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Chitin and chitosan

PART III. SOME ASPECTS OF BIODEGRADATION AND BIOACTIVITY*)

Summary — A review on chitin and chitosan continues with the introduction into their chemistry. This part discusses some aspects of biodegradability and bioactivity of chitosan, chitin and products of their depolymerization. This paper describes the role of enzymes having the susceptibility for chitinous polymer degradation in nature and the hypothetical mechanisms of biological activity of chitosan and its oligomers.
Key words: chitin, chitosan, biodegradation, bioactivity.

Polyaminosaccharides are a group of carbohydrates (*e.g.* chitin, chitosan, muramic or hyaluronic acid, chondtrontitin, heparin) containing amine groups, besides the hydroxyl group in their polysaccharidic carbon backbone; present in animals, principally in crustacean, mollusks, insects as well as in bacteria and fungi.

As it was described in Part I, chitosan is a natural polyaminosaccharide obtained by chemical or enzymatical deacetylation of chitin, which is one of the most abundant polymers, second only after cellulose in amounts produced annually by biosynthesis [1]. Chitin occurs in animals, particularly in marine invertebrates such as crustacean and mollusks or in insects, where it is a major constituent of the exoskeleton, and in the certain fungi forming cell walls as the principal fibrillar polymer [2]. Chitin is a copolymer — poly[β-(1,4)-2-acetamido-2--deoxy-D-glucopyranose] — containing a low percentage of 2-amino-2-deoxy-β-glucopyranose residual units, whereas in chitosan the ratio of glucosamine (GlcN) to the total number of glucosamine and *N*-acetylglucosamine (GlcNAc), so-called degree of deacetylation (*DD*), is higher than 0.5 [3].

BIOSYNTHESIS OF CHITIN

Biosynthesis of chitin usually takes place in the membrane bound protein complex of chitin synthase or in intracellular vesicles called chitosomes, and requires the presence of Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor [4, 5]. Chain elongation occurs by sequential transfer of GlcNAc from UDP-GlcNAc to the non-reducing end of the growing polymer. However, the process of the UDP-GlcNAc synthesis proceeds in presence of several enzymes (Scheme 1) [6].

Chitin synthase operates in tandem with chitin deacetylase; chitin synthase produces chitin by the polymerization of GlcNAc from UDP-GlcNAc and chitin deacetylase hydrolyses the *N*-acetamido bonds in the chitin chains, acting more efficiently on nascent rather than on microfibrillar chitin [7]. One other modification involves the partial and careful degradation by means of chitinases, which weaken chitin in an orderly way and facilitate expansion [5].

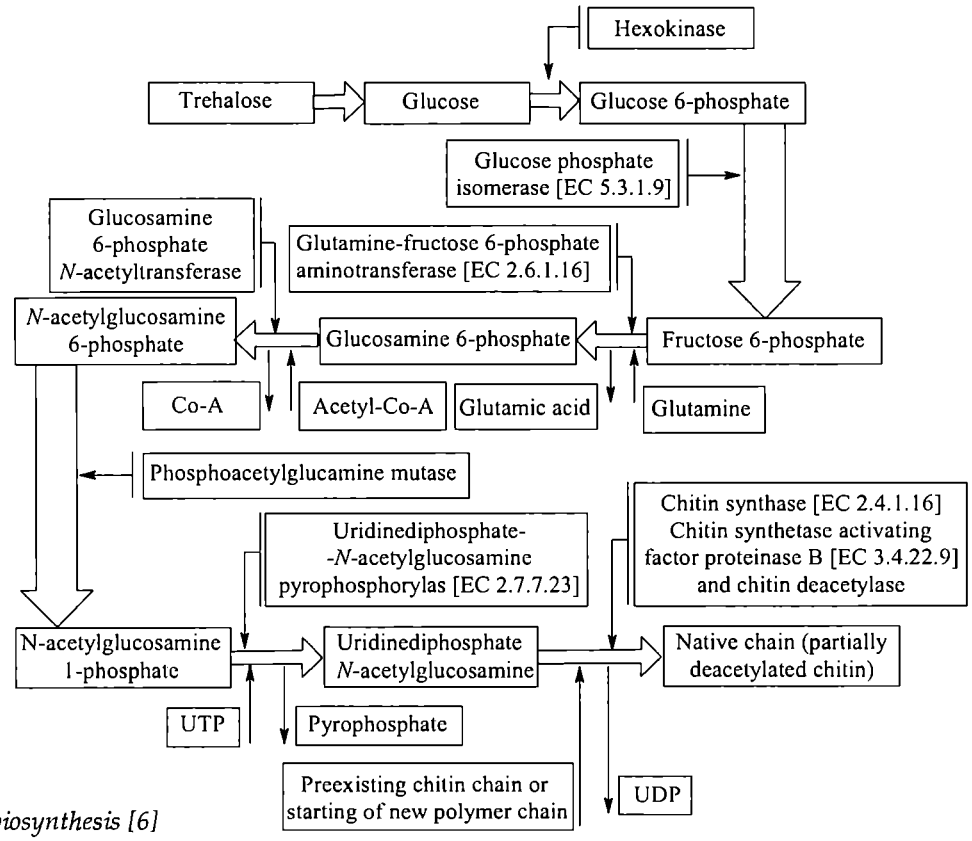
In fungi, sugar chains are extruded to the cell wall space, whereas in insects, chito-oligosaccharides are made intracellular, and then transported across the plasma membrane to the extracellular space where they become associated with lectin-type proteins. This reaction is very important. Normal functions of an insect depend on sclerotization, which involves adducts formation of chitin and proteins with the oxidation products of diphenole residues (*i.e.* *N*-acetyldopamine, *N*-β--alanyldopamine). This results in a reactive *o*-quinone or *p*-quinonemethide derivatives that crosslink proteins and chitin by means of Michael-type conjugate addition and Schiff's base formation [5].

In crustaceans, chitin appears to be synthesized in Golgi system associated with proteins [5].

BIODEGRADATION OF CHITIN AND CHITOSAN

Complete enzymatic hydrolysis of chitin to free GlcNAc is performed by a chitinolytic system based on

*) Part I and Part II *cf.* Polimery 2002, 47, No 5, and 6, respectively.



Scheme 1.
Pathway of chitin biosynthesis [6]

the synergetic and consecutive action. Relevant literature shows that chitinolytic enzymes complex is almost parallel to the cellulytic enzyme system (Table 1).

Table 1. Nomenclature of chinolytic enzymes [2]

Mode of action	Chinolytic enzymes
Random hydrolysis of the chain	Chitinase 1,4-β-poly-*N*-acetylglucosaminidase [EC 3.2.1.14]
Hydrolysis of terminal non-reducing sugar	Chitobiase [EC 3.2.1.29][1)] β-*N*-Acetylglucominidase [EC 3.2.1.30][1) 2)] β-*N*-acetylhexosaminidase [EC 3.2.1.52][3)]
Successive removal of dimer sugar unit from the non-reducing end	Chitobiohydrolase (?)

[1)] Recommendation of the Nomenclature Committee of the IUBMB, 1961.
[2)] Recommendations of the Nomenclature Committee of the IUBMB, 1978, [EC 3.2.1.29] enzyme deleted and it was included with β-*N*-acetylgucosaminidase.
[3)] Recommendations of the Nomenclature Committee of the IUBMB, 1992, [EC 3.2.1.30] deleted and the enzyme was incorporated with β-*N*-acetylhexosaminidase.

The ability to degrade chitin is thought to involve action of at least two enzymes: endo-chitinase [E.C. 3.2.1.14] and β-*N*-acetylhexosaminidase [EC 3.2.1.52] most widely distributed in animals, higher plants, and microorganisms [8]. Endo-chitinase (GlcNAcase) randomly hydrolyses biopolymer chains, chitobiohydrolase existing in fungi *T. Harzianum* [9], releases dimeric units from the non-reducing ends of the chains, whereas β-*N*-acetylhexosaminidase cleaves terminal non-reducing *N*-acetyl glucosamine residue. However, the process may be more complicated than described above. Endo-chitinase shows liquefying and saccharifying effect [9]. The existence of deacetylase [E.C. 3.5.1.41] in media during hydrolysis of chitin produces faster degradation of the polymer. Chitin in the presence of deacetylase is transformed into chitosan cleaved by chitosanases [3.2.1.99].

It has been shown that chitinases have a transglycosidase activity, which may play a role in the linkage of chitin with glucan, thus participating in the macromolecular arrangement of the wall [5].

Chitin and chitosan are also degraded by various lysozymes [EC 3.2.1.17], which are present in plants and animals. A residual number of acetyl groups (degree of deacetylation lower than *ca.* 80%) and the free hydroxy group at C3 of the sugar units are required in order to observe reasonable hydrolysis rates [10].

Chitin deacetylase catalyses the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan [11]. The presence of these enzymes was detected in many fungi and insect species [11]. The purified deacetylases have been isolated and well characterized from fungi *Aspergillus nidulans* [12], *Mucor rouxii* [13], *Absidia coerulea* [14],

Absidia orchidis [15] and *Colletotrichum lindemuthianum* [16]. All these enzymes are glycoproteins and are secreted either into periplasmic region or into the culture medium. Fungal deacetylase performs two biological roles, namely involvement in cell-wall formation and plant-pathogen interactions. The involvement in cell--wall-formation was shown in Scheme 1. An alternative role is to precipitate deacetylase in plant-pathogen interaction, due to the fact that most fungal deacetylases are extracellular and susceptible to chitin oligomers. Note that, contrary to deacetylated oligomers, chitin oligomers (tetramers to hexamers), elicit plant-defense mechanisms such as: callose formation, lignification and synthesis of coumarine derivatives [17, 18]. Finally, a role of deacetylase during the penetration process of the fungus hyphae in plant tissue was proposed [11].

The distribution of chitinases is much wider than chitosanases. Extracellular chitosanases are detected in *Actinomycetes* (especially the species of *Streptomyces*) living mainly in soil [19] and in bacteria (*Bacillus sp., Vibrio sp., etc.*) [20].

Chitinases are constitutively present in plant seeds, tubers, and flower organs and they are associated with induction of self-defense in response to an exogenous attack by plant pathogens and/or contact with chito-oligosaccharides [21]. They have been detected in extracellular, cytosolic, and microsomal fractions from those organisms. In *Insecta*, the chitin cuticle is digested during the molting process by a chitinolytic system [22]. In the Antarctic krill before molting, the chitinase and GlcNAc--ase activities increase shortly to a pronounced maximum, indicating the onset of massive resorption of cuticular material [8]. In fungi, they have autolytic, nutritional, and morphogenetic roles, *i.e.* separation of mother and daughter cells during budding in yeast, local weakening of the wall to permit branching of hyphae or germ tube emergence during spore germination, maintenance of the balance between wall synthesis and lysis at the hyphal tip, and transglycosylation [23]. Chitinolytic enzymes also serve as digestive enzymes in fishes, snails, and other marine animals [8]. Lysosomal enzymes are common in human body fluids such as serum, urine, spleen and tears. The presence of chitinases in human serum plays a defensive role against chitin--containing phatogens [21]. In the fibroblast and some other cell types of higher animals (endothelial cells, lymphocytes, hepatocytes, smooth-muscle cells) no mature forms of lysosomal enzymes are secreted. Macrophages are known to release lysosomal enzymes upon stimulation [8].

Other enzymes degrading chitin and/or chitosan were reported [24, 25]. Papain, used in the food industry for the tenderization of meat by controlled hydrolysis of muscle protein, may be applied as a hydrolyzing agent of chitosan. This gives many advantages, *e.g.* low cost of commercial enzymes, acceptance in the food industry.

This enzyme prefers the degradation of long chains at room temperature, producing low molecular oligosaccharides, which show a number of interesting biological actions and can be used as elicitors of plant defense mechanisms against pathogens, accelerators of the root nodule formation, anti-tumor agents, material for synthesis of biologically active compounds, anti-inflammatory drug, *etc.* [26—30].

In addition, the action of several lipases (except human lipase) [24, 25], cellulases and hemicellulases [24] on chitosan was reported. This phenomenon resulted from the investigation of hyaluronidase, hexosaminidase, glucoronidase, sulfatase, cathepsins and other proteinases to the synergistic degradation of chitinous materials [8].

Classification of chitinases

The classification recommended by Nomenclature Committee of International Union of Biochemistry and Molecular Biology (IUBMB) is not helpful to understand evolutionary dependence of these enzymes among themselves. Based on amino acids sequences of glycosyl hydrolases, chitinases have been divided in three families — 18, 19 and 20 [2]. Both families 18 and 19, comprised of hydrolases, showed endo action and have been isolated from various sources such as: viruses, bacteria, fungi, insects and plants. Endo-β-*N*-acetylhexosaminidase [EC 3.2.1.96] from bacteria *Flavobacterium* are also included in family 18. β-*N*-acetylhexosaminidase [EC 3.2.1.52] from bacterium *Vibrio harveyi* and from humans are grouped in family 20 [31, 32].

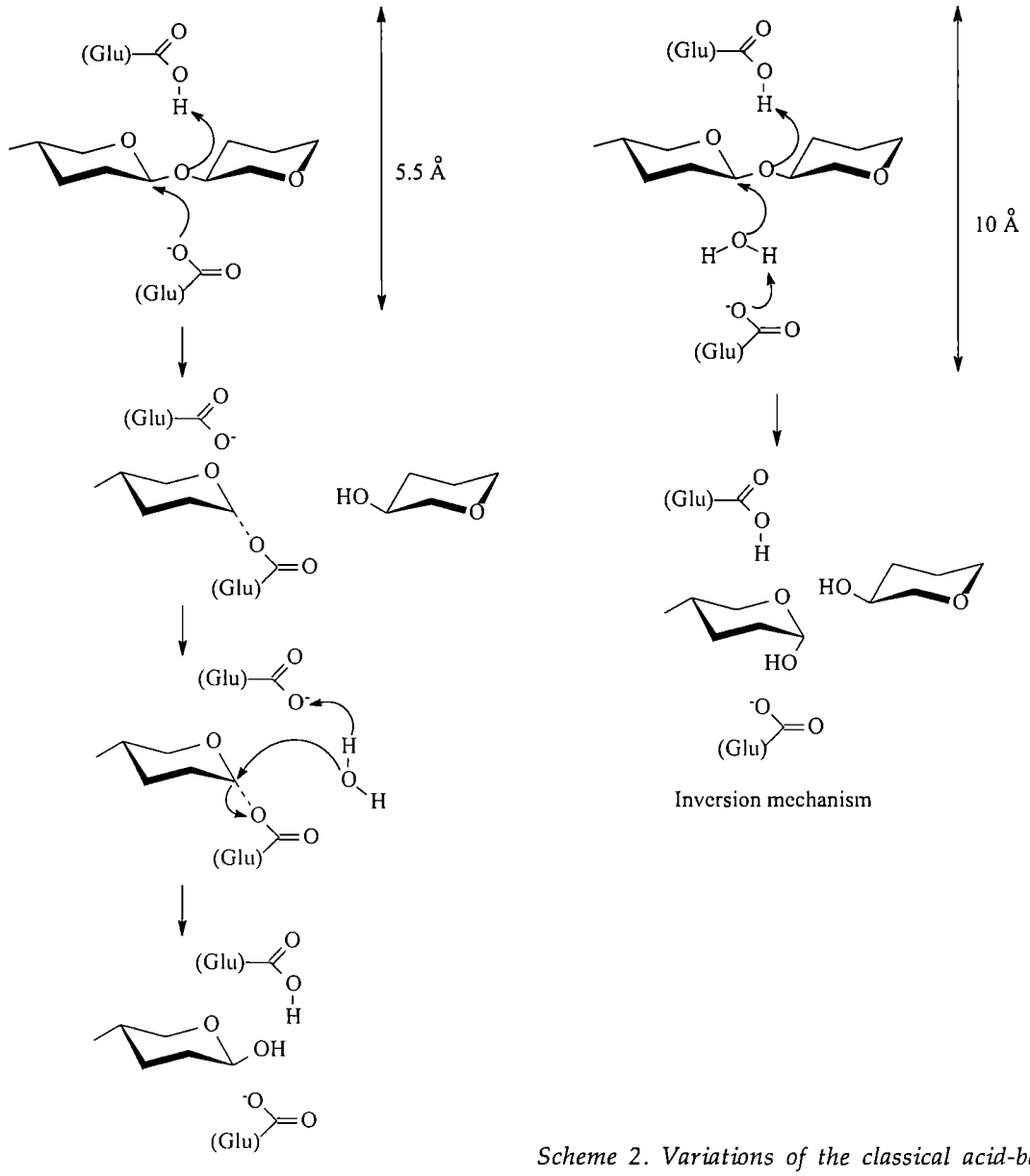
Based on the chitinase sequences of gene, these enzymes are classified into two distinct Classes — I and II, which correspond to families 18 and 19 of glycosyl hydrolases [33].

Plant chitinase isozymes are divided, in accordance with their sequences, into six classes, which differ in *N*--terminal sequences, localization, isoelectric point, signal peptide and the inducer. The chitinases from Class I have been mostly identified as an endoenzymes, whereas Class II are exoenzymes. Chitinases from Class III do not indicate any similarity in sequences to Classes I and II. Although Class IV chitin hydrolases has some similarity to those from Class I, including immunological behavior, they have significantly lower molecular mass as compared with those from Class I. Classes V and VI contain a single example [34].

Catalytic mechanism

Chitinase and chitosanase

Catalytic mechanism of chitosanase and chitinase strongly depends on their molecular structure. Chitinases from plants mainly consist in one catalytic domain only, whereas extracellular yeast chitinase contains four



Scheme 2. Variations of the classical acid-basis catalysis mechanism in hydrolysis of the 1,4-glucosic bond [35]

domains, namely a signal sequence, a catalytic domain, a serine/threonine-rich region, which is an acceptor site for *O*-glucosylation, and a *C*-terminal chitin-binding domain.

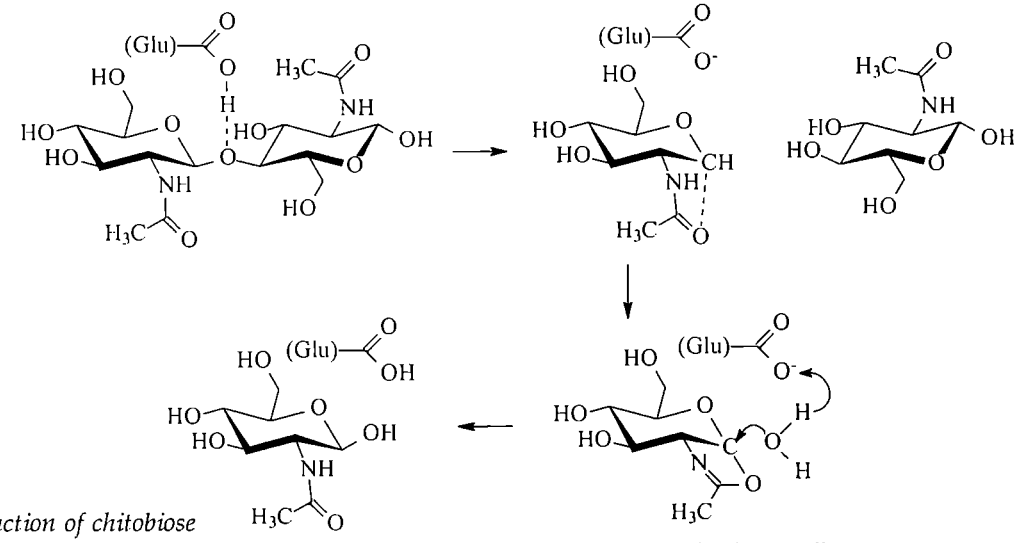
Enzymatic hydrolysis of 1,4-β-glycosidic bonds takes place by acid-base catalysis that requires two critical residues: a proton donor and a nucleophile base. This hydrolysis can result in an ether and overall retention or an inversion of anomeric configuration. Two variations of the classical acid-base catalysis mechanism common for all glycosyl hydrolases were proposed.

The first variation, named "retention mechanism", involves a protonated acidic residue as proton (H^+) donor (*i.e.*, carboxyl group of Glu residue) and negatively charged aminoacid, electrostatically stabilizing the positive charge of the C1 atom formed during catalysis. The carboxyl group of aminoacid donates a H^+ to glycosidic oxygen, causing it to be a better leaving group due to the polarization of the scissile bond. The product leaves and the remaining sugar acquires a positive charge, becoming a carbonium ion or oxocarbonium intermediate. The negatively charged residue is thought to stabilize the oxocarbonium ion intermediate, or to act as a nucleophile to form a covalent intermediate (glycosyl-enzyme). Next, the intermediate reacts with an activated H_2O (OH^-) from the equatorial side, leading to retention of the anomeric configuration of the C1 (Scheme 2).

In the second variation, named "inversion mechanism", the stabilizing base (negative charged residue) is too far from the C1 atom to stabilize the positively charged carbonium ion intermediate. That residue facilitates the polarization of the water molecule from the solution attacking directly from the free axial side, leading

to an inversion of the anomeric configuration (Scheme 2) [35].

In the case of hydrolysis of acetamido sugar derivatives (*i.e.* chitin), this above-described retention mechanism undergoes some modification. The acetamido group of chitin or its oligosaccharides has the particular feature of being able to form an oxazoline intermediate (similar to the chitinases inhibitor allosamidine [36]), from which the reaction proceeds with the retention of the anomeric configuration (Scheme 3). Initial phase of this reaction comprises formation of hydrogen bonding between the catalytic acid (*i.e.*, from Glu) and oxygen responsible for the glycosidic linkage, followed by destruction of this bond. After cleavage of 1,4 glycosidic bonds a *N*-acetylglucosamine unit is released and the second one stabilizes as an oxazolinium ion by the enzyme. The OH group resulting from water molecule completes hydrolysis. The catalytic center (catalytic acid) is recovered by proton when second monomer leaves the enzymes (Scheme 3) [35, 37].



Scheme 3. Hydrolysis reaction of chitobiose by β-N-acetylhexoamidase [37]

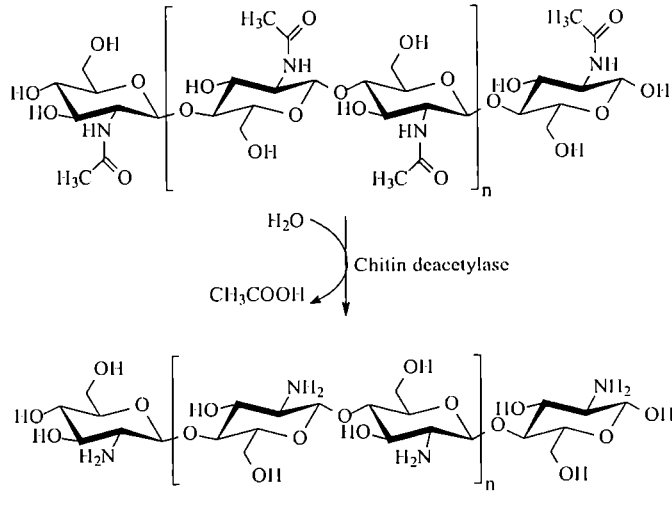
Retention of configuration occurs in result. However, the formation of such an oxazoline ring may be dependent on the three-dimensional structures. The oxazoline cannot form when steric hindrance occurs during the reaction. In such a case, the configuration may invert to the α-anomer [38]. The chitinases of family 19 show inverting mechanism, whereas the family 18 is active in the retaining mechanism. It was reported that mutarotation (α-β anomer transformation) might occur after lytic action of enzyme.

Use of ^{1}H-NMR spectroscopy allowed to show that enzymatic hydrolysis by various chitinases produced the β-anomer in one case subsequently transformed into the α-anomer, whereas in another case, the chitinase produced α-anomer that was transformed into β-anomer by the mutarotation [39].

Deacetylase

The schematic reaction of enzymatic deacetylation of chitin is shown in Scheme 4.

The mode of action of chitin deacetylase from *Mucor rouxii* is through a multiple-attack mechanism with the degree of multiple attack of three, *i.e.* such enzyme forms an enzyme-polymer complex and further catalyses the hydrolysis of three-acetyl groups before it dissociates and forms a new active complex with another polymer chain [11]. The maximum number of consecutive GlcNAc residues that were found the substrate polymer was three [40]. Thus, enzymatic deacetylation of chitin and its oligomers yields block type polymer, whereas chemical deacetylation produces randomly distributed GlcNAc and GlcN residues. Therefore, enzymatic deacetylation offers a possibility to prepare specific novel chitosan polymers.



Scheme 4. Catalytic action of chitin deacetylase

BIOACTIVITY AND BIOCOMPATIBILITY

Chitosan, as well as its degradation products, possess antibacterial, antifungal and antiviral properties.

Bioactivity of chitosan and its derivatives is connected with several phenomena, including biodegradation, induction of natural resistance, membrane effect or direct action against pathogens.

Biocompatibility has been defined as an ability of the biopolymers to perform with an appropriate host response in the specific application [41]. Presence of these phenomena in the creation of chitosan can be related to the type of chitosan forms, pathogens, or organisms attacked. Action of chitosan and its derivatives depends on the type of existing hazards, like infection of phytopathogenic bacteria and fungi, viruses or plant growth stimulation, wound healing, and the structure of chitosanous agents [42].

Chitin and chitosan action against pathogens

Chitin and chitosan are natural components of cell walls of many soil fungi that are harmful to plants. During evolution, plants formed self-defense mechanisms against pathogen fungi. Chitosan is a minor component — about 1 wt. % of carbohydrates of the cell wall, however dormant forms of organisms in the soil contain large accumulations of chitosan [43]. This suggests that an increase of chitosan in fungi may initiate the dormant phase, thereby making it harmless to plants. Moreover, during fungal infection, chitin present in fungal cell wall is converted by extracellular chitin deacetylase to chitosan, which is more resistant to chitinases action [44]. The assumption that enzymatic deacetylation results in protection against plant chitinases is supported by studies, in which mutants of the yeast *Saccharomyces cerevisiae* devoid of chitosan in their ascospore walls were used. It shows higher susceptibility to cell-degrading enzymes than wild types of yeast [45]. However, masking of the fungal structural polymer may not only be due to chitin deacetylase [44]. Enzymatic deacetylation of chitin oligomers that arise from the fungal cell wall and induced plant-defense action results in diminishing of their elicitor activity [18].

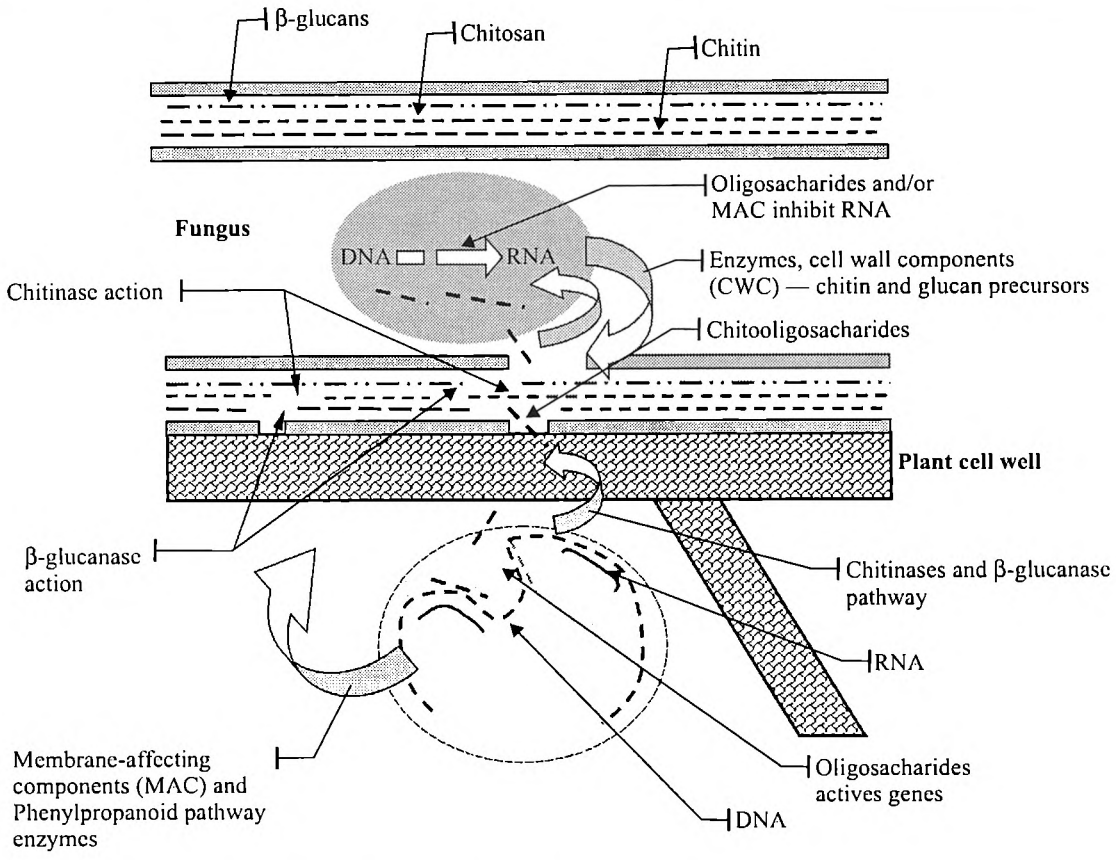
Bioactivation mechanism of chitin or chitosan or their oligosaccharides is not well understood, although preliminary data indicate that it may inhibit the transcription or accumulation of RNA. Chitosan induces various mechanical defensive reactions in plant cells, such as plugging intercellular spaces with amorphous electron opaque substances, accumulation of chitinases, synthesis of proteinase inhibitors, lignification, induction of callous synthesis and the formation of cell wall appositions, which may be implicated in restricting fungal invasion [46, 47].

It is known that the polymer should have a low molecular weight in order to limit the growth of fungus [48]. One possible mechanism is that the positively charged amine groups in chitosan form complexes with the cell DNA [48]. It was reported that the chitosan heptamer action is connected with DNA fragmentation in the cells of the treated tissue [49]. Moreover, chitosan oligomers possess an ability to cleave DNA strands in the presence of Ca^{2+} [48]. Another cationic polymer, poly(L-lysine), can also influence cellular structures like membranes, so perhaps the interaction of chitosan and fungal cells is more complex [43]. However, chitosans having low molecular weight complex DNA more effectively than poly(L-lysine) and protect against nuclease degradation [50]. Poly(L-lysine) shows generally unacceptable cytotoxicity due to the interactions between negatively charged membrane components and the poly(L-lysine) cationic side chains [51, 52].

Moreover, chitosan can induce a set of plant genes known as disease resistance response (DRR) and pathogenesis related (PR) genes and/or their promotores. Some of these genes encode enzymes for secondary pathways of phytoaloxins, hydrolytic enzymes such as RNAase, chitinase and β-glucanase, antifungal cystein--rich peptides called thionins or plant defensins and peroxidases that are directly antimicrobial or capable of generating phenolic polymers *i.e.* lignin [53].

Chitinolytic enzymes are also synergistic with the compounds, which affect cell membranes (membrane--affecting compounds — MAC). MAC include plant proteins (osmotin, zeamitin, and other thaumatin-like protein), killer factors (phytoalexin, chemical fungicides), fungal and bacterial antibiotics, and other compounds [54]. Research using radioisotopes in a system containing pea plant and a fungal pathogen (*Fusarium sp.*) has shown that enzymes (endo-β-glucanase and endo-chitinase), having lower activity in the pea tissue, break down the fungal cell wall, releasing chito-oligosaccharides, which migrate into fungus and plant cells. Degradation of the fungus cell wall is partially balanced by the deposition of new cell wall components (CWC) by the action of chitin and β-glucan synthases. In the pea, the chito-oligosaccharides become localized in the nucleus, where they appear to enhance the synthesis of about 20 major proteins (endo-chitinases, endo-β-glucanase as well as the phenylpropanoid pathway enzymes creating plant's "killer factors", and other membrane-affecting compounds). This activation may be triggered by the ability of oligosaccharides of chitosan to bind to DNA, thereby altering chromatin structure. Chitosan fragments also enter the fungus cell where, in some unidentified way, they block the accumulation or synthesis of RNA responsible for the creation of chitin and β-glucan synthases and other compounds [54—57] (Scheme 5).

Wheat germ agglutinin (WGA), a plant lectin which specifically interacts with chitin oligomers, can bind to the hyphal tips, septa and young spores of a several species of chitin-containing fungi inhibiting the fungal spore germination and colony growth [58]. Moreover, it



Scheme 5. Interaction model of fungus with pea plants proposed by Hadwiger [43, 48]

has strong affinity for zygospores, the hyphal branches involved in sexual reproduction [58].

MAC alter membranes involved in the production of cell wall compounds and reduce the ability to repair cell wall damage. Increase in the activity of plant cellulase in digesting the cell wall facilitates penetration of MAC, whereas increased level of MAC reaching their targets supports action of the lytic enzymes by inhibiting cell wall turn over and repairing mechanisms [54]. It was reported that transgenic tobacco plants transformed to produce barley chitinases and β-1,3-glucanase were more resistant against *Rhizoctonia solani* than plants expressing those genes individually, providing an argument in favor of synergistic action of those enzymes [59].

Initial interaction of polyglucosamine or poly-(*N*-acetylglucosamine) with plant cells or protoplasts may be affected by their degree of polymerization and/or chemical structure. Chitosan oligomers do not induce lignification in wounded wheat leaves, although chitosan and chitin or its oligomers with *DP* higher than 4 are effective. Partial *N*-acetylation or chemical fragmentation of chitosan reduces its ability to elicit callose formation [60]. The same phenomena were observed during viral and bacterial infections in plants and animals. The specific response of plants comprises an interaction between chitosan and the plant rather than between chitosan and the virus. Chitosan degree of polymerization and its chemical structure may affect its interaction with plant metabolism [60]. For example, high average molecular weight chitosan did not inhibit viral infections in the tobacco plant but the products of its degradation indicated high antiviral activity [61, 62]. Chitosan may induce specific substances (or messengers), which are systemically transported through the plant where they activate the defense mechanisms of plants against viral infections. It is important to note that other polysaccharide-inhibitors of viral infections — the fungal glucan and cytoplasmic mycolaminaran — did not induce systemic resistance. Activity of time-related peroxides (PO), involved in virus diseases, was negatively correlated with the level of systemic resistance induced by chitosan. It may be supposed that the increase of PO activity is an expression of the physiological stress caused by chitosan. PO activity is probably not directly involved in the mechanism(s) of chitosan-induced systemic resistance in bean plants [63].

The study [64] carried out using several species of plants such as: cucumber, melon, pumpkin, bean, pea and soybean showed the existence of complementary chitosan receptors linked by the signal transduction chain to a H_2O_2 producing enzyme system. These receptors exhibit binding if some of glucosamine units of chitosan are *N*-acetylated. Moreover, callose synthesis was induced by the chitosan and not by its degradation pro-

ducts, whereas fully deacetylated samples were more potent than partially deacetylated ones. The above study indicated that the induction of callose synthesis requires application of chitosan with high molecular weight due to its high charge density. Polycations interact with general plasma membrane constituents and complementary receptors are not implied in the case of callose induction.

Low-molecular weight microcrystalline chitosan gel-like dispersion (MCCh), as well as chitosan salts with low to medium molecular weight acted with suitably high effectiveness in the inhibition *in vitro* of selected bacteria [65]. In the case of antiviral activity, MCCh or chitosan salts containing cationic charges with a wide distribution low to medium molecular weights show optimum antifungal activity. However, polyanionic chitosan derivatives such as sulfonated and *N,O*-carboxymethylated chitosan totally lost their bioactivity for bacteria growth [42]. Application of *N*-carboxymethyl chitosan reduced aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* by more than 90%, while fungal growth decreased by more than half [66].

Chitosan and its oligomers have been shown to inhibit growth of several fungi and bacteria, especially pathogens [67, 68]. The dependence between the inhibition effect and the degree of polymerization (*DP*) was studied [69]. Chitosan oligosaccharides with *DP* 2—8 possessed stronger inhibitory effect for pathogen growth (such as: *Fusarium oxysporium, Phomopsis fukushi, Alternaria alternata*) than high molecular chitosan.

Chitosan treated bell paper fruit showed the prevention of the maceration of host tissue by *Botritis cinerea* [70]. Chitosan was effective in the decreasing of polygalacturonases production by *B. cinerea* and also caused severe cytological damage to invading hyphae that may explained the limited ability of the pathogen to colonize tissue in the presence of chitosan. In the control, massive fungal colonization was followed by degradation of host cell walls pectin and middle lamella. Decrease in the pectin density was reported in walls distant from invading hyphae.

Medical aspects

The complement activation mechanism of chitosan was studied by change of plasma C3 got-serum concentration in dog and mice after subcutaneous administration [71]. Chitosan induced activation of complement components C3 and C5, but not C4 allowing concluding that activation took place *via* an alternative pathway [72]. It is well-known that anaphylatoxin C3a formed by complement activation stimulates subcutaneous mast cells to produce histamine, leukotrine B4, causes intensive dilation of peripheral vessels and edema, whereas C5a stimulates phagocytes to upregulate endothelial adhesion receptor expression and induces upregulation of the FC receptor for antibodies and the upregulation of the complement receptors CR1 and CR3. Further studies [73] showed that administration of heterogeneous acetylated chitosan indicated stable ability on complement activation as compared with homogeneous acetylated samples where the intensity of the activation decreased with the reduction in degree of deacetylation. Subcutaneous administration caused migration of inflammatory cells and such chemotactic substances were created in serum by the incubation with chitin or chitosan.

Investigations were conducted into the strong inhibitory effect of chitosan salts and MCCh with high average molecular weight on cancer growth by inhibition of lactate formation and a considerable decrease of ATP level in intact Ehrlich ascites tumor (EAT) cells [74]. MCCh with a high degree of deacetylation shows a greater inhibition than chitosan salts with the same viscosity average molecular weight $\overline{M}_v$, and lower *DD*. The removal of neuraminic acid, indispensable for binding of polycations with glycosaminoglycans from the cell membrane or the alteration of environmental pH during the preincubation of EAT cells, eliminates the inhibitory effect of chitosan preparations.

These phenomena indicate that chitosan, acting through the membranes of intact cells, blocks metabolic cascades connected with the transmission of signals from the cell membrane into its interior and leads to a specific inhibition of the tumor cell metabolism [75].

Low average molecular weight chitosan has lipid-lowering effect by means of its controlled absorption on cholesterol [76]. The rise in plasma cholesterol and triglyceride was prevented, when chitosan oligosaccharides of chain length higher than six residues are applied. Oligosaccharides with *DP* lower than five are ineffective [77]. Hypolipidemic influence of chitosan is probably connected with an interruption of enterohepatic bile acid circulation [78].

It can be assumed, that chitosan oligomers with $DP>6$ with moderate degree of deacetylation are effective in blocking absorption of cholesterol and lipids in the intestinal tract. Observed reduction in cholesterol absorption is due to its property of forming gels in the intestinal tract, which entrap lipids and cholesterol. A noteworthy observation is that ascorbic acid enhances gel formation, thereby increasing the lipid binding capacity and potentiating the plasma cholesterol lowering activity of chitosan [77].

Absorption of minerals (*i.e.* calcium) and fat-soluble vitamins such as: A, D, E and K [79, 80] is an important biochemical effect of chitosan and its oligomers treatment. Reduction in vitamin E intake may have adverse metabolic consequences; vitamins D and E should be taken as a food supplement. Absorption of vitamin D and calcium creates a potential risk for pregnant women.

Chitin and chitosan may facilitate wound healing by stimulating granulation tissue formation or reepithelization. Crude preparation of chitin from three fungi: *Aspergillus oryzae, Mucor mucedo* and *Penicilium blakesleeanus*,

promote proliferation of cultured human F1000 fibroblast and provide matrix for their anchorage during the granulation phase of wound healing [81].

Since chitin and chitosan may improve reepithelization and wound healing, they might influence the formation of post-surgical adhesions and the repair of surgical incisions and anstomosis. *N,O*-carboxymethylchitosan delivered as a gel after the operation reduces the size, strength and a number of peritoneal adhesions in the post-surgical uterine horn [82]. It is more effective than hyaluronic acid.

Porous chitosan, glycosaminoglycans-chitosan, dextran sulfate-chitosan and heparin-chitosan complex scaffolds were implanted subcutaneously in rats to evaluate *in vivo* response to these materials [83]. Results showed that when chitosan alone supports cell attachment and growth, glycosaminoglycans-chitosan complex inhibited spreading and proliferation of both endothelial and smooth muscle cells *in vitro*. In contrast, dextran sulfate-chitosan surface supported proliferation of both cells types. Heparin-chitosan and dextran sulfate-chitosan scaffold simulated cell proliferation and formation of a thick layer of dense granulation tissue *in vivo*. Above observation leads to conclusion that the glycosaminoglycans-chitosan complex can be applied to modulate proliferation of vascular cells both *in vitro* and *in vivo*.

In the interaction between cell and non-fibrous extracellular matrix, chitosan may act as a bridging compound between carboxylated and sulfated polysaccharides. Chitosan is capable to generate chito-oligosaccharides that activate macrophages, favorable effect collagen deposition and incorporation into extracellular matrix. It has been observed in bone tissue that the increase in the extracellular Ca^{2+} is perceived by osteoblasts by the specific receptors, which cause mutagenic and chemotactic action [84].

Chitosan solutions have been studied as useful modifiers and binding agents in calcium phosphate cements used for the preparation of injectable biomaterials [85]. It has been reported that chitosan is an effective binder enhancing cement insolubility when the viscosity is high [86].

Emulsion of methylpyrrolidone chitosan with random pyrrolidone rings has been applied in dental surgery to promote osteoconduction [87].

Because of 6-oxychitin similarity in nature and behavior to hyaluronan, which shows in fact morphogenetic activity suitable for correcting bone architecture, it was possible to use 6-oxychitin as a bone regeneration materials [88, 89]. Injected materials made from 6-oxychitin and *N,N*-dicarboxymethyl chitosan induced a good histoarchitectural arrangement into the newly formed bone tissue. However, 6-oxychitin resulted in a more ordered bone structure, based on trabecular bone volume, trabecular thickness and number of trabeculae/mm, reconstructed the correct morphology of bone tissues, even in the presence of important mechanical stress, whereas the application of *N,N*-dicarboxymethyl chitosan resulted in faster healing [84]. It is also expected that 6-oxochitin would be helpful in the healing of the cartilaginous tissue [84].

Implanted titanium plate preliminary plasma-sprayed with hydroxapatite and chitosan reacted with 6-oxochitin with or without carbodiimide indicated excellent bioactivity even in the presence of bone with altered turnover [88]. Prosthetic articles coated with biopolymers enabled to promote colonization of cells, osteogensis and osteointegration [89].

MCCh is a safe and effective form of the biopolymer required to form hemostasis at the puncture site [90]. MCCh has a potential to significantly decrease arterial puncture defects following catheterization.

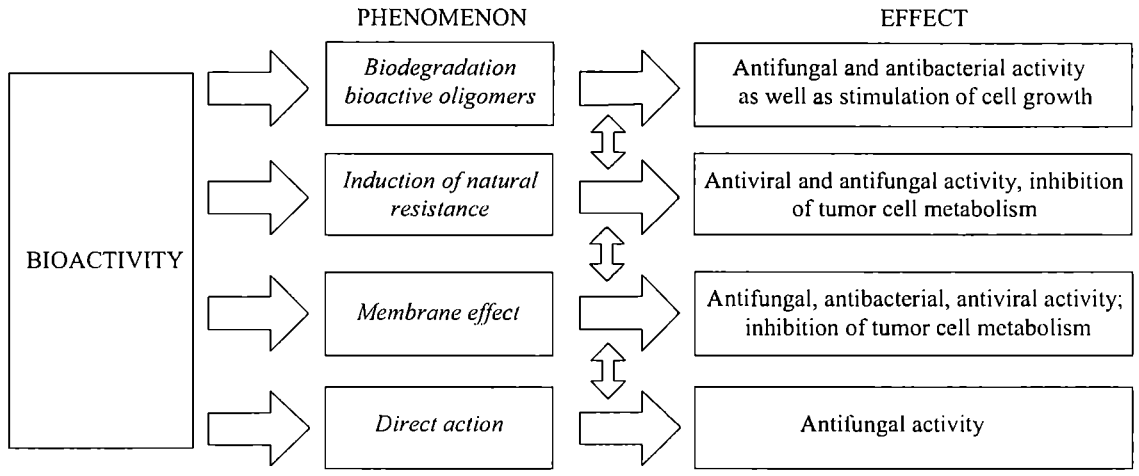
Sulfation of chitin and chitosan yielded derivatives, which posses blood anticoagulant and lipoprotein lipase (LPL)-realizing activity over those of heparin. Such derivatives may become useful as heparinoids for artificial blood dialysis [91].

It was found [92] that chitosan films show excellent compatibility with blood, even better than those obtained for the referenced siliconized glass. Irradiation of samples with fast electrons improved this compatibility even more. Tested materials have been organized by Dutkiewicz [93] in following order from best to worst compatibility with blood:

gamma-irradiated chitosan > silicone > original chitosan > ethylene oxide-treated chitosan > formaldehyde-treated chitosan.

Sulfate-containing chitosan imparted anticoagulant properties due to the introduction of the negative sulfate in the biopolymer chain [92]. However, the thromboresistance of solid chitosan surface could be unique because chitosan itself possesses polycationic behavior due to presence of a primary amino group. Chitosan sample having degree of deacetylation of 50% gave clotting time ratios [*CTR*; calculated by dividing whole blood clotting time (*WBCT*) for a tested sample by *WBCT* for glass] of 3.6, whereas samples with higher *DD* resulted in shorter clotting time ratios. Moreover, *N*-acetylation of chitosan surface did not improve thromboresistance of the modified material. Whole blood is a slightly basic substance and the probability of protonation of amino groups in the chitosan is rather low. Perhaps this was the reason why polycationic character of the biopolymer surface was not decisive in promoting blood-clotting [92].

Addition of aqueous solution of chitosan salts to blood having pH slightly above 7 may result in precipitation of chitosan in the form of microparticles, forming a thin coating on surfaces of cells and complexes with cations, proteins and other biological polymers present in blood plasma. Solutions of chitosan salts prolong only activated partial thromboplastin time (*APTT*) but not thrombin time (*TT*) as compared with the application of heparin that prolonged both *APTT* and *TT* [92].



Scheme 6. Assumed scheme of mechanism for bioactivity of chitosan and its derivatives [42]

Chitosan selectively *N*-acetylated by various carboxylic anhydrides such as acetic, propionic, *n*-butyric, *n*-valeric and *n*-hexanoic reported blood compatibility [93]. The test of blood compatibility *in vitro* of *N*-acyl chitosans, especially *N*-hexanoyl chitosan, indicated best blood compatibility because of surface hydrophobicity and the induction of a balance between hydrophilic and hydrophobic behavior at the surface.

Polyamines such as chitosan show gene carrier behavior as a non-viral vector [94]. In contrast to a viral vector, the advantage is in lower cost, non-infectivity, absence of immunogenicity, good compliance and the possibility of repeated clinical administration. Several forms of chitosan were used as a plasmid carriers, *i.e.*: chitosan complexes chemically modified by addition of ligands [95], self aggregates [96] and nanocapsules [97]. The plasmid/chitosan complex produced better gene expression than plasmid/lipofectin complexes in cell lines: A549, B16, HeLa as well as SOJ cells. Molecular weight of biopolymer and stoichiometry of complex chitosan/plasmid pGL3-Luc (encoding luciferease) strongly affected the expression of plasmid/chitosan complexes. High level of transfection were found when chitosans with molecular weight (M_w) of 40 or 84 kDa in plasmid/chitosan complex were used, whereas the application of chitosans having M_w above or below this range resulted in not detectable expression [94]. Transfection activities of complexes increased when stoichiometry of chitosan's nitrogen and DNA's phosphate is between 3 and 5 and decreased at higher stoichiometries of complexes.

Scheme 6 shows the hypothetical mechanism of the bioactivity of chitosan and its derivatives by means of direct action, affecting membrane, action of degradation products and induction of natural resistance.

CONCLUSIONS

Bioactivity and biocompatibility of chitin, chitosan and its modificates seems to be a function of several phenomena related mainly to their structural parameters, including among other:

— average molecular weight,
— degree of deacetylation,
— polydispersity,
— distribution of both GlcNAc and GlcN in polymer chain,
— crystallinity,
— porosity,
— charge character,
— origin,
— type of existing hazards, *etc.* [98].

The knowledge of above parameters and phenomena could allow preparing chitosan with optimum bioactivity for suitable applications.

Cationic types of chitosan derivatives, for example, are distinguished by a higher biological action against viruses, compared to the sulfonated chitosan being an anionic modification [98]. In turn, anionic types of chitinous derivatives found a number of applications in medicine due their high blood compatibility, trombogenicity and non-toxicity, as well as promotion of cell colonization, osteogensis and osteointegration during bone regeneration.

Moreover, the new uses of their biopolymers appeared. The application of chitosan, *e.g.* as a non-viral vector leads to numerous advantages in genetic modifications, medicine, *etc.* Manufacturing of a new type of biomaterial composite by means of biosynthesis using *Acetobacteriacea* could alter medical application, including *e.g.* wound healing materials or implants.

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