteers who ingested 1−1.5 g each day of NAG or chitin. Glucosamine sulfate has been shown to be a safe and relatively effective treatment for osteoarthritis.[429]

Concerning glucosamine sulfate, well-documented and carefully controlled studies have been completed.[430,431] Sixteen randomized, controlled trials were identified, providing evidence that glucosamine is both safe and effective in the treatment of osteoarthritis. In 13 of them glucosamine was compared to placebo and found superior in all except one.

Glucosamine was compared with ibuprofen for the relief of joint pain as discussed in double-blind randomized controlled trials. Studies on 218 participants compared 1.2 g of ibuprofen with 1.5 g of glucosamine sulfate daily and found glucosamine to be of similar efficacy to ibuprofen. They concluded that glucosamine provides cartilage-rebuilding properties not obtained with simple analgesics and that glucosamine could be used as an alternative to antiinflammatory drugs and analgesics or as a useful adjunct to standard analgesic therapy.[432] The American College of Rheumatology made recommendations for the medical management of patients with lower limb osteoarthritis, and glucosamine was suggested as a first-line agent for patients with knee osteoarthrosis with mild-to-moderate pain.[423,425,427,432−439]

The substance of a number of studies is that the oral ingestion of chitin/chitosan was followed by the appearance of oligomers and monomers of glucosamine and *N*-acetyl glucosamine in the blood that functioned in the chondroprotective processes.[425−428,440,441]

In conclusion, chitosan, glucosamine, and its sulfate salt appears to provide an effective therapy in the prevention and treatment of osteoarthritis. The fact that chitosan provides an effective sustained release of glucosamine can be appreciated. The involvement of glucosamine and its derivatives in preventing inflammatory cascades and oxidative damage in addition to their direct effects on GAG synthesis and degradation is now becoming clear.[442−444]

5.2. Nasal

The obvious disadvantages of injections compared to noninvasive systems are high costs for sterile manufacture and qualified personnel to administer the medicine besides the low patient compliance. The nasal tissue is highly vascularized and provides efficient systemic absorption. Compared with oral or subcutaneous administration, nasal administration enhances bioavailability and improves safety and efficacy. Chitosan effectively enhances the absorption of hydrophilic compounds such as proteins and peptide drugs across nasal and intestinal epithelia. The effect of chitosan on nasal epithelia was studied by varying the molecular weight and degree of deacetylation.[445] Chitosans with low degrees of acetylation (1% and 5%) demonstrated good absorption enhancement at both low and high molecular weights. Chitosan with 35% acetylation and a high molecular weight (170 kDa) exhibited absorption enhancement and low toxicity. It increased apical cell membrane permeability and redistributed cytoskeletal Factin and tight junction protein ZO-1,[446] possibly due to its cationic nature. Its interaction with the cell membrane resulted in the structural reorganization of tight junction-associated proteins followed by enhanced drug permeability.[446,447] Highly quaternized *N*-trimethylchitosan chloride was also deemed to be a potent absorption enhancer.[448] A nasal formulation with improved absorption of macromolecules and water-soluble drugs is still a challenge because of the short retention time in the nasal cavity due to the efficient physiological clearance mechanisms. Chitosan may be a good option in nasal delivery as it binds to the nasal mucosal membrane with an increased retention time and is a good absorption enhancer. These ideas have been borne out by the prolonged release of macromolecules achieved using bioadhesive microspheres in the nose.[449] Systemic bioavailability in test animals demonstrated that as little as 0.5% of chitosan was sufficient to improve plasma insulin concentration significantly.[450] Chitosan binds to mucosal membrane, thus prolonging the retention time of the formulation on the nasal mucosa. The time taken for 50% of chitosan microspheres and solution to be cleared from the nasal cavity following nasal administration to human volunteers was evaluated.[451] When the control was cleared rapidly with a half-life of 21 min, half-lives of 41 and 84 min were recorded for chitosan solution and microspheres, respectively. However, the retention time was dependent on the cross-linking density of chitosan.[452] Illum et al.[453] reviewed their own experiences using chitosan in solution and powder forms as nasal delivery system for vaccines. They discussed three types of diseases, viz., nasal influenza, pertussis, diphtheria, and the vaccination based on chitosan powder and solution.

Chitosan/hyaluronan microparticles for the nasal delivery of gentamicin have been developed.[229] Chitosan glutamate (CH), hyaluronic acid (HA), and a combination of both polysaccharides were used in this study, and the microparticles were prepared by a solvent evaporation method. The developed formulations have been tested in rabbits administered intranasally along with Gentamicin alone as a solution. Physical mixture of Gentamicin with lactose in powder form has been administered intravenously and intramuscularly. Critical analysis of the data suggests poor bioavailability of the drug when administered as a nasal solution and dry powder in comparison to intravenous administration. However, the authors succeeded in their attempts to demonstrate the improved bioavailability of the model drug when used as CH, CH/HA, or HA microparticulate formulation, and the order is CH/HA > CH > HA.[229]

In a recent study, Tengamnuay and co-workers[454,455] reported the effect of chitosan on nasal absorption of salmon calcitonin (sCT), an endogenous polypeptide hormone consisting of 32 amino acids that plays a vital role in both calcium homeostasis and bone remodeling; more importantly, it suffers

from poor bioavailability. β-Cyclodextrin, one of the most commonly used enhancers, was used as a standard for comparison purposes. Two types of chitosan, the free amine and the glutamate salt, were evaluated. The plasma calcium lowering effect in each sCT-treated rat was determined by calculating the total percent decrease in plasma calcium (%D), where plain chitosan showed an increase in %D with the decrease in pH following an increase in ionization and hydration of the free amine chitosan at more acidic pH. However, chitosan glutamate showed an increase in %D with increasing pH, with maximum hypocalcemic effect observed at pH 6.0. The optimal pH was found to be 4 and 6, respectively. The absorption-enhancing effect of both chitosans was concentration dependent and 1.25%.

Hamman et al.[456,457] tested five TMCs with different degrees of quaternization as nasal delivery systems. These polymers were administered together with [^{14}C]-mannitol in the nasal route of rats at pH 6.20 and 7.40, respectively. The absorption was found to be pH dependent as all the tested polymers showed an increase in absorption at pH 6.20; however, at pH 7.40, the absorption is dependent on the degree of quaternization of the polymer. Polymer beyond 36% quaternization exhibited increased activity at pH 7.40, and the increase was observed until 48% and stabilized on further increase. This effect of the degree of quaternization on the absorption behavior was predicted due to steric effects caused by the methyl groups and changes in the flexibility of the TMC molecules and, therefore, led them to conclude that degree of quaternization has a major role in the absorption enhancement of this polymer across the nasal epithelia in a neutral environment.[456]

Cross-linked microspheres for nasal administration of LHRH analogue were prepared by water-in-oil emulsion followed by cross-linking with glutaraldehyde.[458] Chitosan in three different forms along with goserelin as a LHRH analogue were administered nasally to sheep. The bioavailability of the active substance was found to be greatly dependent on the formulation used and followed the order cross-linked microspheres > dry blend > solution.[458]

Alonso and co-workers[184,185] developed freeze-dried insulin−chitosan nanoparticulate formulations in order to reduce plasma glucose levels following nasal administration. Insulin release studies in vitro were performed for the freshly prepared formulations as well as freeze dried. Both formulations behave alike at pH 4.3, exhibiting rapid release due to solubility of insulin as well as chitosan at the working pH. However, due to lack of the solubility of insulin at pH 6.4, further studies at this pH were not carried out. On the basis of these investigations, the authors concluded that solubility is the factor that affects the release of insulin whereas the freeze-drying process had no influence on the release rates. In vivo studies were performed in conscious male New Zealand albino mice and found that these chitosan formulations are able to reduce the plasma glucose levels to a greater extent than the normal insulin−chitosan solutions.

As a part of their extensive studies on chitosan as nasal delivery vehicles, Illum and co-workers[459] investigated the effect on mucociliary transport velocity of 0.25% solutions of five different types of chitosan with varying molecular weights and degrees of deacetylation. The mucociliary transport velocity was determined by monitoring the speed of movement of graphite particles placed on the palate before and after 10 rain exposure times to chitosan solutions using a video camera and a novel image analysis system. The five types of chitosan tested were shown to have no toxic effect on the frog palate clearance mechanism. The transient decrease of mucociliary transport velocity was considered to be the result of an ionic interaction between the positively charged chitosan and the negatively charged mucus. The authors claim that this technique is very efficient, a large number of particles can be tracked simultaneously and accurately, and changes in transport velocity of the particle speed and direction during the recording period can be identified as well. They also studied different types of chitosan solutions as nasal peptide absorption enhancers.[460] The systemic absorption of insulin was monitored by measuring the glucose levels, and it was found that all chitosan solutions studied produce clinically relevant levels of insulin in blood. The effect of 0.25% chitosan solution on the nasal epithelial membranes was also studied using the rat nasal perfusion method, which showed minimal damage by the chitosan used for these studies.[461] The cilia beat frequency (CBF) in guinea pigs after nasal administration of chitosan solution was also studied for 28 days, and none of the chitosans used showed any effect on CBF, suggesting no harm in using various types of chitosan for nasal delivery applications.[451] In continuation of the studies, three different bioadhesive formulations, viz., starch microspheres, chitosan microspheres, and chitosan solution, were administered nasally, and their clearance characteristics in humans was investigated. Using γ-scintigraphy technique, the time taken for 50% of these bioadhesive materials and a control to be cleared from the nasal cavity, after nasal administration to human volunteers, was evaluated. The rate of clearance follows the order control (21 min) > chitosan solution (41 min) > starch microsphere (68 min) > chitosan microspheres (84 min), suggesting that chitosan as well as starch have good bioadhesive characteristics. These results further support the hypothesis that chitosan delivery systems can reduce the rate of clearance from the nasal cavity, thereby increasing the contact time of the carried drug with the nasal mucosa, resulting in an increase in the bioavailability of drugs incorporated into these systems. Similar studies were performed in conscious sheep.[462] They also demonstrated low glucose levels in anesthetized rat and conscious sheep models using chitosan nanoparticles.[463] Extensive work on development of nasal vaccines based on chitosan has been carried out, and the findings were summarized.[464−468]

The possibility to generate both a systemic and local immune response makes the mucosal system an attractive site for immunization; however, the mucosal administration of protein and peptide anti-

gens still suffers poor immune response. The mucosal administration of tetanus toxoid (TT) in the presence of a nonionic block copolymer, Pluronic F127 (F127), with chitosan or lysophosphatidylcholine (LPC) on the systemic and mucosal immune response has been studied.[469] Balb/c mice, immunized intraperitoneally (i.p.) with TT and boosted intranasally (i.n.) with TT in F127/chitosan, showed a significant enhancement in the systemic anti-TT antibody response compared to mice boosted i.n. with TT in PBS or mice boosted i.n. with TT in F127/LPC. The antigen-specific IgA response in the nasal and lung washes of these animals was found to increase in anti-TT mucosal IgA response in the group boosted with TT in F127/chitosan. A similar effect was observed when mice immunized and boosted i.n. with TT in F127/chitosan showed a significant enhancement of their systemic anti-TT IgG and mucosal IgA antibody responses compared to the animals immunized and boosted i.n. with TT in PBS or TT in F127/LPC. Thus, F127/chitosan represents a novel mucosal vaccine delivery system consisting of two components that appear to exert an additive or synergistic effect on the immune response.[469] One of the most recent reviews discusses the intranasal vaccination against plague, tetanus, and diphtheria.[470]

Microspheres composed of hydrophilic polymers (chitosan, PVA, Carbopol, hydroxpropyl methylcellulose) were prepared by solvent evaporation technique and tested for their potential as delivery devices for nasal route.[471] Microspheres prepared by chitosan and PVA appear to be smooth and spherical, whereas the other ones are irregular. The particle size for Carbopol was bigger than that of the others and followed the order Carbopol > chitosan > HPMC > PVA. The order of larger mucoadhesion was Carbopol > chitosan−PVA = HPMC; however, chitosan > HPMC. FITC was encapsulated in the microspheres, and the release rates were found to be much slower in the case of chitosan microspheres than the others. Similar studies were carried out using various particulate carrier systems[472−477] and gels[478] for improved bioavailability of the active substances.

From the foregoing sections it is well understood that chitosans are potent nontoxic absorption enhancers after nasal administration; however, Schipper et al.[479] claims that their effects on the intestinal epithelium in vivo has not been studied in detail. Therefore, they studied the effects of chitosans with varying molecular weights and degrees of acetylation on the absorption of a poorly absorbed model drug (atenolol) in intestinal epithelial cell layers with or without a mucus layer and in an in situ perfusion model of rat ileum. The effects of the chitosans on epithelial morphology and release of lactate dehydrogenase (LDH) into the perfusate were investigated in the in situ model. The observations suggest that chitosans had pronounced effects on the permeability of mucus-free Caco-2 layers and enhanced the permeation of atenolol 10−15-fold, with different absorption kinetics for different chitosans, in accordance with previous results, and this was not the case with atenolol absorption through rat ileum. LDH release from the tissues perfused with chitosans did not increase, indicating that the chitosans were used at nontoxic concentrations. Morphological examination of the perfused ileal tissues revealed more mucus discharge from the tissues exposed to chitosans than from controls, which suggested that the discharged mucus may inhibit the binding of chitosan to the epithelial surface and hence decrease the absorption-enhancing effect. This hypothesis was supported by studies with intestinal epithelial HT29-H goblet cells covered with mucus. The binding of chitosan to the epithelial cell surface and subsequent absorption-enhancing effects were significantly reduced in mucus-covered HT29-H cultures. When the mucus was removed prior to the addition of chitosan, the cell surface binding and absorption-enhancing effects of the chitosans were increased. These extensive studies led them to conclude that the modest absorption-enhancing effects of unformulated chitosan solutions in the perfused rat ileum are a result of the mucus barrier in this tissue. This effect may be overcome by increasing the local concentrations of both chitosan and drug, i.e, through formulation of the chitosan into a particulate dosage form.[479] Chitosan formulations have also been used for the relief of breakthrough pain in the cancer patients.[480]

5.3. Parenteral

Chitin and chitosan were administered parenterally to mice, and their toxicity was evaluated in comparison to orally administered mice.[481] It was found that 5 mg of chitin every 2 weeks on intraperitoneal injections appeared to be normal over a period of 12 weeks; however, loss of body was observed and inactivity started in the fifth week.[482] Histological studies in chitin-administered animals suggests that many macrophages with hyperplasia were observed in the mesenterium and foreign body giant cell-type polykaryocytes were observed in the spleen, which was not the case when administered chitosan. Also, subcutaneous injections of 5 mg of chitin lead to polykaryocytes; however, no other changes and no abnormalities were noticed with chitosan. These investigations suggest that one should be careful while using chitin and chitosan for long-term applications.

Injection of in situ gel-forming biopolymer is becoming increasingly attractive for the development of therapeutic implants and vehicles.[482] Subcutaneous injection of a chitosan hydrogel with human coagulation factor IX triggered sustained release and prolonged plasma levels compared with intravenous and subcutaneous injection.[483] An injectable chitosan gel with morphine sulfate was found to be stable during long-term storage.[484] Beagle dogs that received subcutaneous injection of this gel presented constant plasma concentration compared with conventional morphine sulfate. The antinociceptive actions of morphine were also sustained with these injectable gels compared to the free drug in the experimental rats.[485] Intramuscular injection of progesterone-loaded chitosan microspheres exhibited a constant plasma concentration of approximately 1−2 ng/mL for up to 5 months in rabbits.[486]

The antitumor activity of mitoxantrone−chitosan microspheres was evaluated against Ehrlich ascites carcinoma in mice following intraperitoneal injection.[487] The mean survival time of animals receiving 2 mg of free mitoxantrone was 2.1 days compared to 50 days in the case of animals receiving 2 mg of drug-loaded microspheres. Immunogenicity studies on Wistar rats using diphtheria toxoid-loaded chitosan microspheres exhibited a constant antibody titer for more than 5 months.[488] Clinical therapy with cisplatin−chitosan microspheres in six patients with liver cancer was reported to be successful.[489] Although the plasma concentration of platinum and the area under the curve (AUC) of the microsphere group were lower than that in the free drug solution group, the platinum content in hepatic tissue was 2.92 times greater than that following administration of the free drug solution. The biocompatibility of many chitosan derivatives of different molecular weights intended for parenteral delivery was investigated.[476]

The effect of chitosan on rat knee cartilages using 0.2 mL of 0.1% chitosan solution (pH 6.9) by injecting inside the rat knees articular cavity was studied Figure 38A−E.[490] Histological and histomorphometric studies after 1, 3, and 6 weeks postinjection were performed on undecalcified samples embedded in polymethylmetacrylate. These investigations suggest that after the 1st and sixth weeks chitosan significantly slows the decrease in epiphyseal cartilage thicknesses and significantly increases articular cartilage chondrocyte densities. However, chitosan solution induces a proliferation of fibrous tissue with abundant fibroblasts, fibrocytes, and monocytes inside the joint, and this proliferation is still present after 6 weeks, Figure 38 E. These investigations made the authors suggest that chitosan could act on the growth of epiphyseal cartilage and wound healing of articular cartilage. Biodegradation and distribution of water-soluble chitosan (50% deacetylated) in mice after intraperitoneal (i.p.) injection was studied, whereas in vitro studies were carried out by incubating with lysozyme, murine plasma, and urine.[491] Chitosan and fluorescein isothiocyanate-labeled chitosan have been used for the entire study, and the degradation rates were found to be accelerated in the presence of lysozyme, plasma, and urine, which was confirmed by molecular weight determination using gel chromatography.[491] The body distribution and urinary excretion of FTC−chitosan were investigated at 1, 14, and 24 h after the i.p. injection to mice. Most of the FTC−chitosan was excreted into urine after 14 h due to faster movement to the kidney and urine and was scarcely distributed to the liver, spleen, abdominal dropsy, and plasma. The molecular weight of the excreted FTC−chitosan was as small as that of the product obtained by the long in vitro incubation, suggesting that chitosan is highly biodegradable and easily excreted in urine, and further it is suggested to have no accumulation problem in the body; however, at the same time, chitosan is found not to operate as a polymer support showing long retention in the body.[491]

Kato et al.[492] further investigated succinyl chitosan as a long circulating polymer in the system. The body

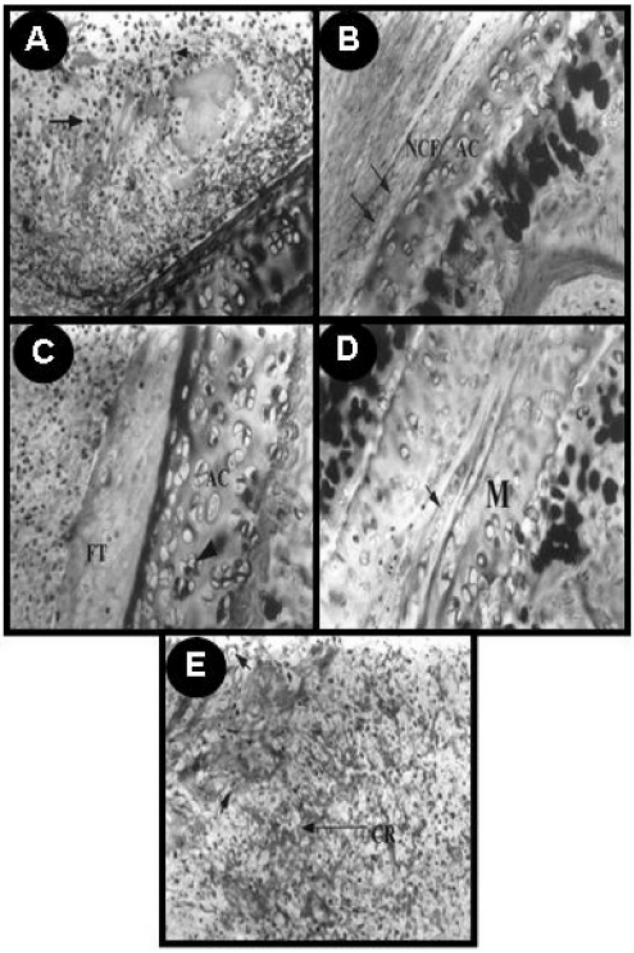


Figure 38. (A) Joint injected with 0.1% chitosan solution at pH 6.9 1 week after injection. Presence of numerous fibroblasts and monocytes trapped into fibrosis (arrows). Presence of possible chitosan residues (CR). Original magnification ×40. (B) Presence of new cartilaginous formation (NCF) in close contact (arrows) with the articular cartilage (AC). Original magnification ×40. (C) Presence of numerous round and hypertrophic chondrocytelike cells (arrow) in the articular cartilage (AC). Fibrous tissue (FT) is not adherent to the cartilage and possibly contains chitosan residues after 3 weeks. Original magnification ×80. (D) Presence of fibrocartilage tissue (arrow) in addition to anatomic fibrous tissue adjacent to knee meniscus (M). Original magnification ×40. (E) Fibrous tissue with abundant fibroblasts (short arrows) and chitosan residues (CR, long arrows) partly fill the articular cavity. Original magnification ×80. (Reproduced with permission from ref 490. Copyright 1999 Elsevier.)

distribution and urinary excretion on a long time scale (24−72 h after i.v. injection) using a fluorescein-labeled succinyl chitosan (Suc-chitosan−FTC) were performed in normal mice as well as Sarcoma 180 tumor bearing mice. Suc-chitosan−FTC was sustained at a high level in circulation over 72 h, that is, the plasma half-life in normal mice was 100.3 h and in tumor-bearing mice 43.0 h, which was longer than those of other long-circulating macromolecules reported to date.[491] As for the tissues except blood circulation, Suc-chitosan−FTC was distributed very little in tissues other than the tumor. Although the total amount of Suc-chitosan−FTC residing in tested tissues decreased gradually, urinary excretion did not increase from 24 h after injection. These observations

suggested that Suc-chitosan−FTC may be eliminated by mechanisms other than in the urine or moved to tissues other than those tested. The ratio of tumor accumulation reached a plateau at 48 h after injection, and the accumulation level, approximately 10%, was similar to those observed for other reported long-circulating macromolecules.[492] The preparation and biodistribution into the intestine, bone, lymph nodes, and male genital organs after i.v. administration of lactosaminated *N*-succinyl chitosan drug carrier was also studied.[493] Similarly, lactosaminated *N*-succinyl chitosan as a liver-specific drug carrier in mice was also studied.[107] Qin et al.[494] studied antitumor activity of water-soluble low molecular weight chitosan prepared by enzymatic hydrolysis.

Domard and co-workers[495] studied the optimal conditions for using chitin-based gels in periodontal surgery. Rheological properties during injection especially the flow through the tip of the syringe, the capacity of adaptation of the solution to the walls of the bone disease, and the injection time are essential factors to be considered. The acetylation parameters found to be key features affecting the injections are as follows: a molar ratio acetic anhydride/glucosamine residue of $R = 1.5$, a temperature of the master solution of 12 °C, a mixture of hydro alcoholic solution/acetylating reactive stirred for 45 s at room temperature, and a chitin concentration of 3.6%. This concentration allows limiting the synthesis to improve the mechanical properties of the gel and obtain a viscosity suitable for injection. Doping of the gel by means of chitosan powder insoluble under these conditions led to an improvement of the biological activity of the gel.

In vitro and in vivo evaluations of sulfated chitosans as blood anticoagulants were performed.[496] Several derivatives with different polymerization and sulfation degrees were evaluated for their potential. The results suggest that low molecular weights (M_w 61−82 KDa, polymerization degree 188−252) and high sulfination patterns (sulfur 15.6−16.9%, sulfination degree 1.58−1.86) were slowly cleared and that there was more antithrombin activity (30−52 IU/mg).[496] The authors observed differences in vivo vs in vitro, especially in activated partial thromboplastin time, therefore, expressing a preference for the in vivo system for evaluating the antithrombin activity of these derivatives.[496]

Thermosensitive gels have been extensively investigated for several applications.[269−273] In a recent study, the potential of thermosensitive chitosan gel for local delivery of paclitaxel was evaluated.[497] The authors claimed that one intratumoral injection of the thermosensitive hydrogel containing paclitaxel was as efficacious as four intravenous injections of Taxol in inhibiting the growth of EMT-6 cancer cells in mice but in a less toxic manner.[497]

5.4. Transdermal

The transdermal delivery of drugs into systemic circulation has generated much interest during the past decade. Transdermal drug delivery systems (TDDS) offer many advantages over the conventional dosage forms or controlled release peroral delivery systems. TDDS provides constant blood levels (1−7 days), avoids first-pass metabolism, increased patient compliance, and dose dumping never occurs. The choice of drugs delivered transdermally, clinical needs, and drug pharmacokinetics are some of the important considerations in the development of TDDS. Although novel approaches such as iontophoresis and ultrasound are gaining importance as a means to increase drug permeation into systemic circulation, clinical products based on these approaches are still far away. The importance of appropriate animal model selection in the development and evaluation of TDDS cannot be ignored. The cost per milligram of drug delivered transdermally is more expensive than peroral route. The added cost could be justified if TDDS improved patient compliance and reduced toxicity.

A chitosan-based transdermal delivery system was developed using cross-linked chitosan membrane and chitosan gel[498] where the membrane was the rate-controlling device and the gel acting as the reservoir: the cross-link density of the membrane dictated the drug permeability. Similarly, results were reported when a combination of chitosan with collagen was used as a rate-controlling membrane.[499] However, alginate gel was used as the drug reservoir instead of chitosan gel. It has been suggested that water-soluble drugs permeate mainly through the pores in the chitosan membrane, whereas hydrophobic drugs permeate by both partition and pore mechanisms.[500] A transdermal film dosage form of captopril was prepared from chitosan plasticized with poly(ethylene glycol).[501] A chitosan patch containing 20 mg of drug improved drug availability when tested on the back skin of male albino rats compared with an oral dose of 4.3 mg/kg. No significant irritant reaction was observed on the skin.

Many previous studies have shown chitosan-mediated decreases in the trans-epithelial electrical resistance. This is accompanied by an increase in the permeability of the monolayers to inert hydrophilic protein markers such as inulin or mannitol. A chitosan-mediated increase in permeability to inert marker points to passive transport that is possible only through the paracellular space.[502−505]

Chitosan has been used as an aid to transdermal drug delivery both in vitro and in vivo in numerous studies. The ability of chitosan to aid the permeation of several drugs across excised skin from numerous species has been shown. The diffusion of buprenorphine, nonivamide, and propranolol hydrochloride from chitosan hydrogels through excised dorsal skin was shown to be greater than passive diffusion from control media.[276,506,507] Delivery of capsaicin across excised nude mouse skin was significantly enhanced by its incorporation into a chitosan gel compared to commercially available creams.[508] This means that chitosan-based transdermal drug delivery systems have the potential to be more effective than other known delivery systems. The use of chitosan as delivery vehicle resulted in improved delivery compared to other polymer hydrogels. Iontophoresis (the application of an electric current to aid the passage of drugs across the epithelium) along with dermal

application of chitosan−drug hydrogels resulted in a greater drug flux across the skin barrier compared to either methods individually, i.e., over a 10-fold increase of transdermal drug flux compared to control. This result may be of clinical interest.

The ability of chitosan to facilitate the systemic availability of transdermally applied drugs has been shown in vivo. Delivery of the drug bromocriptine from chitosan hydrogels across rabbit skin in vivo resulted in a similar concentration of circulating drug as observed following administration of the standard oral dosage.[509] The high fraction of this drug lost by hepatic metabolism following oral administration underlines the need for its delivery by a transdermal route. Transdermal delivery of the β-blocker oxprenolol hydrochloride from chitosan films was shown to have a systemic effect in rats: reduced arterial blood pressure was observed.[510] These data imply that the pharmacological efficacy of drugs is not impaired by delivery in this manner and that chitosan can facilitate the transdermal delivery of drugs for systemic effect.

5.4.1. Roles of Chitosan in Affecting the Skin Barrier

Effective transdermal drug delivery involves permeation of the drug through a number of barriers—the delivery system itself, the epidermis, the dermis, and in the case of systemic drugs endothelial cell walls and into the blood stream. The stratum corneum has been thought to be the primary barrier to drug delivery in the human skin for many years, and the limiting factor of transdermal drug delivery has generally been regarded as the diffusion rate through this structure. Skin that had the stratum corneum removed was highly permeable, whereas an isolated stratum corneum was almost as impermeable as the entire skin. The barrier function of the stratum corneum is thought to be dependent upon the presence of extracellular lipids within the paracellular space. Lamellar granules, lipid containing vesicles, fuse into the plasma membrane, resulting in the extrusion of the lipids into the extracellular space. The lipids are covalently bound to the outer surface of the cell membranes, resulting in the creation of a lipid envelope that renders the stratum corneum relatively impermeable. The presence of large amounts of extracellular lipid means that lipophilic drugs can diffuse down a continuous lipophilic path through the stratum corneum more easily than hydrophilic drugs.

There is evidence that the tight junctions in skin may also contribute toward the skin barrier.[511,512] Given the key role of chitosan-mediated tight junction disruption in trans-epithelial drug delivery in vitro, tight junctions in skin may be investigated in order to understand the action of chitosan in transdermal drug delivery.

Chitosan may increase the permeability of the stratum corneum possibly by occlusion. Second, application of chitosan may result in disruption of the tight junctions immediately below the stratum corneum, allowing permeation of drug through into the underlying layers of the epidermis. The drug would then diffuse down its concentration gradient into the underlying tissues. The permeability of the stratum corneum and the presence of chitosan disrupt the underlying tight junctions, thus increasing drug permeation through both barriers simultaneously. This novel model may provide a new direction to investigate future chitosan-mediated transdermal drug delivery systems.

5.5. Implants

Implantable devices are expected to be intelligent, nontoxic, nonthrombogenic, noncarcinogenic, and easily implantable with adequate storage capacity, drug stability, biodegradability, and sterilizability. Khor and Lim[68] discussed various applications of chitosan implants in a recent review. The current trend of using natural materials as implantable devices is justified by two important reasons: (1) natural materials have been shown to better promote healing at a faster rate and are expected to exhibit greater compatibility with humans and (2) for tissue engineering purposes where cells are seeded onto the biomaterial implants. Chitin and chitosan have been used in orthopedic and periodontal applications.[513,514] Chitosan and hydroxypropyl chitosan presented enzymatic degradation in vitro and following subcutaneous implantation in rats. A combination of these two formulations was studied as film-type implants[515] containing uracil that showed sustained drug release in rats. Chitosan was also tested as local implants in the form of microspheres and fibers as sustained release depots for endothelial growth factors.[516] Endothelial cell growth factors have a relatively short half-life. This angiogenic factor should therefore be administered frequently in order to reach an effective concentration. Drug-loaded matrixes implanted subcutaneously in rats showed a burst effect; however, the release continued for more than 3 weeks. *N*-Succinyl-chitosan−mitomycin C as an implant for controlled drug release, and its antitumor activity has been evaluated.[517]

Sustained release implants of herb extracts using chitosan−gelatin matrix were developed.[518] In vitro and in vivo degradation studies using lysozymes and female Wistar rats were performed, respectively. No side effects on in vivo implants were observed, suggesting their use as medical devices. The biodegradability of these implants is a further advantage, as no surgical removal of the exhausted implants is needed.[518]

Microsphere-based chitosan implants with small particle size, low crystallinity, and good sphericity were prepared by a spray-drying method followed by cross-linking with genipin;[519] glutaraldehyde cross-linked microspheres were used as controls. Histological study of the genipin cross-linked chitosan microspheres injected into the skeletal muscle of a rat model showed a less inflammatory reaction than its glutaraldehyde cross-linked counterparts. On the other hand, the degradation studies suggest that glutaraldehyde cross-linked particles started to degrade by 12 weeks, whereas the genipin cross-linked particles were intact until 20 weeks; this suggests that these implants are suitable for long-term applications.[519]

Most recently, chitosan and sodium hyaluronate implants for controlled release of insulin were stud-

ied.[520] The optimum conditions for polyion complex formation between chitosan and hyaluronate were investigated and found to influence the insulin release; however, the compression exerted to manufacture the implant had no role to play in the release kinetics.[520]

5.6. Ophthalmic Preparations

Traditional aqueous solutions, suspensions, and ointments for ophthalmic use have the disadvantage that the major part of the drug is lost within seconds due to the nasolacrymal duct drainage; therefore, repeated administration is needed. The prime requirement for the controlled delivery of ophthalmic preparations is therefore bioadhesiveness that increases the contact time with the cornea, leading to improved drug absorption at the site.

The viscosity of the polymer solutions might induce one to believe that high viscosity would be an advantage; however, a study involving different fluids having the same viscosity and containing pylocarpine showed that the viscosity relevance is scarce.[521] What is important, on the other hand, is to modify as little as possible the rate of shear of the eye fluid, which has a very wide range ($0.03-28\,500\ s^{-1}$) depending on the movement of the eyelids. As a matter of fact, the chitosan solutions have viscoplastic and pseudoplastic characteristics that do not modify the lacrymal film, thus providing a good patient compliance. In consideration of this favorable aspect and of the chitosan bioadhesiveness, the prime requirement for an effective ocular therapeutic system, attempts were made to take advantage of the cationic property of chitosan in ophthalmic delivery.[522−526] Chitosan- and poly-L-lysine-coated ϵ-polycaprolactone nanocapsules have been tested for their efficacy in ocular delivery exploring the favorable mucoadhesion property of these cationic polymers. An improved interaction between corneal epithelium and the cationic polymers was expected; however, the cationic modification did not alter the drug-release properties. The bioavailability studies suggested no increase in case of poly-L-lysine-coated nanocapsules; however, chitosan coatings improved twice that of uncoated nanocapsules. Therefore, these findings suggest that positive charge is not a contributing factor for the improved bioavailability of the drug, but the specific nature of chitosan is responsible for the enhanced uptake.[527] The difference between PEG and chitosan coatings on ϵ-polycaprolactone nanocapsules for ocular delivery has been studied: the PEG coating accelerates the transport of the nanocapsules across the whole epithelium, whereas the chitosan coating favors retention of the nanocapsules in the superficial layers of the epithelium.[528]

The ability of chitosan as an ocular delivery device has been evaluated by comparing chitosan formulations with commercial collyrium Tobrex. Chitosan as a gel has been tested for the increased precorneal drug residence times, expecting it to slow drug elimination by lacrymal flow by increasing solution viscosity and interacting with the negative charges of the mucus. The influence of the process parameters as well as the effect of the molecular weight and concentration of polysaccharide was thoroughly studied. An ocular irritation test, using confocal laser scanning ophthalmoscopy (CLSO) combined with corneal fluorescein staining, clearly demonstrated the excellent tolerance of chitosan after topical administration onto the corneal surface. γ-Scintigraphic data showed that the clearance of the formulations labeled with ^{99m}Tc-DTPA was significantly delayed in the presence of chitosan with respect to the collyrium, regardless of the concentration and molecular weight of chitosan in solution. At least a 3-fold increase of the corneal residence time was achieved in the presence of chitosan when compared to control.[529]

Alonso and co-workers[187] carried out extensive investigations on chitosan nanoparticles as discussed in the foregoing sections of this article. They also took the challenge to prove the ability of chitosan nanoparticles to be efficient ocular delivery devices for improved bioavailability of the drugs and at the same time preventing the systemic exposure of the administered drug. In vivo experiments were performed in rabbits: therapeutic concentrations in external ocular tissues (i.e., cornea and conjunctiva) were maintained for at least 48 h, and at the same time cyclosporin (CyA) levels in inner ocular structures (i.e., iris/ciliary body and aqueous humor), blood, and plasma were negligible or undetectable.

The ability of chitosan hydrochloride to enhance the transcorneal permeability of the drug has been demonstrated.[530] Poly(ethylene oxide) (PEO) was used as a base material to which ofloxacin-containing chitosan microspheres prepared by spray drying were added and powder compressed, resulting in circular inserts (6 mm). The effect on addition of chitosan has been studied systematically, where 10%, 20%, and 30% responded according to the increase in their concentrations exhibiting accelerated erosion, thereby release of the entrapped drug. PEO molecular weight also has an influence on erosion and drug release, where PEO 900 showed better performance in comparison to PEO 2000. Experiments in the rabbit's eye formed a superficial gel that adhered to the application site, which gradually spread over the cornea and eroded with time. There were signs of irritation in the eye; however, the levels in chitosan-based systems were found to be lower when compared to chitosan-free inserts. The release and bioavailability rates were much higher for the PEO−chitosan−HCl when compared to the plain inserts of either.[530]

Also, Felt et al.[531] reported that co-administration of ofloxacin and chitosan in eyedrops increased the bioavailability of the antibiotic. The authors ascribed this effect to the high viscosity of the chitosan solution. These results are of relevance to the treatment of external ocular infections. It was also reported that chitosan is able to enhance drug penetration through the corneal epithelium, thereby potentially improving topical treatment of endophthalmic infections. Another study ascertained the ability of chitosan·HCl and *N*-trimethyl chitosan chloride (TMC), added to ofloxacin eyedrops, to improve transcorneal antibiotic transport.[532] The polycationic chitosan·HCl and TMC are believed to exert their effects by interacting with the negatively

charged cornea. TMC was more effective because it is soluble, whereas chitosan precipitates at the pH of the tear fluid.

Another chitosan derivative, *N*-carboxymethyl chitosan, did not enhance the corneal permeability; nevertheless, it mediated zero-order ofloxacin absorption, leading to a time-constant effective antibiotic level in the aqueous.[532] Also, *N*,*O*-carboxymethyl chitosan is suitable as an excipient in ophthalmic formulations to improve the retention and bioavailability of drugs such as pilocarpine, timolol maleate, neomycin sulfate, and ephedrine. Most of the drugs are sensitive to pH, and the composition should have an acidic pH to enhance the stability of the drug. Therefore, delivery should be made through an anion-exchange resin that adjusts the pH at around 7 that is physiologically acceptable.[533] Chitosan solutions do not lend themselves to thermal sterilization. A chitosan suspension, however, can be sterilized at 121 °C for 15 min and then treated with sterile solution of a suitable acid (HCl). The presence of mannitol, sorbitol, and glycerol seems to stabilize the sterilized solutions. This preparation was found to be particularly useful for pharmaceutical and ophthalmic compositions.[534]

The favorable characteristics of chitosan have been considered in proposing tear substitutes[535] that appear to be particularly suitable in light of the discovery of chitin-like substances in the human ocular mucus.[536] Chitosan, therefore, has a unique place among the ophthalmic drug delivery systems.[537]

5.7. Gene Delivery

Transfection is the genetic modification of cultured animal cells by the uptake of DNA from the culture medium. The DNA to be transfected is usually in the form of the recombinant plasmid or other type of DNA vector containing the gene of interest. Several methods have been developed to facilitate the process, among which the most widely used are based on calcium phosphate- or diethylaminoethyl dextrin-mediated transfection.

Mumper et al.[78,538] were the first to describe the potential of chitosan as a gene carrier. The low toxicity of chitosan and its nature make it attractive for gene delivery purposes.[449,450,539] In early studies, chitosan has been shown to bind nucleic acids,[540] and it is known that chitosan may actually be endocytosed into the cell.[476]

As for the majority of polycations, the interaction between chitosan and DNA is electrostatic.[541] Such interaction appears to be strong enough that the chitosan−DNA complex does not dissociate until it has entered the cell.[542] Also, it was shown that chitosan plays an important role in both membrane adhesion[543] and lysosomal escape[544] of the encapsulated DNA, providing a plausible explanation for the ability of the polymer in cell transfection. Hayatsu et al.[545] observed that a treatment of DNA/chitosan complex with phosphate-buffered saline resulted in the release of only minute quantities of DNA (0.05%), thereby indicating the formation of a tight and compact complex. Lee et al.[546] examined the potential of a water-soluble and low molecular weight chitosan (LMWC) having an average degree of polymerization of 136 (∼20 kDa) and a degree of deacetylation of 72.5%. Complexes of plasmid/LMWC were prepared by mixing the two components in PBS medium, and the stability of the complexes was judged by gel retardation assay. The complexes were completely retarded at a 1:2 weight ratio (plasmid/chitosan), indicating total condensation. Also, it was demonstrated that plasmid/LMWC formulation efficiently protects the plasmid from DNase degrading enzymes.

The hybrid DNA−chitosan systems can be classified into two categories which differ in their mechanism of formation and morphology: complexes and nanospheres.

(1) Chitosan−DNA complexes: simple mixing of two solutions of DNA and chitosan generated broad particulate complexes with mean sizes between 100 and 600 nm, depending on the molecular weight of the chitosan.[538] Stable complexes are usually formed when chitosan is added in molar excess relative to the negatively charged DNA with zeta potential values between +0 and 20 mV, depending upon the degree of excess.[547]

(2) Chitosan−DNA nanospheres: the method for nanoparticle formation was modified by augmenting the mixing speed and temperature upon blending of the two solutions and incorporating a dilute salt (Na_2SO_4) in the DNA solution and a desolvating agent in the polymer solution.[548] Such modifications induced significant changes in particle morphology, creating a monodisperse, spherical suspension between 200 and 500 nm which sharply differed from the loose rodlike[78] and toroidal[78,547] structures reported for chitosan−DNA complexes. Mao et al.[541] examined the influence of several parameters on the size of chitosan−DNA nanospheres. The particle sizes were not affected by the sodium sulfate concentration in the range of 2.5−25 mM and neither by the plasmid size. On the other hand, chitosan−DNA complex sizes were found to clearly depend on the molecular weight of the chitosan (108−540 kDa) used and N/P ratio.[78,83,549] This nanosphere formulation was found to additionally trap low levels of small molecules such as chloroquine, a well-known lysosomotropic agent, enabling a simultaneous delivery of DNA and drugs. Given their novel properties, these compact structures were considered distinct from the traditional complexes and named chitosan−DNA nanospheres.[541]

At physiological pH, not all amino groups of chitosan are protonated; therefore, the N/P ratio is adopted, where N is chitosan nitrogen and P is DNA phosphate. At a N/P ratio of 2, large-size complexes were obtained with a zeta potential of the complexes close to 0 mV, indicating full retardation of DNA and aggregation of the complex at neutral surface charge.[550]

The electrostatic nature of the interaction of chitosan with DNA was assessed by ethidium bromide competitive studies.[541] When chitosan is added to a solution of ethidium bromide-stained DNA, fluorescence decreases as a result of competitive binding of cationic chitosan to DNA. Ethidium bromide quenching assay in combination with confocal laser scanning

microscopy was also applied to determine the distribution of encapsulated DNA in chitosan nanoparticles.[548] Mumper et al.[538] indirectly demonstrated the absence of free DNA after complex formation by gel electrophoresis.[541] Another method for determining DNA loading is by pico Green assay after digestion with chitosanase and lysozyme. Usually 95% loading of DNA is achieved by simple mixing of chitosan and DNA. Stability studies have shown that the cross-linked chitosan/DNA nanoparticles stored in water remained stable for more than 3 months, whereas un-cross-linked formulation remained stable for few hours only.[8] Also, complexation of DNA with highly purified chitosan having different molecular weights at N/P 1 resulted in almost complete inhibition of degradation by DNase I and DNase II.[551] The resistance to degrading enzymes is probably achieved by change in the tertiary DNA structure, causing steric hindrance.

5.7.1. Chitosan/Plasmid Complexes: Toxicity and Transfection Efficiencies

In recent years, cationic polymers have been in the forefront in the field of gene delivery and cell transfection. This is an attractive choice as simple mixing of plasmid DNA with the polycation in aqueous medium leads to electrostatically driven self-assembly complexes in the nanoscale range.[552] The use of chitosan in the field of gene delivery has several advantages, some of which are listed.[78,538,541,548]

(1) Conjugation of ligands to chitosan−DNA complexes for targeting and receptor-mediated endocytosis.

(2) Incorporation of lysosomotropic agents to enhance the escape from the lysosome and hence reduce degradation of complex.

(3) Coencapsulation of other bioactive agents for multiple plasmids.

(4) High protection against serum nuclease degradation.

(5) Lyophilization of polyplexes for long time storage without comparable loss in the activity.

Mao et al.[541] performed several in vitro transfection experiments and showed that chitosan−DNA nanospheres were able to transfect HEK293, IB-3-1, HeLa cells but with remarkably lower levels than the commercial lipid-based transfecting agent, Lipofectamine. Although, coencapsulation of chloroquine significantly increased the transfection efficiencies of another natural, cationic biopolymer (gelatin), chitosan did not substantially benefit from the same co-incorporation.[541,548] Sato et al.[553] investigated the nature of chitosan−DNA complexes and their transfection efficiencies. The team showed that cell uptake of chitosan−DNA complex was dependent on cell type and the complex mixtures and was far superior to polygalactosamine−DNA complexes in transfecting A549 cells.[69] The optimal charge ratio (N/P) for cell transfection varied from cell to cell type.[550,554−556]

Corsi et al.[557] established optimal conditions for the chitosan−DNA complexation. The complexes were of a mean diameter inferior to 100 nm (Figure 39) with the interior of the particles showing homogeneous DNA distribution. The efficacy of transfection in vitro

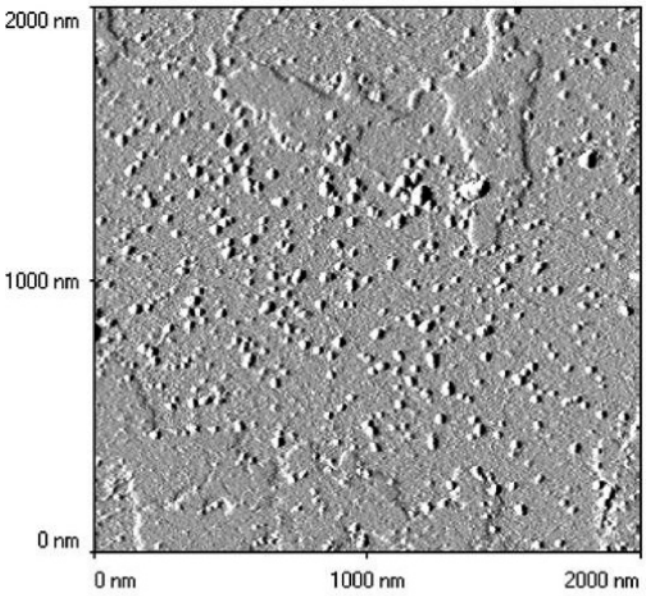


Figure 39. Atomic force microscopy analysis of complexes formed between chitosan and DNA. (Reproduced with permission from ref 557. Copyright 2003 Elsevier.)

on HEK293, MG63, and MSC (Mesenchymal stem cells) demonstrated cell-type dependence, with HEK293 cells having a higher gene expression, however, not surpassing that seen with Lipofectamine. Cell viability studies applying the MTT test confirmed the low toxicity of chitosan, in marked contrast to Lipofectamine which causes approximately 50% toxicity. These results suggest that chitosan−DNA nanoparticles have favorable characteristics for nonviral gene delivery and are cell dependent and not cytotoxic. The transfection efficiencies of chitosan−DNA-based complexes were not affected by the presence of 10% serum in cell culture.[541]

Sato et al.[69] further investigated the effect of the experimental conditions, such as medium pH, serum content, molecular weight of chitosan, and N/P ratio, on the transfection efficiency of plasmid/chitosan complexes. The transfection efficiencies were evaluated in A549 cells applying pGL3 encoding to luciferase. The medium was 10% FBS with two pH values: 6.9 and 7.6. The transfection efficiency at pH 6.9 was remarkably higher than that at pH 7.6. This dependence is probably attributed to the protonation of amines in chitosan. The pK_a of amines in chitosan has been reported to be around 6.3−6.4.[558,559] This means that at a pH lower than 7, amino groups of chitosan are charged and could bind DNA through electrostatic interaction. In contrast, a higher pH value (i.e., 7.6) led to a remarkable decrease of the amino group protonation and hence to a drastic decrease of the transfection efficiencies. On the contrary, when the transfection efficiencies of the Lipofection/DNA, a lipid-based formulation, were tested at various pH values, no remarkable differences in the transfection yields were obtained. This was attributed to the chemical nature of the quaternary ammonium salt of the headgroup of the lipid, which is not affected by the pH of the medium. The transfection efficiency of plasmid/chitosan complex was assessed as a function of N/P ratio. The optimal

ratio for transfection was found to be 5:1. Higher N/P ratios (i.e., 10:1 and 20:1) resulted in a drastic decrease in the transfection yields. Also, it was found that the highest transfection yields were obtained when plasmid concentrations of 5−10 μg/mL were applied. The transfection efficiency was also assessed as a function of the molecular mass of chitosan. The transfection experiments were tested in A548, B16 melanoma, and HeLa cells in 10% FBS. Chitosans of 15 and 52 kDa largely promoted gene expression in all tested cell lines. Heptamer of an average molecular weight of 1.3 kDa did not induce transgene expression in all tested cell lines. Also, it was found that the transfection yield obtained with high molecular weight chitosan (>100 kDa) was less than the transfection efficiency obtained with chitosan of 15 and 52 kDa. As a second control, polygalactosamine (pGalN) was assessed for its transfection efficiencies and found to be inactive in all tested cell lines. The inactivity of pGalN/plasmid complex was attributed to intracellular processes and suggests that the pGalN/plasmid complex is very stable even in the endosome and the plasmid may not release from it. Maximum transfection yield of chitosan/plasmid complex was obtained in 20% FBS medium, in marked contrast to Lipofectin which gave no transfection in this medium. Higher serum contents (i.e., 30% and 50% FBS) resulted in a gradual decrease in the transfection efficiencies.

Richardson et al.[551] prepared highly purified chitosan fractions of <5000 Da (N1), 5000−10 000 Da (N2), and >10 000 Da (N3) by hydrolysis of high molecular weight chitosan and characterized them with respect to their cytotoxicity, ability to cause haemolysis, and ability to complex and protect DNA from degradation. All chitosan fractions displayed little cytotoxicity against CCRF−CEM and L123 cells (IC_{50} > 1 mg/mL), and they were not hemolytic (<15% lysis after 1 and 5 h). Also, chitosan fractions were found to exhibit a stronger interaction with DNA when compared to poly(L-lysine) and resulted in a total inhibition of DNA degradation by DNase II. After intravenous injection of chitosan−DNA complexes, all the chitosan fractions showed rapid blood clearance. The plasma levels at 1 h were 32.2 ± 10.5% of recovered dose for N1 and 2.6 ± 0.5% of recovered dose for N3. Liver accumulation was molecular mass dependent, being 26.5 ± 4.9% of the recovered dose for N1 and 82.7 ± 1.9% of the recovered dose for N3. The observations that the highly purified chitosan fractions used were neither toxic nor hemolytic, that they have the ability to complex and protect DNA from degradation, and that low molecular weight chitosan (N1) can be administered intravenously with minor liver accumulation suggest there is potential to investigate further low molecular weight chitosans as components of a synthetic gene delivery system.[551] Chitosan HCl salt was the most toxic, having an IC_{50} (inhibitory concentration 50%) of 0.21 ± 0.04 mg mL^{-1}, which was only 4-fold less toxic than poly(L-lysine) (IC_{50} of 0.05 ± 0.01 mg mL^{-1}).[7] High molecular weight chitosan was the most toxic, the resulting ranking being chitosan HCl > chitosan hydroglutamate > glycol chitosan > chitosan hydrolactate.

Sato and co-workers[69,560] examined the correlation between complex uptake and degree of gene expression and found a strong proportional correlation in serum-free medium. However, when serum-containing medium was applied, there was no relationship between the transfection activity and cell uptake. This result indicates that cell uptake of the complexes declines as a result of nonspecific interaction with the serum components. Nevertheless, there was the highest transfection activity in the presence of 10% or 20% serum due to cell viability being optimal for transfection under these conditions.

5.7.2. Mechanism of Action of Chitosan/Plasmid Complexes

The mechanism of cell transfection with plasmid−chitosan complexes was investigated by following three processes.[560]

(1) Cell uptake: observation with a confocal laser scanning microscope showed that FITC−plasmid/chitosan complexes (at N/P 5) were taken up by endocytosis. The fluorescence of FITC−plasmid and Texas Red−dextran was observed at the same cellular localization after 1 h postincubation. The uptake of plasmid/chitosan (40 kDa) complexes was monitored also with a flow cytofluorometer. Cell uptake at 37 °C was about 3-fold more than that measured at 4 °C, although the amount of cell uptake of 1 kDa chitosan and DEAE−dextran complexes was virtually constant. This result implied that cell uptake of plasmid−chitosan complex might be thermal and energy dependent and that plasmid/chitosan complexes might be endocytosed. Similar results were reported using transmission electron microscopy observation of plasmid/chitosan complexes (N/P = 5) in HeLa cells.[550]

(2) Release from endosome: the release of plasmid/chitosan complexes from the endosomal compartment may be another rate-limiting step in the transfection process.[78] Protons accumulate into the endosomal vesicles through a vesicular ATPase-driven proton pump. Partially protonated polymers such as polyethyleneimine retain a substantial buffering capacity, so that they can be protonated at virtually any pH.[561] There are two main effects of buffering capacity of cationic polymers in endosome. (I) It may protect the plasmids from lysosomal nuclease due to delay in acidification.[562] (II) It may also perturb the trafficking of endosome for osmotic swelling and subsequent endosome disruption. It is well known from the literature that low molecular weight chitosan (1700 Da) exhibited more effective inhibition of pH fall from pH 7 to 4 than aspartame, phosphate, and monofluorophosphate buffer.[563] Furthermore, it was demonstrated that the extent of protonation of DNA/chitosan (M_w 40 and 110 kDa) complexes from pH 7 to 5 was approximately 2.5-fold larger than that of DNA/PLL complexes. Accordingly, the surface charge of plasmid/chitosan complexes increased from +2 (pH 7) to +21 mV (pH 5). These features may be a cause of pH-dependent transfection efficiency of plasmid/chitosan complexes. At pH 6.5 of the transfection medium, low and negligible transfection yields were obtained, although a certain cell uptake of plasmid/

chitosan complexes was observed at this particular pH. These results are also attributed to the low buffering effect at pH 6.5 of the transfection medium. Moreover, Sato and co-workers[560] found that lysosomal agents such as monensin and chloroquine, which increase the pH of endocytic vesicles, decreased transfection levels with plasmid/chitosan complexes in SOJ cell line. However, these endosomolytic agents improved the transfection levels with plasmid/PLL complexes.[550] It was found from these results that plasmid/chitosan complexes have a high ability for proton accumulation, and thus, endosome buffering may perturb the endosomal membrane and release complexes from endosomes as the pH of endosomes declines. Another factor that seems to limit the transfection activity of plasmid/chitosan complexes is its potential to release DNA after the endosome escape. To have transfection activity with plasmid/chitosan complexes, the complexes have to be first released from endosomes. Next, the chitosan in the complex exchanges with endosomal membranes consisting of anionic lipids via electrostatic interactions. The release of DNA from DNA/chitosan complexes was observed at a higher concentration of sodium dodecyl sulfate (10 times), which is greater than that observed for DNA/PLL complexes. Thus, plasmid/chitosan complexes were not likely to exchange with endosomal membranes and release the intact complexes from endosomes.[560]

(3) Nuclear import: the nuclear import of complexes is still controversial; specifically, the nuclear import of plasmid/chitosan complexes has never been fully examined.[550] FITC-labeled plasmid and Texas Red-labeled chitosan were used to observe cellular localization. At 4 h posttransfection, the two labeled molecules had accumulated in the nucleus and remained localized in the nucleus 24 h posttransfection. On the other hand, FITC−plasmid/(PLL or Lipofectamine) complexes did not display fluorescence images even several hours postincubation. These results illustrated that plasmid/DNA complexes have high nuclear accumulation and resistance toward cellular and nuclear enzymes.[560]

Several chitosan derivatives have been synthesized in the past few years in order to obtain modified carrier with altered physicochemical characteristics.[564] Such modifications include quaternization of amino groups to increase the net positive charge of the complex, ligand attachment for targeting purposes, conjugation with hydrophilic polymers to increase the stability of chitosan−plasmid complex against degrading enzymes, conjugation with endosomolytic peptide to increase the efficiency of transfection, etc. The following summarizes information about important chitosan derivatives and their potential applications.

Deoxycholic-acid-modified−chitosan vector: deoxycholic acid was conjugated to chitosan in methanol/water media using EDC as coupling agent.[564,565] The degree of substitution (DS) was determined to be 5.1 deoxycholic acid groups substituted per 100 anhydroglucose units. Hydrophobically modified chitosan provides stable colloidal self-aggregates in aqueous media having a mean diameter of ca. 160 nm with a unimodal size distribution. Self-aggregates/DNA complexes were formed in aqueous media and found useful in transfecting mammalian cells in vitro. The transfection efficiency of this system was relatively higher in comparison to naked DNA but significantly lower than the Lipofectamine/DNA formulation.

Quaternized chitosan vectors are useful for gene delivery owing to their improved properties in comparison to plain chitosan.[566] Trimethyl chitosan (TMO) derivatives of 40% (TMO-40) and 50% (TMO-50) degrees of quaternization were synthesized and examined for their transfection efficiencies in two cell lines: COS-1 and Caco-2.[81,83,567,568] Plain chitosan as well as DOTAP liposome carriers were applied as positive controls. DOTAP-DNA lipoplexes showed increased transfection efficiencies in COS-1 cells compared to the control group (plain DNA). Chitosan raises the transfection efficiency 2−4 times compared to the control value (i.e., naked DNA). TMO-50 markedly increases the transfection efficiencies from 5-fold (for complexes with DNA/oligomer ratio 1/6) to 52-fold (for ratio 1/14). TMO-40 displays even higher transfection efficiencies ranging from 26-fold (for ratio 1/6) to 131-fold (for ratio 1/14). However, none of the TMO-based vectors was able to increase the transfection efficiency in differentiated cells such as Caco-2. Chitosan and TMO oligomers were found to exhibit significantly lower cytotoxicity than DOTAP.

Modification of chitin and chitosan with hydrophilic polymers such as poly(ethyleneglycol) would be expected to result in hydrophilic chitin or chitosan while keeping the fundamental skeleton intact. Multiple methods have been developed for the grafting of hydrophilic polymers onto chitin or chitosan[569−572] to improve affinity to water or organic solvents. PEG−chitosan derivatives with various molecular weights (M_n = 550, 2000, 5000) of PEG and degrees of substitution were synthesized, and the water solubility of these derivatives was evaluated at pH values of 4, 7.2, and 10.[103] Almost all PEG−chitosan derivatives were soluble in acidic buffer (pH 4). Furthermore, some derivatives dissolved in neutral (pH 7.2) and alkaline buffers (pH 10). The weight ratio of PEG in the derivatives seems to dominate its water solubility. Higher molecular weight PEG was found to enhance water solubility to chitosan with a lower degree of substitution of PEG in comparison with the lower molecular weight PEG. PEG modification was found to minimize aggregation and prolong the transfection potency for at least 1 month in storage. Intravenous injection of nanoparticles and PEGylated nanoparticles resulted in a majority of nanoparticles localizing in the kidney and liver within the first 15 min. The clearance of PEGylated nanoparticles was slightly slower in comparison to non-PEGylated nanoparticles.

Although in many cases the uptake of chitosan−DNA nanoparticles appears to occur in the absence of ligand−receptor interaction, Park et al.[573,574] prepared galactosylated chitosan−graft−dextran−DNA complexes. Galactosyl groups were chemically bound to chitosan for liver-targeted delivery, and dextran was grafted for enhancing the complex stability in

aqueous media. The extensive investigations lead to the conclusion that the charge ratio of the complex has an important effect on compaction of DNA. The GC−DNA complex had an extended and aggregated form, whereas the GCD−DNA complex had a dense spherical shape. It was also observed that the GCD−DNA complex was smaller and more compact than that of GC−DNA.[573] In parallel work, galactosylated chitosan−graft−PEG (GCP)[111] was developed for the same purpose. GCP−DNA complexes were found to be stable due to hydrophilic PEG shielding and increased protection against DNase. Also, GCP−DNA complexes were found to enhance transfection in HepG2 cells having ASGR, indicating galactosylated chitosan will be an effective hepatocyte-targeted gene carrier. They also carried out extensive investigations in visualizing the transfection of hepatocytes by galactosylated chitosan−graft−poly(ethylene glycol)/DNA complexes by confocal laser scanning microscopy.[575] The efficacy of GCP/plasmid complex in gene transformation of HepG2 cells bearing ASGR on cells and HeLa and CT-26 cells without ASGRs was demonstrated. The hepatocyte-specific delivery of rhodamine-labeled GCP (R−CGP)/fluorescein-labeled plasmid (F-plasmid) complex was compared with those of CP/F−DNA one or F-plasmid using CLSM. They also investigated the cell-type dependence transfection using primary hepatocytes and HepG2 human hepatocarcinoma cell line. Further, they also checked whether the complex existed in the cytosol or was only attached to the plasma membrane of the cell. A galactosylated chitosan−graft−poly(vinyl pyrrolidone) (GCPVP) was also synthesized[112] and showed improved physicochemical properties over the unmodified chitosan. Erbacher et al.[550] synthesized lactosylated-modified chitosan derivatives (having various degrees of substitution) and tested their transfection efficiencies in many cell lines. However, the in vitro transfection was found to be cell-type dependent. HeLa cells were efficiently transfected by this modified carrier even in the presence of 10% serum, but neither chitosan nor lactosylated chitosans have been able to transfect HepG2 and BNL CL2 cells.

Transferrin/KNOB/endosomyltic proteins conjugated chitosan vector: transferrin receptor responsible for iron import to the cells is found on many mammalian cells.[576] As a ligand, transferrin could efficiently transfer low molecular weight drugs, macromolecules, and liposomes through a receptor-mediated endocytosis mechanism.[577] Transferrin has also been used as a ligand to deliver plasmid DNA and oligonucleotide.[578−582] Mao et al.[541] explored two strategies to bind transferrin onto the surface of chitosan−DNA complex Figure 40. In the first strategy aldehyde groups were introduced in transferrin (glycoprotein) after oxidation with periodate and thereafter allowed to react with chitosan amino groups via the formation of Schiff-base linkages (Figure 40a). The transfection efficiencies of transferrin-modified chitosan carriers (at varying degrees of modification) were examined in HEK293 cell line and found to produce a 2-fold transgene expression in comparison to unmodified chitosan carrier. In the

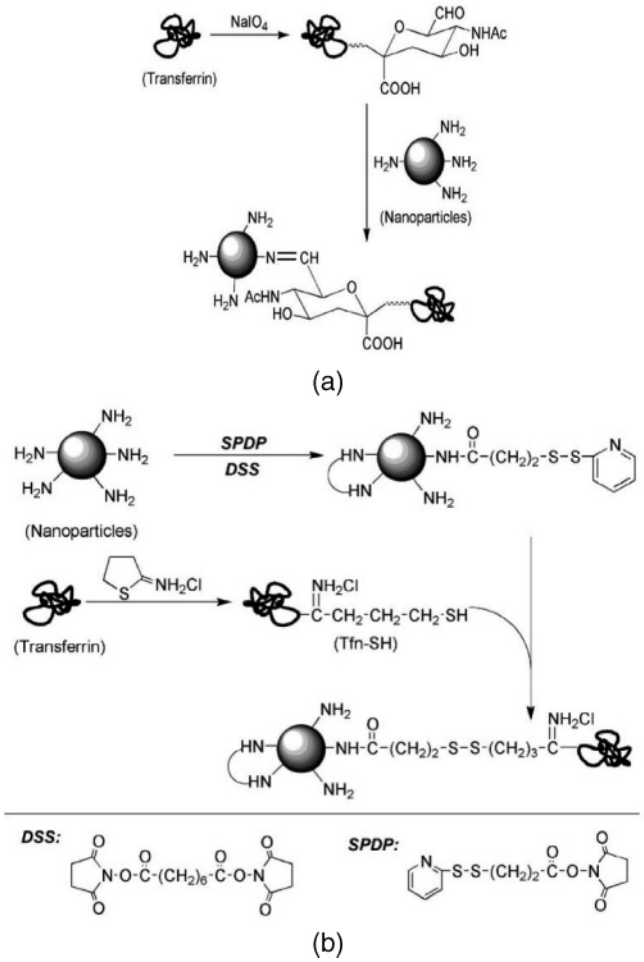


Figure 40. Conjugation of transferrin through periodate oxidation. (b) Conjugation of transferrin through a reversible disulfide linkage. (Reproduced with permission from ref 541. Copyright 2001 Elsevier.)

second strategy transferrin was introduced to the nanoparticle surface through a disulfide bond (Figure 40b). The transferrin-conjugated carrier only resulted in a maximum of 4-fold increase in transfection efficiency in HEK293 cells and only 50% increase in HeLa cells. The negligible increase in the transgene efficiencies as a result of ligand modification (i.e., galactose and transferrin) led the investigators to speculate that chitosan nanospheres may enter the cell via a unique endocytic pathway.[111,573−575,579,583−585]

To further enhance the transfection efficiency, KNOB (C-terminal globular domain on the fiber protein) was conjugated to the nanoparticle surface via a PEG spacer as shown in Figure 41. The KNOB conjugation to the nanoparticles could improve gene expression level in HeLa cells by approximately 130-fold. Also, inclusion of pH-sensitive endosomolytic peptide GM227.3 in the formulation enhanced the level of expression in vitro. Expression of a plasmid/chitosan/GM225.1 formulation in rabbits after administration in the upper small intestine and colon was observed in contrast with naked plasmid which gave no expression. The lipidic formulation DOTMA/DOPE was used as a control and was not expressed to as high an extent as the chitosan/lytic peptide formulation.[78]

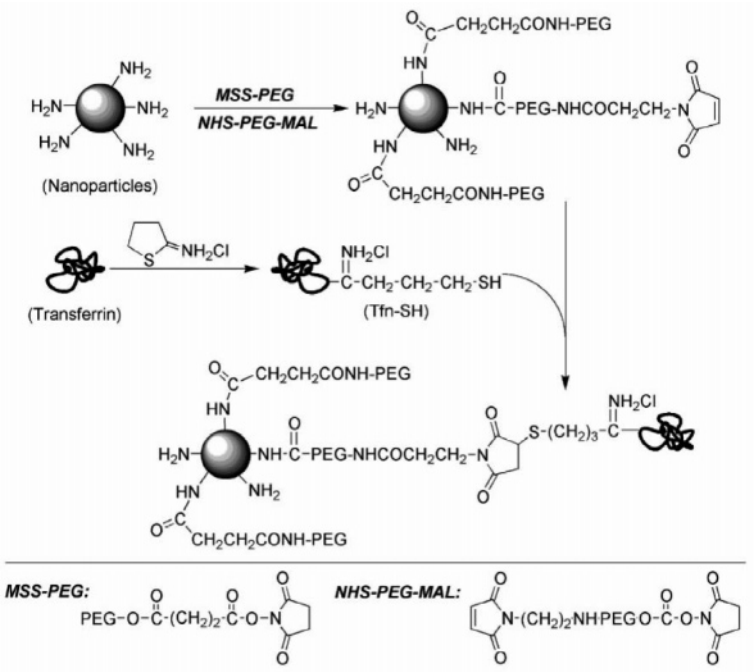


Figure 41. PEGylation of chitosan−DNA nanoparticles and conjugation of transferring through a PEG spacer. (Reproduced with permission from ref 541. Copyright 2001 Elsevier.)

6. Dressings, Scaffolds, and Radiopharmaceuticals

6.1. Wound Dressing Materials

Modified chitins have been administered to humans in the form of dressings for wounded soft and bone tissues.[12,586−590] The most peculiar characteristics of chitins and chitosans are hemostatic action, antiinflammatory effect, biodegradability, biocompatibility, and similarity to hyaluronan besides antimicrobial activity, retention of growth factors, release of glucosamine and *N*-acetylglucosamine monomers and oligomers, and stimulation of cellular activities.

6.1.1. Hemostatic Action

In the wound healing process hemostasis is the first step. Platelets, the protagonists in blood coagulation, release some cytokines that enhance the healing. The mechanism by which chitin and chitosan act as hemostatics has been investigated, and many authors have concluded that chitosan influences platelets. Okamoto et al.,[591] making use of fine powders (2.8 and 6.9 μm) of chitin and chitosan, found that the blood coagulation time was reduced by 3.7 and 4.7 min (control 12 min) in a dose-dependent manner by chitin and chitosan, respectively, and platelet aggregation was influenced by the granulometry. Chitin and chitosan enhanced the release of the platelet-derived growth factor-AB and the transforming growth factor-β-1.

6.1.2. Biodegradability

Biodegradable and nontoxic materials capable of activating host defenses to prevent infection and accelerate healing of the wound are highly desirable. Chitin is degraded by enzymes such as lysozyme[588] *N*-acetyl-D-glucosaminidase, and lipases.[592] It is not excluded that NO also plays a role in a chemoenzymatic degradation process. Evidence has been collected that significant portions of chitin-based dressings are depolymerized and that oligomers are further hydrolyzed to *N*-acetylglucosamine, a common aminosugar in the body which either enters the innate metabolic pathway to be incorporated into glycoproteins or is excreted as carbon dioxide.[593]

Chitin sutures developed in the early 1980s undergo relatively rapid biodegradation in vivo: the resistance to traction of their knots falls to 74% after 5 days and 18% after 15 days. Increased knot resistance is claimed for *N*-acyl chitosans, particularly *N*-pentanoyl chitosan (degree of substitution < 0.20), which is expected to last for 2 years or longer.[594]

The biodegadability, verified in many kinds of dressings, is independent of the degree of acetylation of the chitins. This implies that lysozyme is not the only enzyme involved in the degradation or that the substrate is modified in situ.[595]

6.1.3. Hyaluronan Synthesis

Hyaluronan, a non-sulfated glycosaminoglycan of D-glucuronic acid and *N*-acetylglucosamine, is found in extracellular matrixes of various connective tissues. In fresh wounds, chitins seem to enhance its production, resulting in a reduced risk of scar formation and related complications such as keloids, intraperitoneal adhesion, and intestinal structures.[596] Evidence was produced for the presence of DG42 protein (a chitooligomer synthase) during embryogenesis, yielding chitooligomers capable of acting as primers in the synthesis of hyaluronan. Overexpression of DG42 in mouse cells leads to the synthesis of chitooligomers, and hyaluronan synthase preparations also contain chitin synthase activities; therefore, chitooligomers act as templates for hyaluronan synthesis.[597,598] Hyaluronan has been shown to pro-

mote cell motility, adhesion, and proliferation and to have important roles in morphogenesis, inflammation, and wound repair.[599]

6.1.4. Biocompatibility

The in vitro biocompatibility of wound dressings in regard to fibroblasts has been assessed and compared with three commercial wound dressings made of collagen, alginate, and gelatin. Methylpyrrolidinone chitosan and collagen were the most compatible materials.[600,601] The ranking of biocompatibility was established by various laboratories to be chitosan hydrochloride < chitosan glutamate < glycol chitosan < chitosan lactate < methylpirrolidone chitosan. Rat erythrocyte lysis was observed after 24 h contact with soluble chitosan salts and most evidently with chitosan glutamate. Berscht et al.[600] observed that after 72 h incubation with fibroblasts the chitosan salts inhibited cell growth by 70−80%; in contrast, methylpyrrolidinone chitosan caused only 35% inhibition.[476] Chitosan hydrochloride is however 4-fold less toxic than poly-L-lysine. Glutaraldehyde cross-linked chitosans are cytotoxic. These studies confirm that chemical modification does not only impart novel chemical characteristics, but also modifies the biochemical prerogatives of chitosan.

Combinations of basic FGF with methylpyrrolidinone chitosan or β-FGF with hydroxypropyl chitosan have an especially advantageous influence on impaired wound healing because the chitosans prevent excessive scar formation and β-FGF induces fast wound closure.[602,603] The stability of these associations is satisfactory, the half-life being 90 days at 25 °C and 22 days at 37 °C. Exogenous β-FGF is in fact a potent inducer of angiogenesis and granulation tissue formation that stimulates wound healing.

Chitosan has been associated with other biopolymers and with synthetic polymer dispersions to produce wound dressings. Biosynthetic wound dressings composed of a spongy sheet of chitosan and collagen, laminated with a gentamicyn sulfate-impregnated polyurethane membrane, have been produced and clinically tested with good results. Mosbey[604] proposed a wound-filling gel obtained from chitosan malate, polypropylene glycol, guar gum, and locust beam gum.

6.1.5. Wound Healing

Wound healing consists of a complex series of biochemical processes regulated by humoral factors and antiinflammatory mediators, resulting in rebuilding of the tissue and protection against infection.[605] Regulating factors include biochemical substances, growth factors, and immunologic mediators whose influence can be decisive particularly during the early phase of tissue rebuilding.[606]

The wound healing process essentially consists of the following stages. First, inflammatory cells from the surrounding tissue move toward the lesion site. Subsequently, fibroblasts appear and begin to produce collagen fibers that impart tensile strength to the regenerated tissue. Simultaneously, numerous capillaries begin to form to supply the site with nutrients and oxygen, and epithelial cells at the edge of the wound start filling in the area under the scab. In the final stage the new epithelium forms and the wound is healed.

Several preclinical studies on chitin and chitosan biomaterials show that they are endowed with biochemical significance not encountered in cellulose, starch, and other polysaccharides.[607] In general, chitin-based products provide improved healing of surgical wound by first intention in all cases, the healing process is faster, and smooth scars are obtained. They can be considered a primer on which the normal tissue architecture is organized. Key factors in the rebuilding of physiologically effective tissues exerted by chitosans are an enhanced vascularization and a continuous supply of chitooligomers to the wound that stimulate correct deposition, assembly, and orientation of collagen fibrils and are incorporated into the extracellular matrix components.[608] The chitooligomers are released by hydrolytic action of lysozyme and *N*-acetyl-β-D-glucosaminidase.[609]

It is interesting to note that a partially deacetylated chitin called water-soluble chitin, i.e., a polysaccharide midway between chitin and chitosan, has been found to be particularly effective as a wound healing accelerator. This chitin can be prepared via alkaline treatment of chitin and ultrasonication: chitin is suspended in 40% NaOH aqueous solution, and the resulting alkali chitin is dissolved by stirring with ice, then the solution is further stirred at 25 °C for 60 h (so-called homogeneous deacetylation), and then neutralized with HCl. Insolubilization of the product can be promoted with acetone. The product has M_w = 1.64 MDa that drops to 795 kDa after ultrasonication at 225 W for 1 h, and the degree of deacetylation is 0.50. It is therefore highly susceptible to lysozyme and soluble in slightly acidic solutions. Histological findings on wounded skin at 7 days postoperation indicated that collagen fibers were fine in the wounds and more mature than in the control and their arrangement was similar to that in normal skin. The tensile strength was clearly superior compared to controls. At 7 days the wounds were completely re-epithelialized, granulation tissues were almost replaced with fibrosis, and hair follicles were almost healed.[610]

6.1.6. Macrophage Activation

Macrophage activation is the major effect of the application of chitin-based dressings. Macrophages from laboratory animals are activated by chemically modified chitin.[62,94] Chitosan activates macrophages for tumoricidal activity and for the production of interleukin-1 (IL-1). Moreover, chitosan shows immunopotentiating activity.[94] This mechanism involves, at least in part, the production of interferon-γ (IFN-γ).[611] Chitosan also has an in vivo stimulatory effect on both macrophage nitric oxide production and chemiotaxis.[612−614] Chitosan-based dressings also modulate the peroxide production.[615]

In the early days of implantation of spongelike chitin in animals, polykaryocyte cells, derived from macrophages, appear in the granulation tissue and disappear within 4 weeks after surgery; the absence

of polykaryocytes in control lesions suggests that chitin induces their formation.

Granulation is accelerated by interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), or fibroblast growth factor (FGF).[616] In particular, IL-1 and TNF-α produced by macrophages[617] are known to activate fibroblasts. Peritoneal macrophages obtained from laboratory animals are activated by chitin.[62,94] The amount of IL-1 in the exudate taken from around the areas where chitin−nonwoven fabrics were implanted showed a 2-fold increase in comparison with control.

Chitin/chitosan items administered intravenously to mice become bound to macrophage plasma membrane mannose/glucose receptors that mediate their interiorization. In mice preconditioned with a small dose of IFN-γ, a higher level of priming is induced by chitin.[611]

Fibroblast growth factor-2 stimulates the proliferation of fibroblasts and capillary endothelial cells, thus promoting correct wound repair via angiogenesis; however, this factor has a short half-life in vivo and high diffusibility. A way to keep it on the desired position is to use a photo-cross-linkable chitosan in the form of a viscous solution that becomes an insoluble gel upon UV irradiation and has better adhesion than fibrin glue. High molecular weight chitosan was reacted with *p*-azidebenzoic acid and lactobionic acid and, upon exposure to UV light for 10 s, cross-linked by reaction of azide and amino groups. The factors retained in the hydrogel remained biologically active and were released upon in vivo degradation of the chitosan. Wound contraction was induced, and wound closure was accelerated in healing-impaired diabetic mice compared to controls. The application of a chitosan hydrogel as an occlusive dressing was recommended because chitosan would be more effective in this form in order to protect and contract the wound in a suitably moist healing environment.[91,618]

In addition, it should be noted that chitins and chitosans induce IL-8 release from fibroblasts in vivo, which leads to angiogenesis and migration of neutrophils. IL-8 is a potent neutrophil chemotaxin.[619]

6.1.7. Antiinflammatory Action and Angiogenesis Stimulation

Chitin accelerates the first phase of wound healing where inflammation is accompanied with infiltration of mononuclear and polymorphonuclear cells without any side effects such as high temperature and pain. In vivo histological studies in animal models also highly support the fact that the chitosan fibrils stimulate migration of these cells.[620,621]

N-Acetylglucosamine is itself an antiinflammatory drug and is synthesized in the human body from glucose and incorporated into glycosaminoglycans and glycoproteins. It has been administered to human volunteers by intravenous, intramuscular, and oral routes for pharmacokinetic studies.[622] *N*-Acetylglucosamine diffuses very rapidly in most tissues and organs, even after oral administration, and accumulates in the articular tissue and bone.[623]

Morphological examination performed at the site of a lesion treated with chitin shows an increase in vascularization during wound healing.[62] The newly formed granulation tissue around chitin + nonwoven fabric composite implants is actively invaded with new blood vessels, a phenomenon not observed in controls.

Among chitin derivatives, the phosphate esters proved to exhibit the greatest antiinflammatory properties, which suggests that chitin phosphate ester can be used as a drug for the treatment of inflammation. In the acute toxicity test for chitin, the LD50 was reported as over 10 g/kg subcutaneously in mice: because dosages would be in the mg/kg range, chitin and chitin derivatives are considered to be safe compounds for medical administration.[624]

6.1.8. Granulation and Scar Formation

Granulating tissue can generally be divided into healthy and unhealthy granulating tissue. The healthy one develops only in the absence of bacteria and debris. Chitins can induce healthy granulating tissue in the early stage of wound healing, with a mechanism that is still unclear. Chitosan is superior to chitin in its effect on the acceleration of granulation tissue.

The formation of a scar is a serious problem in the wound healing process. However, mild fibroblast activation is observed in the area close to the chitosan implant in experimental animals, and minimum scar formation takes place in the wound. Several reports document an increase of tensile strength without an increase in collagen synthesis in the presence of chitin, suggesting that *N*-acetylglucosamine may reinforce wounded tissues without excessive inflammatory reaction. However, recent studies show that in the chitin-treated lesions many histiocytes are present and that fine collagen fibers are produced. The presence of histiocytes might be induced by chitin and might promote the proliferation of fibroblasts which produce fine collagen. In contrast, little histiocyte invasion and thick collagen fibers were observed in the controls. Therefore, in chitin-treated wounds, synthesis of collagen will accelerate in the early wound healing stage but synthesized collagen will be degraded to an appropriate rate in the subsequent healing stages by a variety of collagenases from granulocytes, macrophages,[625] epidermal cells, neutrophils, and fibroblasts.[626] Chitin promotes keratin production and facilitates rapid and effective regeneration of oral mucosa. In chitosan treatment of purulent digit disease, epidermization is observed with a short convalescence and no antibiotic administration.[627]

6.1.9. Dressing Materials and Specific Uses

Several wound dressings have been commercially available for a few years, mainly in Oriental countries: they are mentioned in Table 4. However, they are not available in Europe, which could be due to marketing choices. Most of the experimental results have been obtained with freeze-dried modified chitosans, such as methylpyrrolidinone chitosan, reacetylated chitosan, or treated chitins, as mentioned below.

Table 4. Chitin-Based Dressings

Tegasorb, 3M	dressing contains chitosan particles that swell, absorbing exudate and producing a soft gel; a layer of waterproof Tegaderm film dressing covers the hydrocolloid; for leg ulcers, sacral wounds, chronic wounds; reportedly superior to Comfeel and Granuflex
Beschitin, Unitika	nonwoven material manufactured from chitin; available in Japan since 1982
Chitipack, S. Eisai Co.	spongelike chitin from squid; favors early granulation, no scar formation; for traumatic wounds, surgical tissue defects
Chitipack, P. Eisai Co.	dispersed and swollen chitin supported on polyethylenetherephthalate; for the treatment of large skin defects; favors early granulation; suitable for defects difficult to suture
Chitopack, C. Eisai Co.	cotton-like chitosan obtained by spinning chitosan acetate salt into a coagulating bath of ethylene glycol, ice, and NaOH; fibers washed with water and methanol; complete reconstruction of body tissue, rebuilding of of normal subcutaneous tissue, and regular regeneration of skin

6.1.10. Gel-Forming Freeze-Dried Sponges

The efficacy of chitosan in the treatment of leg ulcers stems from its antiinflammatory action and stimulation of epithelialization. Chitosans stimulate the granulation process and epidermis formation, thus accelerating healing. Among the many patients so far treated, the case of L. A., aged 84, suffering from chronic venous insufficiency, is worth mentioning as an exceptionally difficult one: prolonged treatment with methylpirrolidinone chitosan led to complete healing.[628]

Re-acetylated chitin gels were used to treat leg and decubitus ulcers in paraplegic subjects.[608] Selected preparative conditions permitted obtaining a self-sustaining gel useful for this use. The treatment periods were 63−182 days, and complete healing was obtained.[629] It was observed that the gel form permitted frequent changes without disturbing the newly formed tissues, this being the key aspect especially at the beginning of the treatment.

6.1.11. Skin Substitutes

Cultured skin from human cells is extremely thin and needs mechanical support that can conveniently be provided by biopolymer complexes. A typical example is a skin substitute made of type I and type III collagen, chitosan, and chondroitin 4- and 6-sulfate (72% collagen, 20% chitosan, and 8% glucosaminoglycan). The cross-linking between the chitosan primary amine and the sulfate groups are essential in imparting insolubility and mechanical resistance. This skin substitute includes human fibroblasts on the "dermal side" and keratinocytes on the "epidermal" side. The latter, once exposed to air during the last stage of culture, generates skin similar to the natural one in which the epidermal layers including the stratum corneum are present. The differentiation processes include expression of filaggrin and keratin and formation of a structure similar to the basal lamina.

Chitosan is indispensable for preparation of this skin substitute not only for providing insolubility but also for increasing the production of collagen and regulatory factors by fibroblasts. The addition of chitosan provides good adhesion (better than collagen alone) without proliferation problems. The dermal substitute does not cause antigenic incompatibility and allows controlled vascularization and fibroblastic colonization, yielding an organized matrix and limited formation of granulation and hypertrophic scar.[593,630]

Healing at skin graft donor sites dressed with chitosan has been studied and found to be superior to the results obtained with alginates and other products. Chitosan facilitated rapid wound re-epithelialization and the regeneration of nerves within a vascular dermis. In addition, donor site scars demonstrated an earlier return to normal skin color at chitosan-treated areas.[631]

6.1.12. Nerve Regeneration

For application in neurosurgery, macrocapsules and hollow fibers made of polyacrylonitrile−poly(vinyl chloride) (PAN−PVC) were filled with PC12 cell suspension in chitosan solution. The chitosan prevents extensive cell clumping and necrosis, which is known to take place in alginate gels and other encapsulation devices. When microencapsulated with chitosan, the PC12 cells attached successfully to the precipitated chitosan and extended neurites upon exposure to nerve growth factor (NGF). Differentiation of neuronal cells was also supported by the chitosan matrix.[632]

Advances have been recently made by Suzuki et al.[633] with crab tendon chitin tubes harvested from *Macrocheira kaempferi*, demineralized, partially deacetylated, and molded in triangular cross section. Chitosan is able to bind peptides covalently when reacted in the presence of water-soluble carbodiimides and in particular to bind laminin to the inner tubular surface: laminin is a glycoprotein that promotes neural cell attachment, differentiation, and neurite outgrowth.[634] The so prepared tendon tube is hydrophilic and water permeable, but its pore size does not permit cells to pass through. With the polysaccharide-aligned structure preserved, it has remarkable mechanical resistance and its three flat surfaces favor cell attachment. An acute inflammatory reaction occurred for 2 weeks, but the overall tissue response was mild. Encapsulation of the tube was not observed. Although cationic groups on a material surface evoke a severe inflammation reaction upon implantation, the surface of the chitosan tube is not recognized as a foreign material. Laminin disappeared as soon as the tube disintegrated within 12 weeks postoperation, leaving the newly formed neural tissue that bridged the surgically amputated peripheral nerve in rats.

6.1.13. Cartilage and Bone Tissue Regeneration

Chitosan freeze-dried fleeces support chondrocyte attachment and synthesis of extracellular matrix.[635]

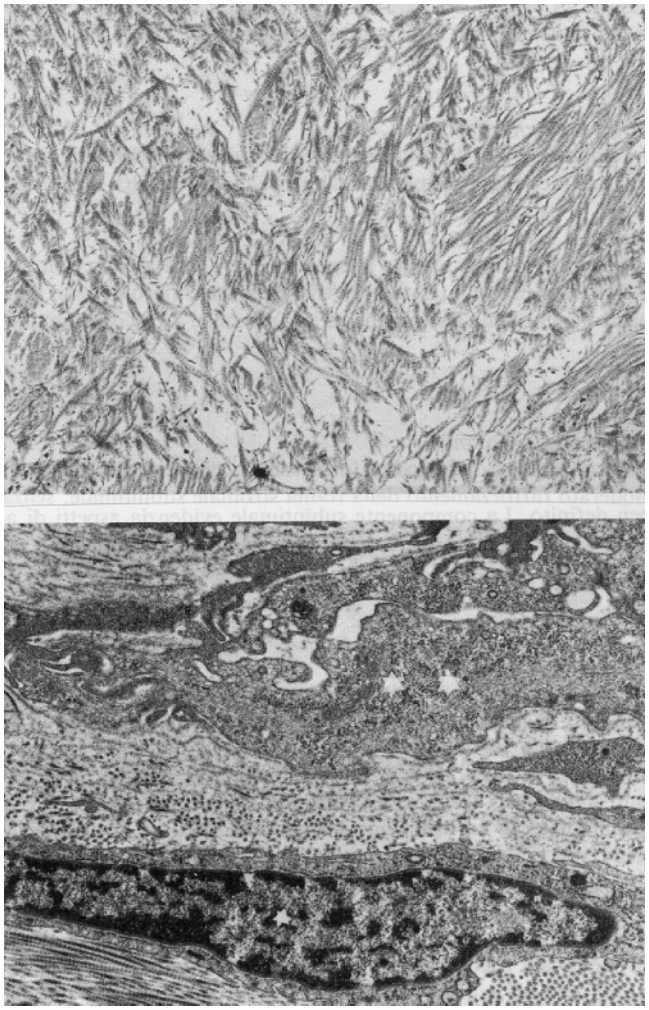


Figure 42. Healing of the meniscal lesions in the rabbit. (top) Control showing the poorly organized collagen network. (bottom) After medication with methylpyrrolidinone chitosan (45 days a.s.), vascular structures and fibroblastic elements lead to complete repair.

Chitosan was used to assist the spontaneous tissue repair of the meniscus, which is usually difficult, because it is well tolerated at the articular synovial level. It favors and stimulates the repair processes that do not take place spontaneously in the meniscus. Its initial angiogenetic action appears to be effective enough to stimulate the repair of the meniscus by providing the latter with the necessary tissue elements and humoral factors (Figure 42).[636]

Several studies dealing with the reconstruction of the periodontal tissue with chitosan were a prelude to the discovery of the osteoinductive properties of chitosan.[637] Surgical wounds from wisdom tooth avulsions were treated with freeze-dried methylpyrrolidinone chitosan, which promoted bone regeneration. The polysaccharide is depolymerized by lysozyme and is no longer detectable 6 months after surgery. Methylpyrrolidinone chitosan was found to be useful in apicectomy as well. None of the patients reported adverse effects over 3 years of observation.[638]

The existence of osteoprogenitor cells in the wound site offers the possibility of regenerating the periodontal, peri-implant, and alveolar ridge bone tissue simply with the aid of chemical mediators from chitosan. The amount of bone-forming colonies is almost doubled in the presence of chitosan. Chitosan also stimulates the differentiation of osteoprogenitor cells and thereby facilitates the formation of bone.[639]

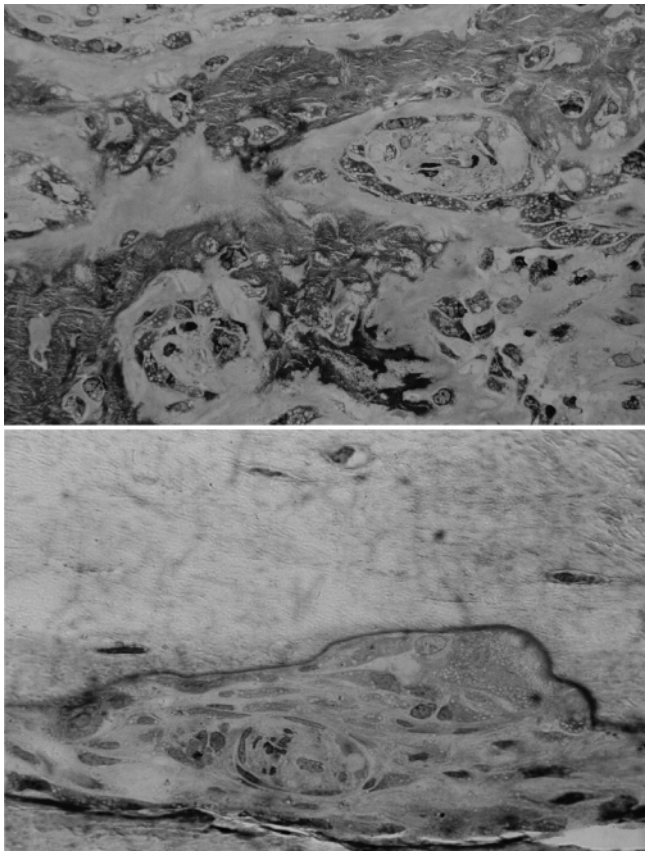


Figure 43. Bone regeneration in the rabbit. (top) Control showing absence of newly formed bone and scarce cells. (bottom) After medication with methylpyrrolidinone chitosan, the tissue shows regular cells with numerous cytoplasmic granules indicative of high functional activity.

Bone defects surgically produced in sheep and rabbit models have been treated with freeze-dried methylpyrrolidinone chitosan (Figure 43).[638,640−642] Histological examination performed 60 days after surgery showed a considerable presence of neoformed bone tissue as compared to control. Endosteal−periosteal and bone marrow osteoblast-like precursors, stimulated by growth factor entrapped in the polysaccharide, generated intramembranous bone formation. Bone osteoid formation was followed by mineralization. Osteoinduction was also observed in rabbit endochondral bones.[643]

Experimental studies on rabbit and sheep were performed in order to evaluate the possibility of improving bone tissue reconstitution with chitosan associated with calcium phosphate. Microscopic and histological analyses showed the presence of an osteogenic reaction moving from the rim of the surgical lesion toward the center. In control lesions dense fibrous tissue without the characteristic histoarchitecture of bone was observed.

The pattern of bone regeneration has been studied in an osteoporotic experimental model with the bone morphogenetic protein (BMP) linked to chitosan. The biodegradation of chitosan leads to controlled release of BMP, providing a synergistic effect on bone formation. Morphometric and morphological analyses show

that bone tissue regeneration in a surgical bone defect is improved using this combination. This important result also proves the validity of a biochemical approach to the therapeutical correction of various affections in the elderly.[644]

Dicarboxymethyl chitosan (DCMC) and 6-oxychitin sodium salt applied to femoral surgical defects for 3 weeks produced a good histoarchitectural order in the newly formed bone tissue. The spongious trabecular architecture was restored in the defect site. The association of the chitin derivatives with the osteoblast cells seemed to be the best biomaterial in terms of bone tissue recovery. The 6-oxychitin sodium salt gave better osteoarchitectural reconstruction than DCMC as well as in the presence of added osteoblasts. Healing seemed to be slower, but the spongious trabecular architecture was superior. 6-Oxychitin, therefore, represents an advance in the experimental study of the osteoinduction process and preludes to novel applications intended to reconstruct the correct morphology of bone tissues, even in the presence of important mechanical stress.[645]

6.1.14. Bone Substitutes

The use of degradable polymers as matrixes forms a major approach to the development of bone composites when compared to the nondegradable systems. Degradable matrixes maximize the osteoconductive behavior of hydroxyapatite, allowing bone ingrowth into the implant to occur as the matrix is resorbed with time. In this respect, the degradable matrixes act as a binder to prevent migration of hydroxyapatite from the implant site. The chitosan-bonded hydroxyapatite bone-filling paste was made as follows: chitosan (0.5 g) was dissolved in malic acid (0.5 g) solution made with saline, and a chitosan film was formed by mixing this solution with hydroxyapatite powder (2 g), followed by neutralization with 5% sodium polyphosphate. To help cells and blood vessels to penetrate this material, the tensile strength and elongation were optimized.[646−648]

Similarly, a composite of hydroxyapatite and a network formed via cross-linking of chitosan and gelatin was made with glutaraldehyde.[649] The presence of hydroxyapatite did not retard the formation of the chitosan/gelatin network.

Calcium phosphate cements are suitable for the repair and reconstruction of bone because they are osteostransductive, i.e., after implantation in bone defects they are rapidly integrated into the bone structure, after which they are transformed into new bone thanks to the activity of osteoclasts and osteoblasts. These cements have the advantage that they can be molded during the operation and are injectable, i.e., they adapt immediately to the bone cavity and permit subsequent good osteointegration.

To make the cement injectable, several additives can be incorporated; however, the properties of the cement should be preserved: setting times suited to a convenient delay with surgical intervention, limited disintegration in aqueous medium, and sufficient mechanical resistance. Lactic acid, glycerol, glycerophosphate, and chitosan are adjuvants[650] in terms of injectability, setting time, disintegration, and toughness. Chitosan alone improved injectability, increased setting time, and limited the evolution of the cement toward hydroxyapatite by maintaining the octacalcium phosphate phase; therefore, only slight disintegration was observed. Because octacalcium phosphate is considered one of the precursors in bone tissue formation, the adsorption of chitosan on this salt was considered advantageous.[651,652]

Studies were carried out on the effects of dicarboxymethyl chitosan on the precipitation of a number of insoluble salts, and calcium phosphate is one of them.[653] The chelating ability of this modified chitosan interfered effectively with the physicochemical behavior of magnesium and calcium salts. Dicarboxymethyl chitosan mixed with calcium acetate and disodium hydrogen phosphate in suitable ratios yielded clear solutions from which an amorphous material ca. 50% inorganic was isolated. This compound was used for treatment of bone lesions in experimental surgery and dentistry. Bone tissue regeneration was promoted in sheep, leading to complete healing of otherwise nonhealing surgical defects. Radiographic evidence of bone regeneration was observed in human patients undergoing apicectomies and avulsions. The DCMC−calcium phosphate chelate favored osteogenesis while promoting bone mineralization.

The in situ precipitation route toward obtaining composites of polymer and calcium phosphate is similar to the strategy employed in naturally occurring biocomposites and may prove a viable method for the synthesis of bone substitutes.[654]

6.1.15. Effects of Oral Delivery on Wound Healing

The restorative effects of orally administered N-acetyl-D-glucosamine and glucuronic acid on experimentally produced cartilaginous injuries have been studied in rabbits. The massive proliferation of matured cartilaginous tissue was observed, surrounded by proliferating chondroblast cells. In the regenerated tissue, matured cartilage substrate was also observed, while in the control and glucose groups the injured parts were covered by fibrous connective tissues.[655] Exogenous glucosamine can be transported into the cell and acted on by a kinase which phosphorylates it at the 6 positions, enabling it to enter the pathway of glycosaminoglycan synthesis.[656] Exogenous glucosamine is readily incorporated into hyaluronan by cultured fibroblasts. It seems that the glucosamine 6-phosphate synthesis is the rate-controlling step.

Enhanced availability of exogenous glucosamine is beneficial for the synthesis of hyaluronan because endogenous glucosamine is insufficient to achieve the high concentration of UDP−*N*-acetyl glucosamine necessary to optimize synthase activity. Thus, the administration of glucosamine (as well as chitosan) in general is beneficial for wound healing.[596]

The nephrotic syndrome is often associated with hypercholesterolemy and anaemia. Human erythropoietin is used for treatment of patients with anaemia nephrotic syndrome undergoing haemodialysis, but its administration must continue long term with risks of many side effects.

The oral administration of chitosan for 12 weeks to patients with chronic renal failure produced the elevation of serum hemoglobin. Other expressions of improvement of renal function were the reduced serum levels of urea, creatinine, and cholesterol.[657]

The biological significance of chitosan biomaterials in the human body depends largely on the actions that certain hydrolases exert on them.[658] The resulting chitooligomers stimulate various cells, while the released monomers are phosphorylated and incorporated into hyaluronan, keratan sulfate, and chondroitin sulfate, components of the intracellular matrix and connective tissue.

6.2. Tissue Engineering

Tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure−function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions. In other words, tissue engineering involves the use of cells and extracellular components, either synthetic or natural, to create replacement, implantable parts to restore, maintain, or repair the function of damaged/diseased tissues and organs.[659] This approach may be applied toward several goals, among which are providing replacement parts or cellular prostheses for the human body, providing vehicles to deliver engineered cells to an organism, creating tissue models with cells to study the states of diseases using aberrant cells, surfacing nonbiological apparatus, and providing acellular parts capable of regenerating tissues.

The first important phase of any tissue engineering endeavor is to evaluate the types, ratios, and organization of the cells necessary to produce the tissue or organ of interest. In general, one chooses a substrate material upon which to culture the cells that will enhance their organization in three dimensions and possibly provide initial mechanical integrity to the cell−polymer construct. This material should be biocompatible, be sterilizable, and have a large surface area on which new tissue can grow.[660] Applications may also require the scaffold material to be biodegradable. Second, the cells of the tissue or organ of interest are seeded onto the substrate and cultured for a period of time in vitro. Finally, the cell−substrate construct may be implanted at the site of tissue damage in vivo.

This general approach is currently being used in many tissue engineering research labs around the world and has proven to be clinically successful, as evidenced by commercially available tissue-engineered skins. Other engineered tissues and organs such as cartilage, heart valves, blood vessels, bone, intestine, and tendon, among many others, also have promise for clinical success. Obviously the more complex the tissue or organ, the more complex the engineering process, but the same general methods are still employed. One tissue that is very well suited for tissue engineering methods is articular cartilage because of its fairly simple composition, one cell type, and lack of vascularity. Francis and Matthew,[661] recently, reviewed the application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering.

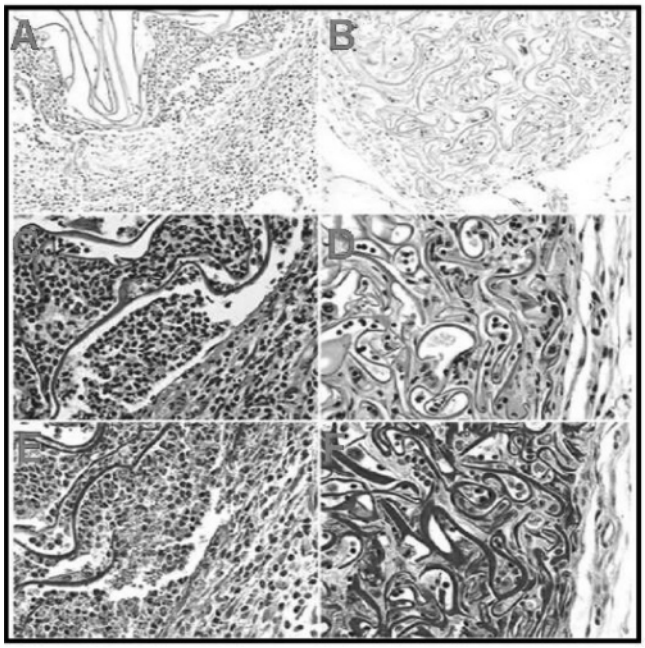


Figure 44. Histology of IP implants: Wright's stain (original magnification ×10) displaying cells (blue) and chitosan (pink) at (a) week 1 and (b) week 12. Carazzi hematoxylin and eosin stain (original magnification ×40) at (c) week 1 and (d) week 12. Masson's trichrome staining (original magnification ×40) indicating deposition of collagenous material (blue) was deposited within the implant at (e) week 1 and (f) week 12. (Reproduced with permission from ref 662. Copyright 2002 Wiley-Liss, Inc. (http://www3.interscience.wiley.com/cgi-bin/jabout/30035/ProductInformation.html))

Chitosan biocompatibility was investigated in mice model by implantation of porous chitosan scaffolds and their evaluation. Histological assessment indicated neutrophil accumulation within the implant, which resolved with time, and there were minimal signs of any inflammatory reaction to the material itself, Figure 44. Cellular immune responses indicated a very low incidence of chitosan-specific reactions. Collagen presence indicated that a connective tissue matrix was deposited within the chitosan implant.[662]

One of chitosan's most promising features is its excellent ability to be produced into porous structures for use in cell transplantation and tissue regeneration. Porous chitosan structures can be formed by freezing and lyophilizing chitosan salt solutions in suitable molds. During the freezing process ice crystals nucleate from solution and grow along the lines of thermal gradients. Ice removal by lyophilization generates a porous material, the mean pore size of which can be controlled by varying the freezing rate. Pore orientation can be directed by controlling the geometry of the temperature gradients during freezing. Madihally and Matthew[663] studied bulk scaffolds and also performed systematic studies demonstrating the effects of freezing temperature and chitosan concentration on the mean pore diameter of cylindrical chitosan scaffolds. Bulk scaffolds were also prepared in a planar geometry by freezing

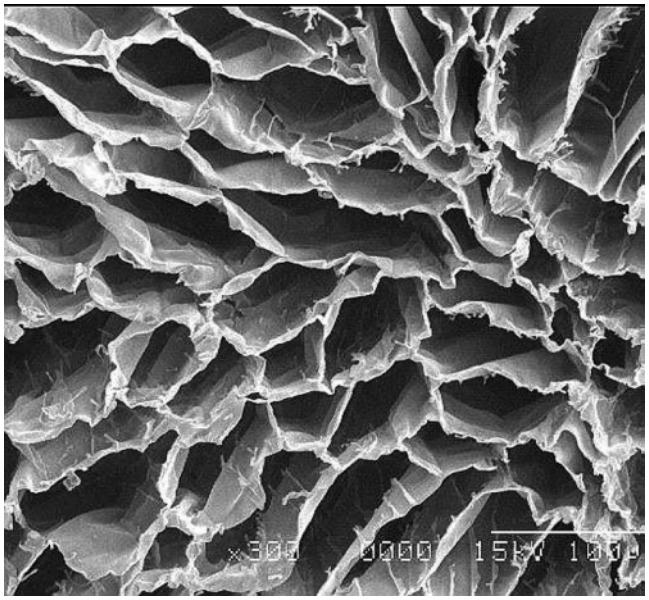


Figure 45. SEM image of a bulk scaffold frozen in a planar geometry. A 2% chitosan solution was frozen in a shallow polystyrene dish by contact with dry ice. The lyophilized sample was sectioned parallel to the sample plane. (Reproduced with permission from ref 663. Copyright 1999 Elsevier.)

chitosan solutions in shallow dishes, resulting in a planer scaffold ~5 mm thick with perpendicularly oriented, thin walled pores Figure 45. The freezing was accomplished by liquid nitrogen in the case of cylindrical scaffolds, whereas dry ice was used for the shallow scaffolds. The mean pore diameter could be controlled within the range 40−250 μm by varying the freezing temperature and hence the ice crystal size. A lesser effect of chitosan solution concentration was also observed, with smaller pores being formed at higher concentrations. Since ice crystal growth and pore diameters are functions of the temperature gradient, pore diameters can be expected to vary with radial position in cylindrical scaffolds. In fact, pore diameter increased significantly, moving from the edge toward the center of the sample.

The effect of polyanionic modification of chitosan scaffolds was also studied.[663] Chitosan scaffolds were complexed to heparin via ionic interaction. Significant swelling of the material was observed during rehydration; however, SEM examination of subsequently dehydrated samples confirmed that the internal porous microstructure was retained, where as the outer surfaces of the scaffolds appeared to be partially sealed in some regions by an ionic complex membrane. Removal of the membrane revealed the porous structure beneath. Directly frozen chitosan microcarriers were also investigated, which were prepared by extruding the droplets of chitosan into a beaker of liquid nitrogen or dry ice cooled methylene chloride under stirring, and to prepare gelled microcarriers, the droplets were extruded in 0.1 M NaOH and transferred to a freezing bath after washing.[5] In both cases the frozen microcarriers were cooled to −78 °C. Both techniques produced totally different pore microstructures, Figure 46. These extensive studies reveal the possibilities of fabricating chitosan scaffolds with varied pore sizes and with

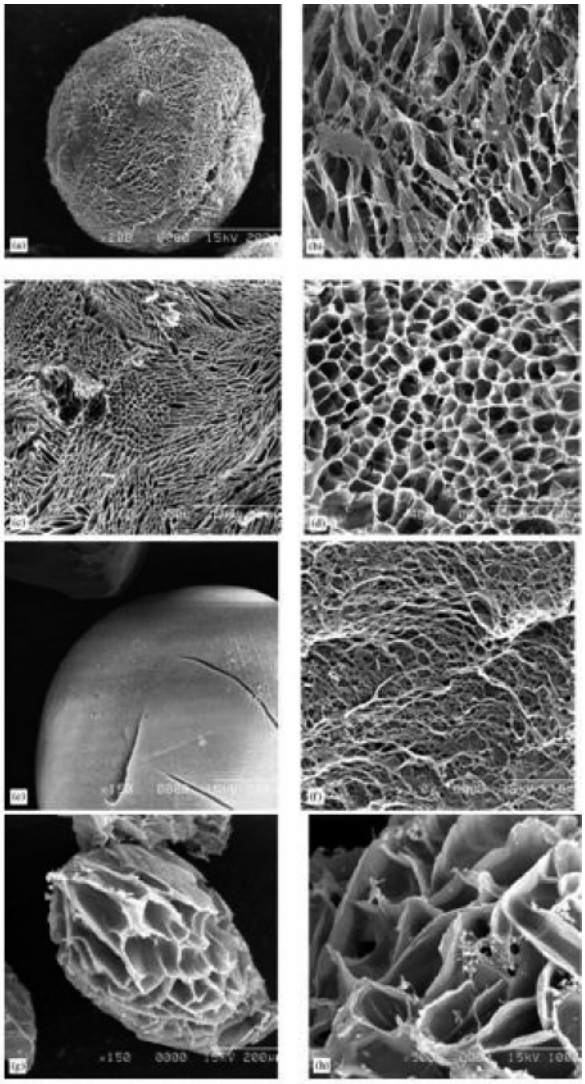


Figure 46. SEM micrographs of porous chitosan microcarriers. Micrographs show low- and high-magnification views of microcarriers formed by various freezing protocols: (a, b) chitosan droplets directly frozen in liquid nitrogen, (c, d) interior views of directly frozen microcarriers, (e, f) chitosan gel beads directly frozen in liquid nitrogen, and (g, h) chitosan gel beads directly frozen in methylene chloride at 53 °C. (Reproduced with permission from ref 663. Copyright 1999 Elsevier.)

control over the pore morphology.[663] Chow et al.[664] produced porous chitin matrixes with pore sizes ranging from 100 to 500 μm using the same technique. In an attempt to extend the upper pore size limit of chitin matrixes obtained by lyophilization, they developed a blowing method. This was achieved by adding $CaCO_3$ to the chitin solution, casting into a mold, leading to $CaCO_3$−chitin gel that was subsequently submerged in 1 N HCl solution to generate gaseous CO_2.[68,664] The mechanical properties of chitosan scaffolds formed by the lyophilization technique are mainly dependent on the pore sizes and pore orientations. Tensile testing of hydrated samples shows that porous membranes have greatly reduced elastic moduli compared to nonporous chitosan membranes. The extensibility (maximum strain) of porous membranes varied from values similar to nonporous chitosan (~30%) to greater than 100% as a function of both pore size and orientation.[663]

Chitosan scaffolds support the attachment, morphology, and proliferation of various kinds of cells, including chondrocytes,[635] dermal fibroblasts,[665] hepatocytes,[666] and adrenal chromaffin cells.[667]

The combination of chitosan with other polymers appears to be common in various reports. Chitosan−gelatin scaffolds were prepared by Mao et al.[668,669] as artificial skin, where the pore size is directly proportional to the freezing temperature. Co-culturing keratinocytes with fibroblasts on the scaffolds resulted in a construct of an artificial bilayer skin in vitro, which was flexible and had good mechanical properties.[668,669] Risbud et al.[670] studied chitosan−gelatin hydrogels and their cell interactions. The hydrogel did not exert a cytotoxic effect on macrophages, and primary human respiratory epithelial cells cultured on the hydrogel showed proper attachment and normal morphology and growth. Chitosan−gelatin scaffolds were also evaluated as a support for inducing chondrocyte growth and differentiation. Chondrocytes were observed to attach and exhibited differentiated phenotype with proper cell−cell contact.[671] Zhao et al.[672] prepared a hydroxyapatite/chitosan−gelatin scaffold with 90% porosity. The scaffolds were used to examine the proliferation and functions of neonatal rat calvaria osteoblasts. Histological and immunohistochemical staining and scanning electron microscopy (SEM) observation indicated that the osteoblasts attached to and proliferated on the scaffolds. Chitosan collagen scaffolds were examined for their ability to regulate cellular activity: SEM analysis indicated that the addition of chitosan to the collagen matrix reinforced the structure and increased pore size. In addition, cell function based on cytokine release was augmented.[673]

Macroporous chitosan/calcium phosphate (β-tricalcium phosphate and calcium phosphate inverted glass) scaffolds have also been prepared. Here the role of chitosan was to provide a scaffold form, while calcium phosphates' bioactivity presumably encouraged osteoblast attachment and strengthened the scaffold. The composite scaffold was found to be stronger, bioactive, and biodegradable, the effect being dependent on the ratio of chitosan to the two types of calcium phosphate. Human osteoblast-like MG63 cells were cultured on the scaffolds and enhanced the phenotype expression of the cells, in comparison with chitosan scaffolds.[674,675] Lee et al.[676] seeded osteoblastic cells on chitosan−tricalcium phosphate scaffolds and found them to support the proliferation and differentiation of the cells in three-dimensional structures. Chitosan−poly(vinylpyrrolidone) hydrogels have been evaluated and found not to have significant interactions with endothelial cells.[677]

Zhu et al.[678] utilized the reaction between the amino group of chitosan and the carboxylic acid group of amino acids to attach various amino acids (lysine, arginine, aspartic acid, phenylalanine) onto chitosan. These amino acid functionalized chitosan moieties were subsequently entrapped onto PLA surfaces. The amino acid−chitosan−PLA membranes demonstrated good cyto-compatibility to chondrocytes, behaving much like glycosaminoglycans found in tissue. Cai et al.[679] reported similar results using a carbodiimide process to link chitosan onto the PLA surface. To improve endothelial cell adhesion and growth on chitosan, cell adhesive peptide Gly-Arg-Gly-Asp was photochemically grafted to its surface and found to support the proliferation of human endothelial cells compared to chitosan.[110,680]

Sodium alginate and chitosan sponges were prepared via freeze-drying process in order to assess the utility of mixed sponges as matrixes for tissue engineering. The sponges had a flexible yet strong texture, as assessed macroscopically. Measurement of the resistance to compression ('hardness') indicated that the chitosan sponges were the 'hardest' while the alginate sponges showed the least resistance to compression, with all sponges showing a high degree of recovery. SEM studies indicated that the mixed systems had a less defined microstructure than the single-component sponges. This was ascribed to the two polysaccharides interacting in aqueous solution via Coulombic forces, leading to a more randomly ordered network being formed on freezing.[224]

Yang et al.[681] prepared porous scaffolds of alginate/galactosylated chitosan (ALG/GC) by lyophilization for liver-tissue engineering. The ALG/GC sponges using GC-10, GC-100, and GC-200 (−20 °C, 20 mM $CaCl_2$, 5% (w/v) GC) were observed to be highly porous structures with interconnected pores. The addition of GC in the system decreased the average pore sizes of the alginate sponge. The pore sizes of the ALG/GC sponges were found to decrease with the increase of molecular weight of chitosan. Primary hepatocytes in ALG/GC sponges showed higher cell attachment and viability than in alginate alone, owing to the specific interaction of the asialoglycoprotein receptors on hepatocyte with the galactose residues on ALG/GC sponges. Improvements in spheroid formation and long-term liver-specific functions of the immobilized hepatocyte were also observed in ALG/GC sponge.[681]

Park et al.[109] emphasized on galactose moiety in the GC for the specific adhesive ligand to ASGR in the two-dimensional cell culture. Hepatocytes adhered to a GC-coated dish at a low concentration of GC (0.05 mg/mL) was very well demonstrated by microphotographs, Figure 47. Cells adhered to the surface showed spreading shapes after 24 h in the absence of EGF, whereas it took 10 h in the presence of EGF, indicating that EGF enhances the spreading shapes of adhered hepatocytes. Chitosan−calcium phosphate composites were investigated as injectable resorbable scaffolds for bone tissue regeneration. Figure 48 shows phase-contrast microphotographs of hepatocytes adhered to GC-coated dish at a high concentration of GC (5 mg/mL). Cells adhered to the surface showed round shapes and exhibited spheroid formation after 24 h in the absence of EGF, whereas in the presence of EGF cells exhibited a round shape and many spheroid formation.[109] Chitosan solution gel in response to pH changes from slightly acidic to physiological: at pH lower than 6.5, the chitosan−calcium phosphate suspension is a paste-like moldable/injectable system, and at physiological pH the polymer undergoes a phase transition, resulting in

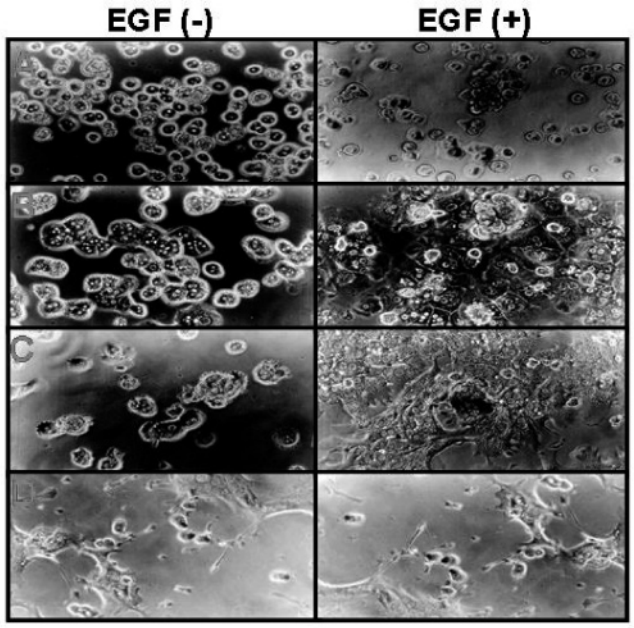


Figure 47. Phase-contrast microphotographs of hepatocytes adhered to a GC-coated dish at a low concentration of GC (0.05 mg/mL): (a) 3, (b) 10, (c) 24, and (d) 48 h. EGF (−) and EGF (+) represent without EGF and with EGF, respectively. (Reproduced with permission from ref 109. Copyright 2003 Elsevier.)

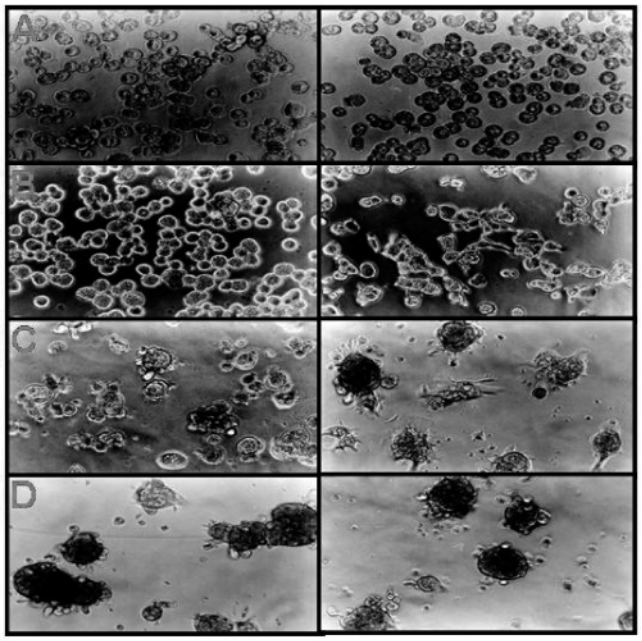


Figure 48. Phase-contrast microphotographs of hepatocytes adhered to a GC-coated dish at a high concentration of GC (5 mg/mL): (a) 3, (b) 10, (c) 24, (d) 48 h. EGF (−) and EGF (+) represent without EGF and with EGF, respectively. (Reproduced with permission from ref 109. Copyright 2003 Elsevier.)

entrapment of calcium phosphate component within the reversible gel matrix.[682] More recently, Li et al.[683] described fructose-modified chitosan scaffolds for hepatocytes.

Kast and Bernkop-Schnürch[89,684] reported chitosan−thioglycolic acid (TGA) conjugate as a new scaffold material for tissue engineering. TGA was attached covalently to the primary amino groups of chitosan under the formation of amide bonds. The degradation rate was controlled by thioglycolic acid conjugation.[89] They compared two formulations for the cell growth, one being the chitosan−TGA gel and the other being chitosan−TGA sheets, and found that L-929 mouse fibroblast cells grew much better on sheets rather than gels (Figure 49) because of the more porous nature of the sheets. Kim et al.[685] evaluated porous scaffolds containing microspheres loaded with growth factor for cartilage tissue engineering.

6.3. Radiopharmaceuticals

There is interest in selectively targeting radionuclides to cancer cells in order to destroy them. The possibility to bind a radionuclide to chitosan resides in the chelating capacity of the latter as well as in its capacity to retain metal ions already in the form of complexes: for instance, chitosan becomes blue upon chelation of copper ions, and after exposure of the blue chelate to ammonia, a mixed chitosan ammonia violet chelate forms. The first report on the chelating capacity of chitosan was published by Muzzarelli[686] and followed by an article that included data on lanthanides,[687] the series of elements to which the most useful radionuclides belong.

6.3.1. Gadolinium-157

Gadolinium neutron capture therapy (Gd-NCT) is a cancer therapy that utilizes γ-rays emitted during the reaction ^{157}Gd(n, γ)^{158}Gd to kill tumor cells. The advantages of ^{157}Gd are its large neutron capture cross-section, the long range (<100 μm) of its γ-rays within the cancer or in its proximity, and Auger electrons that destroy DNA. Gd has also been used as a contrast agent in magnetic resonance imaging (MRI), and it is possible to integrate Gd-NCT with MRI diagnosis. The in vivo performance of Gd-NCT has not been sufficiently established yet because commercially available Gd compounds such as Magnevist and Gadovist are eliminated rapidly from tumor tissues even after intratumor injection, probably due to their high hydrophilicity.

The bioadhesive characteristics of chitosan and its capacity to recognize, to a certain extent, the tumor cells prompted research on the delivery of Gd with the aid of chitosan. For this purpose, chitosan nanoparticles were prepared by emulsion-droplet coalescence. Fully deacetylated chitosan was dissolved in 10% gadolinium diethylenetriaminepentaacetic acid (GdDTPA) solution so as to obtain 2.5% chitosan concentration. One milliliter of this solution was added to liquid paraffin (10 mL) with Arlacel 5% and stirred with a homogenizer to form a water-in-oil emulsion. Separately, a similar emulsion was prepared with NaOH, and then the two emulsions were combined to solidify the chitosan droplets. Washing was done with toluene, ethanol, and water. The mean particle diameter was ca. 400 nm, and the Gd content was ca. 9%.

The GdDTPA chelate was strongly retained by chitosan, so that Gd was not released to an isotonic phosphate buffer over 7 days. Actually the Gd concentration in the tumor tissue was about 100

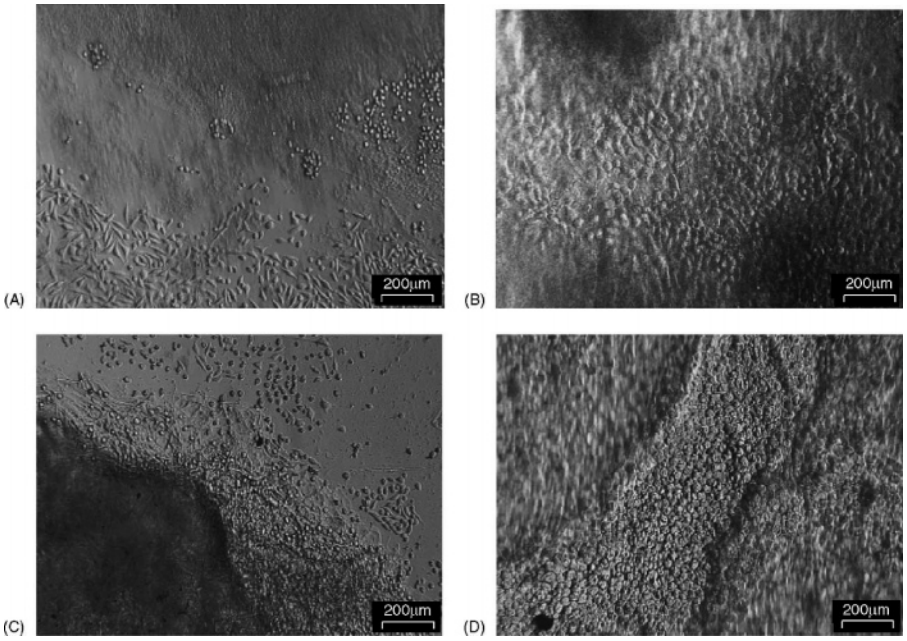


Figure 49. L-929 mouse fibroblasts seeded onto chitosan−TGA gel after (A) 6 and (B) 24 days of incubation. L-929 mouse fibroblasts seeded onto chitosan−TGA sheet after (C) 6 and (D) 24 days of incubation. (Reproduced with permission from ref 684. Copyright 2003 Elsevier.)

times higher compared to Magnevist controls at the time of the thermal neutron irradiation (neutron flux on the tumor surface was 6.32×10^{12} neutron·cm^{-2}).

Endocytic uptake of nanoparticles, strongly holding GdDTPA, was suggested by transmission electron microscopy studies that indicated that GdDTPA has a high affinity to the cells, contributing to the long retention of Gd in tumor tissue. The treatment led, in fact, to suppression of tumor growth in the in vivo studies. In the controls the increase of the tumor volume was 20−25%, 14 days after irradiation, while the tumor was suppressed in mice treated with the Gd preparation; also, the survival time was remarkably prolonged.[688,689]

MRI was used to make in vivo qualitative assessments of articular cartilage in the rabbit knee. Proteoglycan loss and disruption of the collagen framework in cartilage are early events associated with osteoarthritis. The kinetics of tracer diffusion may be used as an informative marker of proteoglycan loss, in addition to other parameters. When applied to a relevant osteoarthritis model, GdDTPA MRI may help in identifying new active compounds during efficacy studies on cartilage protection.[690]

The chitosan Gd-nanoparticles incorporating GdDTPA were prepared by the emulsion-droplet coalescence technique. Their releasing properties and their ability for long-term retention of GdDTPA in the tumor indicated that Gd-nanoparticles might be useful as an i.t. injectable device for Gd-NCT.[691,692] Some noticeable differences were found between different chitosans used in the preparation. The highest Gd was possible with 100% deacetylated chitosan in 15% Gd-DTPA aqueous solution, and the particle size was 452 nm, whereas lower deacetylated chitosan produced much larger particles with decreased Gd-DTPA content.

Gadolinium-loaded chitosan particulate devices for the gadolinium neutron-capture therapy of cancer were further described. First, cross-linked Gd−chitosan microspheres were prepared by a conventional method using glutaraldehyde. The increase in glutaraldehyde applied contributed to a size reduction and to the formation of a reservoir structure via preferential surface hardening with glutaraldehyde but competitively led to a decrease in the gadolinium content of GdDTPA chitosan microspheres.

The average diameter of GdDTPA chitosan microspheres was 1.9 μm, and the Gd content was 61%, equivalent to a GdDTPA content of 21.2%. Next, a novel emulsion-droplet coalescence technique was developed in order to prepare an injectable gadolinium-loaded chitosan particulate system without cross-linking agents. This method is based on neutralization of water-in-oil emulsion droplets containing chitosan and GdDTPA and subsequent precipitation of the chitosan−GdDTPA complex caused by coalescence with water-in-oil emulsion droplets containing sodium hydroxide, Figure 50. The gadolinium-loaded chitosan micro- and nanoparticles produced using this technique hardly released GdDTPA in an isotonic phosphate-buffered solution over 7 days despite the high water solubility of GdDTPA, thus suggesting a strong interaction between chitosan and GdDTPA. The optimized process conditions facilitated production of gadolinium-loaded chitosan nanoparticles with an extremely high GdDTPA content (45.3%) and a suitable size for i.v. injection (452 nm). Gadolinium-loaded chitosan nanoparticles displayed prolonged retention in tumor tissue after intratumoral injection in vivo. Consequently, this led to intensified tumor-growth suppression in vivo in the gadolinium neutron-capture therapy by intratumoral injection.[693]

6.3.2. Holmium-166 and Samarium-153

^{166}Ho is utilized in medical radiotherapeutic applications due to its properties which include high-

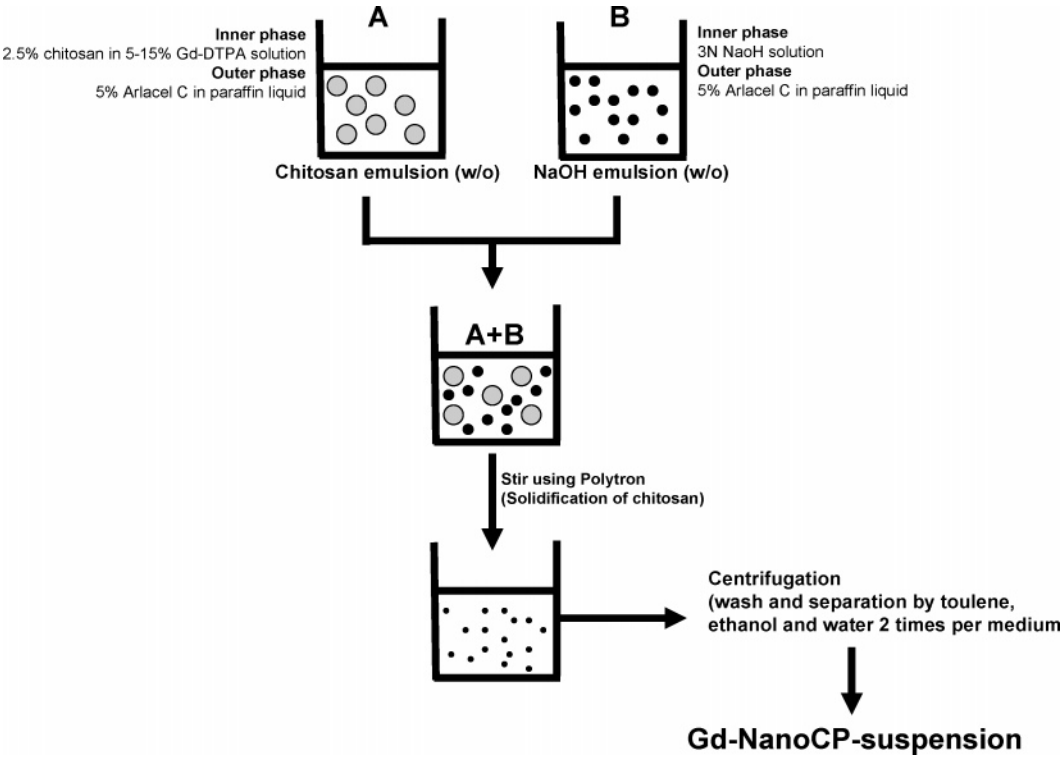


Figure 50. Preparation of Gd-nanoCPs by emulsion-droplet coalescence technique.

energy β-radiation (1855, 1776, 666 keV), 26.4 h half-life, and decay to a stable daughter. In addition, ^{166}Ho has chemical characteristics suitable for labeling with bifunctional chelates. ^{166}Ho emits low-intensity and low-energy γ-rays useful for imaging and can be used without external irradiation of other individuals. ^{166}Dy serves as a source of high specific activity ^{166}Ho.

MRI was used to demonstrate the effect of radiation synovectomy after the intra-articular injection of ^{166}Ho−chitosan complex for the treatment of rheumatoid arthritis of the knee. Fourteen patients aged 36−59 years were included in the study based on the absence of observable improvement after three or more months of treatment of the knee with disease-modifying antirheumatic drugs. MR images were acquired both prior to and after 4 months treatment, with 10−20 mCi of ^{166}Ho−chitosan complex. Clinical evaluation included the use of visual analogue scales to assess pain, and the circumference of the knee and its range of motion were also determined. MR evaluation included measurement of the volume of synovial enhancement and wall thickness, the amount of joint effusion, and quantifiable scoring of bone erosion, bone edema, and lymph nodes. Visual analogue scale readings decreased significantly after radiation synovectomy; MRI showed that joint effusion decreased significantly. In conclusion, the decreased joint effusion noted at a 4 month follow-up resulted from radiation synovectomy of the rheumatoid knee by means of intra-articular injection of ^{166}Ho−chitosan complex.[694]

Previous animal studies established that the intra-articular injection of ^{166}Ho−chitosan complex causes effective necrosis of the inflamed synovia with little leakage of radioactivity from the injected joint. A total of 16 patients, who had knee synovitis refractory to antirheumatic drug treatments longer than 3 months, were randomly assigned to three treatment groups with different radiation doses: 370, 555, and 740 MBq. Following the intra-articular injection, the blood radioactivity was little changed from the baseline measurement and the accumulated radioactivity excreted in urine was minimal. γ-Scan indicated that most of the injected radiochemical was localized within the injected joint cavity and that the extra-articular leakage was negligible 24 h after injection. Major adverse events were transient postinjection knee joint pain and swelling. Therefore, the ^{166}Ho−chitosan complex might be a safe agent for radiation synovectomy, particularly for the treatment of knee synovitis of rheumatoid arthritis.[695]

Preparation of the ^{153}Sm−chitosan complex for radiation synovectomy was also described: it was obtained by mixing acidic solutions of chitosan and $^{153}SmCl_3$, When a solution of this complex was injected into the knee joints of rabbits, minimal extra-articular leakage was observed. This was attributed to the rapid change in the pH of the complex solution from acidic to neutral, resulting in the formation of gel followed by the subsequent retention in the administered site.[696]

^{166}Ho−chitosan complex [0.75 mg of $Ho(NO_3)_3 \cdot 5H_2O$ and 1 mg of chitosan/head] was administered intrahepatically to male rats. To determine the effects of chitosan in ^{166}Ho−chitosan complex, ^{166}Ho alone [0.75 mg of $Ho(NO_3)_3 \cdot 5H_2O$/head] was intrahepatically administered to male rats, and radioactive concentrations in blood, urinary, and fecal excretion and radioactive distribution were examined. Intratumoral administration of ^{166}Ho−chitosan complex [0.075 mg of $Ho(NO_3)_3 \cdot 5H_2O$ and 0.10 mg of chitosan/head] was also investigated. After administration of ^{166}Ho−chitosan complex, the radioactive concentrations in blood were low and cumulative

urinary and fecal excretions over a period of 0−72 h were 0.53% and 0.54%, respectively. Most of the administered radioactivity was localized at the administration site, and very little radioactivity was detected in the liver, spleen, lungs, and bones. On the other hand, results of intrahepatic administration of ^{166}Ho alone showed high radioactive concentrations in the blood, and the whole-body autoradiographs showed that the administered radioactivity was distributed in many organs and tissues. These results strongly suggest that ^{166}Ho is retained at the administration site only when it forms a chelate complex with chitosan. It was concluded that administered ^{166}Ho−chitosan complex is retained at the administration site after either intrahepatic or intratumoral administration to rats or tumor-transplanted nude mice.[697]

This was supported by further work indicating that chitosan decreases the distribution of ^{166}Ho into other tissues when applied intrahepatically and led to clinical works on patients.[698]

One hundred eighteen patients (100 male, 18 female, mean age 57 years) with hepatocellular carcinomas were enrolled in this study. ^{166}Ho−chitosan complex was injected at 20 mCi/cm of tumor diameter through a 3F microcatheter. After the injection, γ camera imaging was obtained to depict the radioactivity within the tumor. Follow up CT scans and angiography showed complete tumor necrosis in 72 patients (63.2%), >50% necrosis in 18 (15.8%), <50% necrosis in 8 (6.8%), and no response in 16 (14.0%).

Intra-arterial administration of ^{166}Ho−chitosan complex was effective and safe for the treatment of hepatocellular carcinomas and could be a new treatment modality to control the inoperable hepatocellular carcinomas.[699]

More applications are those concerning the percutaneous sclerotherapy of renal cysts with ^{166}Ho−chitosan complex and the local control of prostate cancer by intratumoral injection of ^{166}Ho−chitosan complex.[700−702]

7. Concluding Remarks

The literature published in the last trimester of 2003 includes a number of articles dealing with associations of chitosan with polysaccharides, viz., carrageenan,[703] alginate,[704] hydroxypropylguar,[705] hyaluronan,[520,706−709] cellulose,[710,711] gellan,[712] arabinogalactan,[713] and xanthan,[714] all of them more or less directly related to the preparation of drug vehicles. Chitosans were also modified with pendant lactosyl, maltosyl, and galactosyl groups for a better targeting to certain cells,[31,32,715−717] and a review on succinyl chitosan, a long-lasting chitosan for systemic delivery, has been published.[718] Other reviews deal with the antimicrobial activity of chitosans[719] and DNA−chitosan complexes.[720] A number of articles considered the association of chitosan with polylactic acid or similar compounds;[721−723] another group of articles presented new data on highly cationic chitosans.[56,57,724−727] More data have also been made available on the delivery of growth factors[728] and ophthalmic drugs,[528,729] on the activation of complement, macrophages,[730−732] and fibroblasts,[733] and on mucoadhesion.[734] The delivery of drugs and the interactions with living tissues seem therefore to be major topics in the current research on chitosan.

8. References

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