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Chitosan-Based Polyelectrolyte Complexes: A Review

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Abstract—This review focuses on the formation of polyelectrolyte chitosan complexes with biologically active compounds and the prospects of use thereof. The possibility of obtaining low-molecular-weight, water-soluble batches of chitosan, which differ in their degree of acetylation, is discussed, with emphasis on their use for binding nucleic acids into complexes.

Polysaccharides, nucleic acids, and proteins constitute three major classes of biopolymers. Polysaccharides play diverse roles in living organisms and nature as a whole. Studies of the last decade demonstrate that glycopolymers are key elements of fine biological processes. Amino polysaccharides, which are the most interesting in this respect, have been the most studied representatives of this group [1].

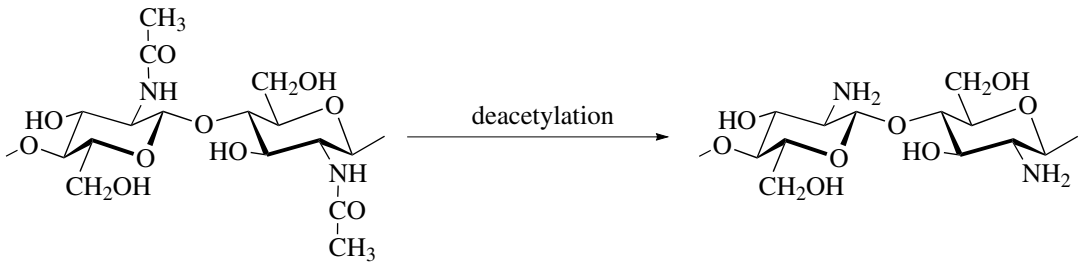
Chitin and its deacetylated derivative chitosan (amino polysaccharides) have received much recent attention from researchers working in various fields. The reason for this is twofold: (1) the unique chemical, physicochemical, and biological properties of chitin and chitosan; and (2) an unlimited (renewable) source of raw materials for their production. As of today, there are two major sources of chitin and chitosan, i.e., shells of marine crustaceans and fungi.

The polysaccharide nature of these polymers accounts for their affinity to living organisms, and the presence of reactive functional groups ensures the ability to undergo diverse chemical modifications (either increasing the extent to which the properties of parent compounds are pronounced in the derivatives, or conferring new characteristics, in compliance with the goal of the study).

Chitosan is the simplest chitin derivative. Structurally, chitin appears as poly-1⟶4, β-*D*(*N*-acetyl-2-amino-2-deoxyglucopyranose; on heating in alkaline media, its acetamide groups (at position 2 of the carbon backbone of each glucopyranose unit) are transformed into primary amino groups (with concomitant cleavage of acetic acid; see the scheme). The resulting chitosan carries an additional reactive function, which is partially protonated in weakly acidic aqueous solutions. At pH 4.0, the protonation is complete: $NH_2 + H^+ \rightleftharpoons NH_3^+$.

Thus, the molecule of chitosan is present in solutions in a cationic polyelectrolyte form, which opens the possibility for interactions with negatively charged molecules (anions and polyanions) [2–4]. For example, metal anions (Mo VI and Pt II) [5] and molecules of β-glycerophosphate tripolyphosphate [6–8] are used for crosslinking chitosan hydrogels; such ionic crosslinking offers a simple and sparing means of obtaining the hydrogels. In contrast to covalently crosslinked gels, ionic complexes retain the main properties of chitosan (biodegradability and low toxicity) [9, 10]. If an ionic complex is subjected to chemical crosslinking (for the purpose of increasing its stability), further purification is needed, because the crosslinking agents are toxic. Moreover, even if such complexes retain biocompatibility and biodegradability, the question of safety of adducts formed in the course of crosslinking remains open to debate.

Recent interest in the chemistry and physical chemistry of polyelectrolytes is due to the ability of chitosan to form specific complexes with polyanions of various natures; the formation of such complexes involves electrostatic and dipole–dipole interactions, as well as



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hydrogen and hydrophobic bonds. The formation of the complexes depends directly on the degree of ionization of cation- and anion-polymers (pH and ionic strength of their solutions), the density of their charges (chain conformations), their concentrations and ratios in the reaction medium, and duration and temperature of the interaction [11–17].

At present, polyelectrolyte complexes are widely used as carriers of drugs [18–24], non-viral vectors of transferred genes [25–29], biospecific sorbents [30, 31], films [32–34], gels [35–38], etc.

CHITOSAN–DNA COMPLEXES

The use of chitosan-based polyionic complexes as non-viral vectors transporting DNA molecules into cells is viewed as a promising field of research. Positive results are useful for further development of gene therapy, which assumes introduction of specific gene constructs with defined properties into the organism of a patient. Therapeutic effects are achieved via two possible mechanisms involving, respectively, expression of the introduced gene or suppression of the defective gene (which may be partial or complete). Efficient delivery of the gene into target cells and maintenance of its functional activity therein (i.e., setting conditions for its unhampered operation) constitute the most important condition of successful gene therapy.

Polycations used thus far as non-viral gene vectors are limited to polyethyleneimine, polyphosphazene, and polyamidoamine (polylysine) [39–41]. Potential advantages of these systems are obvious (ease of synthesis and low immunogenicity). Their use is restricted by the low efficiency of delivery and poor biocompatibility (all synthetic polycations listed above are toxic, which precludes their extensive use). Cato *et al.* [42] studied the efficiency of polygalactosamine as a non-viral vector targeting luciferase plasmid (pGL3) to tumor cells; the result obtained was unsatisfactory. In order to overcome the problem of low efficiency, new carriers and chitosan-based modified polycations are developed, which are nontoxic but biodegradable and biocompatible. Subsequent research allowed Cato *et al.* to conclude that chitosan is several times more active than polygalactosamine in this respect; the efficiency of chitosan depended on its molecular weight. Chitosans with molecular weights (MWs) of 15 or 52 kDa increased luciferase activity (i.e., facilitated the gene delivery), whereas those with MWs no lower than 100 kDa exhibited low efficiency, as well as oligochitosan (MW, 1.3 kDa).

Chitosan has been used for gene transfer by several researchers [43–45]. The formation of complexes is associated with neutralization of the negative charge of DNA; as a result, the molecule of DNA becomes more compact, and this both protects it from degradation by restriction endonucleases and facilitates permeation of the cell membrane.

Particular attention is paid to effects of the size of the molecule, its positive charge, and the density of its distribution over the length of the polymer chain. Studies of the process of complex formation, involving chitosan oligomers (with a degree of polymerization equal to 6, 8, 10, 12, 14, and 24) and fluorescence-labeled DNA demonstrated that the complexes formed were stable when the degree of polymerization of chitosan was equal to 24 (which roughly corresponds to an MW of 5 kDa). This complex was also characterized by the highest ability to penetrate cells [46]. However, the mechanism of this process has not been adequately explored. Crossing of model cell plasmalemma (lipid bilayer) by a low-molecular-weight (4.2 kDa) chitosan was studied using a variety of methods. These experiments demonstrated that chitosan is capable of disrupting lipid bilayers in a concentration-dependent way [47]. Japanese researchers studied penetration of cells by chitosan-associated fluorescence-labeled plasmids [48]. Their results confirmed the critical roles of the MW of chitosan, the ratio of charges, and pH. It turned out that, prior to penetration of the cells, the complex forms aggregates of 5–8 μm, which are adsorbed to the cell surface; the aggregated are internalized by endocytosis, after which hydrolytic enzymes of lysosomes release them from the endosomes. In this study, accumulation of the complex in the nucleus of the target cell was visualized by confocal scanning laser microscopy.

Chinese researchers reported that *N*-alkylated chitosan forms polyelectrolyte complexes if the ratio of positive and negative charges is equal to 1 : 4, whereas, for native chitosan, the value of this ratio should be 1 : 1; the introduction of an alkyl radical (C_8) facilitated cell permeation by the complex due to the hydrophobic character of this substituent [49].

In order to increase the specificity of DNA targeting to the cells, chitosan is modified by low-molecular-weight compounds (e.g., galactose residues), and this facilitates recognition of chitosan (and its DNA complexes) by liver cells; the authors indicate that the toxicity of the complex was very low [50]. Using confocal scanning laser microscopy, Korean researchers studied the interaction with hepatocytes of a complex formed by galactosylated chitosan and DNA [51]. Due to the availability of specific galactose receptors on the surface of hepatocytes, the complex rapidly crossed the cell membrane, but its transport from the cytoplasm to the nucleus constituted a rate-limiting step. The authors noted that maximum rate of transport was observed in human cells (HepG2 line).

COMPLEXES OF CHITOSAN WITH PROTEINS AND GLYCOSAMINOGLYCANS

In addition to collagen, gelatin, and glycosaminoglycans, chitosan plays a leading part in tissue engineering as a biostructuring material (temporary replacement of skin and bone tissue), a component of artificial blood vessels and valves, and as an implant in

cosmetic surgery [52–54]. Varying the conditions of freezing and lyophilic drying of chitosan-based solutions and gels made it possible to generate porous structures with controlled pore size (1–250 μm), which were further used for obtaining ionic complexes [55]. An application of this approach in medicine is given by the use of porous elastic sponges for treating wounds. The sponge contains collagen fibrils and chitosan fibers; of note, the fibrils are fixed in space by means of a filamentous network formed by chitosan molecules and the structure-forming agent. In the tissue regeneration phase, it is most reasonable to use coatings based on biodegradable polymers that stimulate wound healing. Chitosan is one such polymer constituent in the preparation. The contribution of chitosan to wound healing involves two potential mechanisms: induction of an immune response (by macrophage stimulation, which triggers other biological processes) and utilization of *N*-acetylglucosamine as a precursor in the synthesis of chondroitin sulfate, keratin sulfate, hyaluronic acid, etc. Keratin sulfate (a mucopolysaccharide) is a component of a proteoglycan complex capable of associating with hyaluronic acid; this complex plays an important role in the formation of blood vessel walls [56, 57]. Domard *et al.* noted that, irrespective of the degree of acetylation (DA), chitosans are compatible with keratinocytes and fibroblasts; on increasing the value of DA, however, the cells become less adhesive. Adhesion is an important parameter: fibroblasts are twice as adhesive as keratinocytes, but the proliferation of the latter is inversely related to the value of the DA [58].

Heparin is known for its differential effects on cell proliferation (some cells are stimulated to proliferate, whereas, in others, proliferation processes are suppressed); it also facilitates wound healing by acting on certain phases of this process (stabilization of growth factors). It is not infrequent that the considerable surface area of wounds requires the use of heparin at high concentrations and for significant periods. Due to this, the use of therapeutic concentrations of heparin necessitates its immobilization onto a biodegradable base, and chitosan is the best material in this respect. Water-soluble polyelectrolyte complexes of chitosan (MW, 200 kDa; DA, 10%) and deaminated heparin have been reported [59–61]. It is interesting to note related studies in which chitosan formed complexes with sulfated chitosan or polystyrene sulfate [62]. The structure of sulfated chitosan and some of its properties (biological activity) mimic natural heparin. The effects of the DA and MW on the structural density of such complexes were studied by the method of static light scattering in dilute solutions of variable ionic strength. The stability of the complexes turned out to be inversely related to their MW and DA; a similar pattern was recorded for chitosan–alginate complexes [63].

CHITOSAN COMPLEXES WITH LIPOPOLYSACCHARIDES

Complexes of chitosan with lipopolysaccharides are of undoubted interest to researchers. Lipopolysaccharides constitute the main component of the outer membrane of gram-negative bacteria, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Each lipopolysaccharide contains a highly variable O-specific chain and a less variable core oligosaccharide bound to a structurally conserved lipid portion known as lipid A. Lipopolysaccharides play a significant part in the function of the cell membrane: first and foremost, they account for the low membrane permeability and its resistance to hydrophobic agents; in addition, they affect the growth and viability of bacteria and constitute the main target of antibacterial preparations and immunoglobulins. In higher organisms, lipopolysaccharides exert a broad spectrum of biological effects. The presence of lipid A makes it possible to detect causative agents of gram-negative infections (using the so-called endotoxin tests). Of related interest are studies of polyionic complexes formed by chitosan and endotoxins. The mechanism of binding was studied by sedimentation analysis and centrifugation in CsCl density gradients. The process of complex formation involved several stages and depended on both the time of incubation and the temperature of the medium. A stable complex was formed only after incubation of the reaction components at 37°C (which favored dissociation of lipopolysaccharide molecules and their binding by chitosan). After the formation of the complex, further dissociation of lipopolysaccharide molecules took place, resulting in chitosan–endotoxin complexes with MW values lower by an order of magnitude; these complexes retained stability in solutions with high ionic strength [64]. The degree of polymerization (DP) of chitosan was another important characteristic. For example, a preparation of chitosan with an MW of 20 kDa and a DP of 100 showed higher affinity to lipopolysaccharides than its counterpart with an MW of 140 kDa and a DP of 700. Overall, interactions with chitosan decreased lipopolysaccharide toxicity [65]; this important result lays the groundwork for further research.

The cell walls of bacteria (lipopolysaccharides) are also disrupted by lipases. Due to the waxlike nature of lipids of *M. tuberculosis*, this bacterium is not digested by macrophages. It is conceivable that the problem of lipid amenability to the hydrolytic effects of lipases may be solved by immobilizing it on an appropriate sorbent capable of crossing the membrane barrier. Microparticles of chitosan, obtained by ionic gelatinization (using solutions of chitosan and calcium chloride or tripolyphosphate as a source of anions) and subsequent lyophilization, may be used for this purpose. Compared to alginate-based microparticles prepared in a similar way, the microparticles of the chitosan-immobilized enzyme were five times more active [66]. It was

noted [67] that the ability of chitosan to serve as a carrier of the enzyme depends on both the DP (which should be in the range 10–30%) and the MW (low-molecular-weight chitosan is more readily dissolved). Combining the two processes (i.e., preparation of chitosan particles and immobilization of lipase) may be a promising approach. For example, noncovalent immobilization of lipase on Chitoxan (a preparation of polyionic particles of a chitosan–xanthane complex) increased the activity of the enzyme twofold [68]. Analysis of the microstructure of this preparation of the enzyme demonstrated nonuniform distribution of lipase throughout the particles. The highest concentration of the enzyme was observed at their outer surface (contacting the membrane of bacterial cells).

EFFECTS OF CHITOSAN PROPERTIES ON THE PROCESS OF COMPLEX FORMATION

Because polyelectrolyte complexes are formed in aqueous solutions, studies of the complex formation should take into account data on the MW of chitosan, its mass distribution, DA, the location of free and acetylated amino groups in the polymer chain, chain length, and conformation of molecules in solution. As a rule, the effects of the above characteristics are studied in dilute, weakly acidic solutions of chitosan. Under such conditions, chitosan is readily soluble (by virtue of amino group protonation) and acts as a true polycationic polyelectrolyte (although a weak one). Depending on the value of the DA, pK_0 of chitosan varies in the range 6.46–7.32 [69]. The conformation and size of chitosan chains in dilute solutions also depend considerably on the DA and ionic strength. At low DA values (0–20%), electrostatic repulsion of the protonated amino group predominates, resulting in chain extension. The observed rigidity of the polymer chain, observed in the case of weakly acetylated molecules, depends on the ionic strength. Addition of salts to chitosan solutions exerts protective effects, decreasing (without complete inhibition) the electrostatic repulsion of the positively charged amino groups throughout the polymer chain. As the DA increases from 20 to 50%, acetyl residues take part in intermolecular interactions (via the formation of hydrogen bonds), and this further limits the rotation of the six-membered structural units of chitosan around β-glycoside bonds. In addition, the acetamide group (the volume of which is higher than that of the amino function), is responsible for steric hindrances that limit the chain flexibility; as a consequence, the rigidity of chitosan chains should increase with the DA. At DA values of 50% or higher, the solution becomes a dispersion of highly solvated microgels containing no polymer chains. As a result, local concentrations of polymeric segments increase (compared to the initial concentration), further strengthening the hydrophobic interactions. In one report, an increase in the DA decreased the polydispersion [70], which was an unexpected observation, because the process of acetylation [71] did not degrade the polymer chain. Two possible causes were identified which may account for these findings. First, the range of pH within which chitosan is soluble increases with the DA. Multiple washing steps that follow each acetylation could contribute to the loss of low-molecular-weight fractions and thereby decrease the polydispersion. Second, the tendency to form aggregates also increases with the DA. Retention of the aggregates in the chromatographic column could decrease the efficiency of the chromatographic separation.

The majority of natural polymers tend to aggregate in solutions in a concentration-dependent way, and chitosan is not an exception. The mechanism of formation of these associations is not fully understood. It is possible that hydrophobic interactions between acetyl groups and glycoside units play an important role in aggregate formation. This process was studied using viscometry and fluorescence spectroscopy in two samples of chitosan (MW, 190 kDa; DA, 12%), one of which was modified by introduction of a hydrophobic function ($C_{12}H_{25}$, 4%). Hydrophobic domains were formed in both samples of chitosan. Because the addition of urea or ethanol resulted in only partial disruption of hydrophobic interactions, the authors concluded that chitosan contains two types of hydrophobic domains, one being inherent in chitosan and polysaccharides in general, and the other, resulting from introduction of hydrophobic substituents [72].

Polyelectrolyte complexes are usually produced from low-molecular-weight water-soluble chitosan species. The original chitosan, however, is a high-molecular-weight compound that is poorly, if at all, soluble in water. For this reason, the first step preceding the formation of complexes involves decreasing the MW two to three orders of magnitude. One of the ways to achieve such a decrease involves the use of enzymatic hydrolysis, but the lack of inexpensive enzymes exhibiting high chitinolytic activity prompts researchers to use commercially available enzyme preparations devoid of specific activity [73–75]. The ability of such preparations to depolymerize chitin and chitosan was the subject of a separate report [76]. It would be tempting to speculate that the active preparations may contain admixtures of chitinolytic enzymes; this is improbable, however, given the fact that the enzymes were isolated from multiple sources (bacteria, fungi, plants, mammals, etc.). In addition, pH optima of these enzymes differ from the pH values which ensure optimum operation of the majority of chitinases. Of still greater importance, neither consequent nor simultaneous use of two nonspecific preparations produces any synergistic effects.

Our studies of enzyme-catalyzed hydrolysis of high-molecular-weight chitosan demonstrate that the best results have been achieved with chitinolytic complexes produced by bacteria of the *Streptomyces*, *Serratia*, and *Bacillus* genera [77–80], as well as with Celloviridin,

an enzymatic preparation exhibiting cellulose, β-glucanase, and xylanase activities [81, 82].

We obtained preparations of low-molecular-weight chitosan, differing in medium-viscosity MW values (2.0, 4.1, 7.8, 13.6, 19.0, and 31.0 kDa; 85% amino groups) and the content of amino groups (46, 66, 75, and 85%; MW, 14.6 kDa), and used them in forming complexes with nucleic acids. Small volumes of the original aqueous solution of chitosan were added under stirring to a salt-supplemented aqueous solution of nucleic acid. Absorption spectra were recorded after each addition.

The critical concentration of each chitosan, required for the formation of dispersions of DNA–chitosan complexes, was determined from characteristic changes in the shape of absorption spectra (230–450 nm). Attainment of a critical concentration was heralded by the appearance of a peak in the vicinity of 320 nm (where neither DNA nor chitosan absorb) and an elevation of the peak normally occurring at 260 nm. These changes in the spectrum reflect the formation of dispersions of DNA–chitosan complexes, the microparticles of which scatter UV radiation.

Effects of the MW of chitosan and pH and ionic strength of solutions, as well as theoretical descriptions of interactions of DNA–chitosan complexes, resulting in the formation of liquid crystals, are dealt with in a series of reports [83–85]. The phase transition efficiency of DNA molecules (and acquisition of the liquid crystal state) depends on a number of factors, which makes it hardly possible to compare results generated by individual researchers. This means that, in selecting the conditions of transfection, it is necessary to take into account the properties of liquid crystal dispersions (formed by DNA–chitosan complexes). Moreover, it is advisable to standardize such conditions.

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In conclusion, the formation of polyelectrolyte complexes directly depends on (1) the degree of ionization of cation- and anion-polymers, (2) the density of charges, (3) charge distribution over the polymer chain, (3) concentration of polymers, and (4) their ratio, as well as on (5) the duration of the interaction and (6) the temperature of the reaction medium. Chitosan-based complexes may be used as carriers of drugs, non-viral gene vectors, biospecific sorbents, films, gels, etc.

REFERENCES

1. Janes, K.A., Calvo, P., and Alonso, M.J., *Adv. Drug. Deliv. Rev.*, 2001, vol. 47, no. 1, pp. 83–97.
2. Mochizuki, M., Kadoya, Y., Wakabayashi, Y., Kato, K., Okazaki, I., Yamada, M., Sato, T., Sakaira, N., Nishi, N., and Nomizu, M., *FASER J.*, 2003, vol. 17, no. 8, pp. 875–877.
3. Berger, J., Reist, M., Mayer, J.M., Felt, O., Peppas, N.A., and Gurny, R., *Eur. J. Pharm. Biopharm.*, 2004, vol. 57, no. 1, pp. 19–34.
4. Cerchiara, T., Luppi, B., Bigucci, F., Orienti, I., and Zecchi, V., *J. Pharm. Pharmacol.*, 2002, vol. 54, no. 11, pp. 1453–1459.
5. Brack, H.P., Tirmizi, S.A., and Risen, W.M., *Polymer*, 1997, vol. 38, no. 10, pp. 2351–2362.
6. Mi, F.I., Shyu, S.S., Wong, T.B., Jang, S.F., Lee, S.T., and Lu, K.T., *J. Appl. Polym. Sci.*, 1999, vol. 74, no. 5, pp. 1093–1107.
7. Chenite, A., Buschmann, M., Wang, D., Chaput, C., and Kandani, N., *Carbohydr. Res.*, 2001, vol. 46, no. 1, pp. 39–47.
8. Li, J. and Xu, Z., *J. Pharm. Sci.*, 2002, vol. 91, no. 7, pp. 1669–1677.
9. Noble, L., Gray, A.I., Sadiq, L., and Uchegbu, I.F., *Int. J. Pharm.*, 1999, vol. 192, no. 2, pp. 173–182.
10. Berger, J., Reist, M., Mayer, J.M., Felt, O., and Gurny, R., *Eur. J. Pharm. Biopharm.*, 2004, vol. 57, no. 1, pp. 35–52.
11. Sabnis, S. and Block, L.H., *Int. J. Biol. Macromol.*, 2000, vol. 27, no. 3, pp. 181–186.
12. Kim, Y.H., Gihm, S.H., Park, C.R., Lee, K.Y., Kim, T.W., Know, I.C., Chung, H., and Jeong, S.Y., *Bioconjug. Chem.*, 2001, vol. 12, no. 6, pp. 932–938.
13. MacLaughlin, F.C., Mumper, R.J., Wang, J., Tagliaferri, J.M., Gill, I., Hinchcliffe, M., and Rolland, A.P., *J. Control. Release*, 1998, vol. 56, nos. 1–3, pp. 259–272.
14. Kim, T.H., Ihm, J.E., Choi, Y.J., Nah, J.W., and Cho, C.S., *J. Control. Release*, 2003, vol. 93, no. 3, pp. 389–402.
15. Evdokimov, Yu.M., Salyanov, V.I., Semenov, S.V., Il'ina, A.V., and Varlamov, V.P., *Izv. Akad. Nauk SSSR, Ser.: Biol.*, 2002, vol. 36, no. 2, pp. 532–541.
16. Evdokimov, Yu.M., *Khitin i khitozan*, (Chitin and Chitosan), Skryabin, K.G, Vikhoreva, G.A, and Varlamov, V.P, Eds., Moscow: Nauka, 2002.
17. Fredheim, G.E. and Christensen, B.E., *Biomacromolecules*, 2003, vol. 4, no. 2, pp. 232–239.
18. Singla, A.K. and Chawla, M., *J. Pharm. Pharmacol.*, 2003, vol. 53, no. 8, pp. 1047–1067.
19. Tapia, C., Escobar, Z., Costa, E., Sapag-Hagar, J., Valenzuela, F., Basualto, C., Nella, G.M., and Yazdani-Pedram, M., *Eur. J. Pharm. Biopharm.*, 2004, vol. 57, no. 1, pp. 65–75.
20. Murra, P., Zerrouk, N., Mennini, N., Maestrelli, F., and Chemtob, C., *Eur. J. Pharm. Sci.*, 2003, vol. 19, no. 1, pp. 67–75.
21. Murdan, S., *J. Control. Release*, 2003, vol. 92, nos. 1–2, pp. 1–17.
22. Vila, A., Sanchez, A., Janes, K., Behrens, I., Kissel, T., Vila-Jato, J.L., and Alonso, M.J., *Eur. J. Pharm. Biopharm.*, 2004, vol. 57, no. 1, pp. 123–131.
23. Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A.N., and Daviss, S.S., *Adv. Drug. Deliv. Rev.*, 2001, vol. 51, nos. 1–3, pp. 81–96.
24. Dyer, A.M., Hinchcliffe, M., Watts, P., Castile, J., Jabbal-Gill, I., Nankervis, R., Smith, A., and Illum, L., *Pharm. Res.*, 2002, vol. 19, no. 7, pp. 998–1008.
25. Borchard, G., *Adv. Drug. Deliv. Rev.*, 2001, vol. 52, no. 2, pp. 145–150.

26. Okamoto, H., Nishida, S., Todo, H., Sakakura, Y., Iida, K., and Danjo, K., *J. Pharm. Sci.*, 2003, vol. 92, no. 2, pp. 371–380.
27. Thanou, M., Florea, Bi., Geldof, M., Junginger, H.E., and Borchard, G., *Biomaterials*, 2002, vol. 23, no. 1, pp. 153–159.
28. Nagasaki, T., Hojo, M., Uno, A., Satch, T., Koumoto, K., Mizu, M., Sakurai, K., and Shinkai, S., *Bioconjug. Chem.*, 2004, vol. 15, no. 2, pp. 249–259.
29. Erbacher, P., Zou, S., Bettinger, T., Steffan, A.M., and Remy, J.S., *Pharm. Res.*, 1998, vol. 15, no. 9, pp. 1332–1339.
30. Sashiwa, H., Thompson, J.M., Das, S.K., Shigemasa, Y., Tripathy, S., and Roy, R, *Biomacromolecules*, 2000, vol. 1, no. 3, pp. 303–305.
31. Sharma, A., Mondal, K., and Gupta, M.N., *J. Chromatogr., A*, 2003, vol. 995, nos. 1–2, pp. 127–134.
32. Sasaki, C., Kristiansen, A., Fukamizo, T., and Varum, K.M., *Biomacromolecules*, 2003, vol. 4, no. 6, pp. 1686–1690.
33. Park, S.I. and Zhao, Y., *J. Agric. Food Chem.*, 2004, vol. 52, no. 7, pp. 1933–1939.
34. Shu, X.Z., Zhu, K.L., and Song, W., *Int. J. Pharm.*, 2001, vol. 212, no. 1, pp. 19–28.
35. Kofuji, K., Akamine, H., Qian, C.J., Watanaba, K., Togan, Y., Nishimura, M., Sugiyama, I., Murata, Y., and Kawashima, S., *Int. J. Pharm.*, 2004, vol. 272, nos. 1–2, pp. 65–78.
36. Ruel-Gariepy, E., Chenite, A., Chaput, C., Guirguis, S., and Lerous, J., *Int. J. Pharm.*, 2000, vol. 203, nos. 1–2, pp. 89–98.
37. Ramanathan, S. and Block, L.H., *J. Control. Release*, 2001, vol. 70, nos. 1–2, pp. 109–123.
38. Vinogradov, S.V., Bronich, T.K., and Kabanov, A.V., *Adv. Drug. Deliv. Rev.*, 2002, vol. 54, no. 1, pp. 135–147.
39. Koping-Hoggard, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Varum, K.M., and Artursson, P., *Gene Ther*, 2001, vol. 8, no. 14, pp. 1108–1121.
40. Luten, J., Steenis, J.H., Someren, R., Kemmink, J., Schuurmans-Nieuwenbroek, N.M., Koning, G.A., Crommelin, D.J., Nostrum, C.F., and Hennink, W.E., *J. Control. Release*, 2003, vol. 89, no. 3, pp. 483–497.
41. Smedt, S.C., Demeester, J., and Hennink, W.E., *Pharm. Res.*, 2000, vol. 17, no. 2, pp. 113–126.
42. Sato, T., Ishii, T., and Okahata, Y., *Biomaterials*, 2001, vol. 22, no. 15, pp. 2075–2080.
43. Mansouri, S., Lavigne, P., Corsi, K., Benderdour, M., Beaumont, E., and Fernandes, J.C., *Eur. J. Pharm. Biopharm.*, 2004, vol. 57, no. 1, pp. 1–8.
44. Mao, H.Q., Roy, K., Troung-Le, V.L., Lin, K.Y., Wang, Y., August, J.T., and Leong, K.W., *J. Control. Release*, 2001, vol. 70, no. 3, pp. 399–421.
45. Richardson, S., Kolbe, H., and Duncan, R., *Inter. J. Pharmaceutics*, 1999, vol. 178, no. 2, pp. 231–243.
46. Koping-Hoggard, M., Mel'nikova, Y.S., Varum, K.M., Lindman, B., and Artursson, P., *J. Gene Med.*, 2003, vol. 5, no. 2, pp. 130–141.
47. Yang, F., Cui, X., and Yang, X., *Biophys. Chem.*, 2002, vol. 99, no. 1, pp. 99–106.
48. Ishii, T., Okahata, Y., and Sato, T., *Biochim. Biophys. Acta*, 2001, vol. 1514, no. 1, pp. 51–64.
49. Liu, W.G., Zhang, X., Sun, S.J., Sun, G.J., Yao, K.D., Liang, D.C., Guo, G., and Zhang, J.Y., *Bioconjug. Chem.*, 2003, vol. 14, no. 4, pp. 782–789.
50. Gao, S., Chen, J., Xu, X., Ding, Z., Yang, Y.H., Hua, Z., and Zhang, J., *Int. J. Pharm.*, 2003, vol. 255, nos. 1–2, pp. 57–68.
51. Park, I.K., Kim, T.H., Kim, S.E., Park, Y.H., Kim, W.J., Akaike, T., and Cho, C.S., *Int. J. Pharm.*, 2003, vol. 257, nos. 1–2, pp. 103–110.
52. Gutowska, A., Jeong, B., and Jasionowski, M., *Anat. Rec.*, 2001, vol. 263, no. 4, pp. 342–349.
53. Mao, J.S., Zhao, L.G., Yin, Y.J., and Yao, K.D., *Biomaterials*, 2003, vol. 24, no. 6, pp. 1067–1074.
54. Sun, J.K. and Matthew, H.W., *Biomaterials*, 2000, vol. 21, no. 24, pp. 2589–2598.
55. Madihally, S.V. and Matthew, H.W., *Biomaterials*, 1999, vol. 20, no. 12, pp. 1133–1142.
56. Denuziere, A., Ferrier, D., and Domard, A., *Ann. Pharm. Fr.*, 2000, vol. 58, no. 1, pp. 47–53.
57. Kuberka, M., Heschel, I., Glasmacher, B., and Rau, G., *Biomed. Tech.*, 2002, vol. 47, no. 1, pp. 485–487.
58. Chatelet, C., Damour, O., and Domard, A., *Biomaterials*, 2001, vol. 22, no. 3, pp. 261–268.
59. Kweon, D.-K., Song, S.-B., and Park, Y.-Y., *Biomaterials*, 2003, vol. 24, no. 9, pp. 1595–1601.
60. Tsai, C.-C., Chang, Y., Sung, H.-W., Hsu, J.-Ch., and Chen, Ch.-N., *Biomaterials*, 2001, vol. 22, no. 6, pp. 523–533.
61. Lin, W.C., Liu, T.Y., and Yang, M.C., *Biomaterials*, 2004, vol. 25, no. 10, pp. 1947–1957.
62. Berth, G., Voigt, A., Dautzenberg, H., Donath, E., and Mohwald, H., *Biomacromolecules*, 2002, vol. 3, no. 3, pp. 579–590.
63. Yan, X.L., Khor, E., and Lim, L.Y., *J. Biomed. Mater. Res*, 2001, vol. 58, no. 4, pp. 358–365.
64. Davidova, V.N., Yermak, I.M., Gorbach, V.I., and Solovieva, T.F., *Membr. Cell Biol.*, 1999, vol. 13, no. 1, pp. 49–58.
65. Davydova, V.N., Ermak, I.M., Gorbach, V.I., Krasikova, I.N., and Solov'eva, T.F., *Biokhimiya*, 2000, vol. 65, no. 9, pp. 1278–1287.
66. Betigeri, S.S. and Neau, S.H., *Biomaterials*, 2002, vol. 23, no. 17, pp. 3627–3636.
67. Alsarra, I.A., Betigeri, S.S., Zhang, H., Evans, B.A., and Neau, S.H., *Biomaterials*, 2002, vol. 23, no. 17, pp. 3637–3644.
68. Magnin, D., Dumitriu, S., Magny, P., and Chornet, E., *Biotechnol. Prog.*, 2001, vol. 17, no. 4, pp. 734–737.
69. Sorlier, P., Denuziere, A., Viton, C., and Domard, A., *Biomacromolecules*, 2001, vol. 2, no. 3, pp. 765–772.
70. Schatz, C., Viton, C., Delair, T., Pichot, C., and Domard, A., *Biomacromolecules*, 2003, vol. 4, no. 3, pp. 641–648.
71. Sorlier, P., Viton, C., and Domard, A., *Biomacromolecules*, 2002, vol. 3, no. 6, pp. 1336–1342.
72. Philippova, O., Volkov, E., Sitnikova, N., and Khokhlov, A., *Biomacromolecules*, 2001, vol. 2, no. 2, pp. 483–490.
73. Tsai, G.J., Zhang, S.L., and Shieh, P.L., *J. Food Prot.*, 2004, vol. 67, no. 2, pp. 396–398.
74. Kumar, A.B., Gowda, L.R., and Tharanathan, R.N., *Eur. J. Biochem.*, 2004, vol. 271, no. 4, pp. 713–723.

75. Kittur, F.S., Vishu-Kumar, A.B., and Tharanathan, R.N., *Carbohydr. Res.*, 2003, vol. 338, no. 12, pp. 1283–1290.

76. Vishu-Kumar, A.B., Varadaraj, M.C., Lalitha, R.G., and Tharanathan, R.N., *Biochim. Biophys. Acta*, 2004, vol. 1670, no. 2, pp. 137–146.

77. Ilyina, A.V., Tatarinova, N.Yu., and Varlamov, V.P., *Proc. Biochem. Soc.*, 1999, vol. 34, no. 9, pp. 875–878.

78. Ilyina, A.V., Tikhonov, V.E., Albulov, A.I., and Varlamov, V.P., *Proc. Biochem. Soc.*, 2000, vol. 35, no. 6, pp. 563–568.

79. Ilyina, A.V, Varlamov, V.P, Gabdrakhmanova, L.A, Yusupova, D.V, and Albulov, A.I, in *Progress on Chemistry and Application of Chitin and its Derivatives*, Struszczyk, H., Ed., Lodz: Polish Chitin Soc., 2001, vol. 7, pp. 57–63.

80. Il'ina, A.V., Varlamov, V.P., Melent'ev, A.I., and Aktuganov, G.E., *Prikl. Biokhim. Mikrobiol.*, 2001, vol. 37, no. 2, pp. 160–163.

81. Il'ina, A.V., Tkacheva, Yu.V., and Varlamov, V.P., *Prikl. Biokhim. Mikrobiol.*, 2002, vol. 38, no. 2, pp. 132–135.

82. Il'ina, A.V. and Varlamov, V.P., *Prikl. Biokhim. Mikrobiol.*, 2003, vol. 39, no. 3, pp. 273–277.

83. Salyanov, V.I., Il'ina, A.V., Varlamov, V.P., and Evdokimov, Yu.M., *Izv. Akad. Nauk SSSR, Ser.: Biol.*, 2002, vol. 36, no. 4, pp. 699–705.

84. Evdokimov, Yu.M., Salyanov, V.I., Skuridin, S.G., and Dembo, A.T., *Izv. Akad. Nauk SSSR, Ser.: Biol.*, 2002, vol. 36, no. 4, pp. 706–714.

85. Yevdokimov, Yu.M. and Salyanov, V.I., *Liq. Cryst.*, 2003, vol. 30, no. 9, pp. 1057–1074.