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Chitosan and its derivatives: vectors in gene therapy

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The review deals with the use of chitosan and its derivatives as vectors for delivery of nucleic acids in gene therapy. Factors influencing the transfection efficiency of chitosan-based polyplexes (molecular weight, the degree of deacetylation, pH, type of chemical modification, type of transfected cells) are discussed. Particular attention is paid to methods of chemical modification of chitosan (covalent hydrophilic and hydrophobic modification and non-covalent modification), which are able to increase the transfection efficiency and to provide targeted delivery of nucleic acids.
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I. Introduction

Gene therapy is among the most promising and vigorously developing trends of modern medicine. The principal concept of this therapy is correcting defects caused by mutations in the DNA sequence or endowing cells with new functions. Considerable attention has been lately paid to the search for the optimal vehicle for drug delivery that would possess the whole set of properties required for clinical applications.[1 – 4]

Initially, viral systems have been used as vectors, and they are still most popular in gene therapy. Currently, about 70% of clinical trials carried out in gene therapy employ viral vectors.[1] Although viral systems are very efficient and are widely used in clinical practice, they have several drawbacks, including toxicity (the most severe case is insertional mutagenesis[5]), immunogenicity, inflammatory potential, low carrying capacity for the genetic material (the largest nucleic acid size is 34 000 bp), the cell specificity inherent in viruses and complicated industrial production.[6, 7]

Non-viral delivery systems are less immunogenic, eliminate the risk of insertional mutagenesis and accommodate larger amounts of nucleic acids; they are also much more economically feasible and safe in production.[8] It is also possible to increase the cell specificity of vectors by attaching the desired chemical sequences to the polymer.[9] An ideal non-viral agent for nucleic acid delivery must possess the following properties: form nanoparticles upon coupling with a nucleic acid, protect the nucleic acid from the action of nucleases, promote endocytosis into the target cells, efficiently escape from endosomes and lysosomes, release

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the nucleic acid at the site of action (but prevent the nucleic acid–polymer complex from too early decomposition) and possess low toxicity and relatively low production cost.[10]

Among non-viral systems, of most interest are complexes based on cationic lipids (lipoplexes) and polymers (polyplexes). Polyplexes based on synthetic polymers are efficient for transfection, but have rather high toxicity, resulting in cell death. Therefore, it appears pertinent to search for efficient vectors based on low-toxicity natural polymers, among which a prominent place belongs to chitosan, a non-toxic polyaminosaccharide possessing a unique set of physicochemical and biological properties. The pristine chitosan has some shortcomings for this application, in particular, low solubility in water at physiological pH and relatively low transfection efficiency.[11,12] However, easy chemical modification of chitosan makes it possible to obtain derivatives with the desired properties.

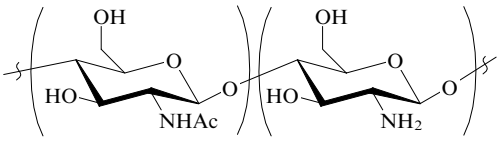
Targeted nucleic acid delivery systems are fabricated using non-modified chitosan of various molecular weights (MW) and degrees of deacetylation (DD). The transfection efficiency of chitosan can be improved by non-covalent modification *via* the formation of complexes with polyanions or by covalent modification using various functional groups for increasing the cationic density of chitosan, changing the hydrophilic–lipophilic balance and conferring the capability for targeted delivery.

In this review, we consider issues related to the targeted nucleic acid delivery by means of non-modified chitosan and discuss in detail the chemical strategies used for chitosan modification in order to enhance the transfection efficiency. The published reviews on this subject mainly deal with the biological and pharmacological aspects and barely concern the chemistry of chitosan and its derivatives.

II. Properties of chitosan

Chitosan (**1**) is the product of complete or partial deacetylation of chitin and represents a linear polysaccharide consisting of randomly distributed *N*-acetylglucosamine and glucosamine units. The proportion of glucosamine units (DD) varies in the range from 50% to 100%.[13] The chitosan amino groups have a relatively low basicity ($pK_a = 6.5$).[14] Chitosan is soluble in dilute aqueous solutions of many organic and mineral acids owing to protonation of the amino groups of glucosamine units, but it is insoluble in alkaline or neutral media.[15] It is noteworthy that the solubility of chitosan polymers depends also on the molecular weight: chitosans with low MW are more soluble.[16] The presence of a positive charges in acidic solutions allows chitosan to form complexes with negatively charged nucleic acids.[15]

Structure 1



1

Unlike many synthetic cationic polymers, chitosan is virtually non-toxic: the median lethal dose (LD_{50}) of chitosan is 16 g kg^{-1} for oral administration to mice (for comparison, the LD_{50} of NaCl is 3 g kg^{-1}).[17] According to *in vitro* studies, chitosan has a relatively low toxicity towards various cell lines.[18–20]

Oral administration of chitosan (up to 6.75 g day^{-1}) to healthy human volunteers demonstrated its safety and the absence of side effects.[21] The safe dose for intravenous injection into rabbits was 4.5 mg kg^{-1} day^{-1} (Ref. 22). Chellat *et al.*[20] studied the effect of chitosan–DNA nanoparticles on the induction of metalloproteinases and cytokines in THP-1 leukemia cells. The results demonstrated that chitosan–DNA nanoparticles did not induce the release of the pro-inflammatory cytokines, whereas the synthesis of a matrix metalloproteinase (MWP-9) substantially increased. In order to investigate the stability in the amniotic fluid *in vitro*, chitosan-based vectors for the fetal gene therapy were constructed.[23] Chitosan successfully protected plasmid DNA (pDNA) from enzymatic degradation, although it is prone to aggregation in the amniotic fluid.

Sgouras and Duncan[24] noted an increase in chitosan toxicity with increasing MW. However, later the toxicity was found to depend only on the polymer concentration, and the dependence on the polymer MW was disproved.[18] The chitosan cytotoxicity is also affected by the DD value: the toxicity increases with increasing DD.[25]

Chitosan molecules undergo enzymatic hydrolysis under the action of chitinases, chitosanases, *N*-acetylglucosaminidase, hexosaminidase and other non-specific hydrolytic enzymes, for example, lysozyme,[26] which is a constituent of many human biological fluids, in particular, it occurs in blood serum and leucocytes. Chitinase is also present in human tissues, being a part of body's protection system against pathogenic fungi.

Zhang and Neau[27] noted that chitosan is degraded by the bacterial flora that is normally present in human intestines, the polymer degradation being more pronounced for lower MW and DD chitosans. Tomihata and Ikada[28] performed *in vitro* and *in vivo* experiments demonstrating that the chitosan biodegradability increases with increasing proportion of acetamide groups in the polymer. Unlike chitin, fully deacetylated chitosan almost does not decompose. These data were later confirmed by Lavertu *et al.*[29]

The intermediate products of chitosan degradation include chito-oligomers and monomers, which can activate macrophages and fibroblasts.[30] As a result of complete hydrolysis by various enzymes, chitosan is converted to glucosamine and is metabolized to final products (urea, carbon dioxide and water).[31,32]

III. Factors affecting the delivery of nucleic acids by chitosan

The amino groups of chitosan are positively charged in acidic media and are capable of electrostatic interaction with negatively charged nucleic acids,[33] which makes chitosan a suitable vehicle for the delivery of nucleic acids, thus protecting them from nucleasing and increases the transfection efficiency.

However, the transfection efficiency of chitosan should be considered with allowance for the MW and DD. These characteristics influence the biological and physicochemical properties of the polymer and, finally, the efficiency of nucleic acid delivery into the cell. The DD of chitosan considerably affects a number of its properties such as the charge density, solubility and ordered structure.[28,34] Molec-

ular weight is an important parameter, which determines the size of the nanoparticles formed, the stability of polymer complexes with nucleic acids, the efficiency of cellular uptake of polyplexes and release of nucleic acids from the complexes to the cytoplasm and thus affects the overall transfection efficiency.

It should be noted that MW has an ambiguous influence on the transfection efficiency. There are examples in the literature describing the advantages of using chitosan of either high or low MW. For example, it was found in a number of works that the transfection efficiency and the stability of the DNA – chitosan system increase with increasing MW and DD.[29, 35] Kiang *et al.*[36] have shown that particle destabilization caused by a decrease in the DD leads to a decrease in the luciferase expression levels in the HeLa (cervical carcinoma), SW756 (squamous cell carcinoma of the cervix) and HEK293 (human embryonic kidney) cells. However, *in vivo* transfection into muscle tissue carried out by the same research team showed that chitosans with lower DD (70% and 62%) are twice as efficient in cell transfection as chitosan with DD = 90%.[37] According to this study, the results obtained for *in vitro* transfection do not always correlate with the results obtained in *in vivo* experiments.

Lavertu *et al.*[29] deem that the effect of DD on the properties of complexes should be considered together with other factors, for example, with the polymer MW. The highest expression level can be attained either by decreasing MW and increasing DD or by decreasing DD and increasing MW. This work demonstrates the importance of balancing the DD and MW for the preparation of stable nanoparticles that would have high capacity for transfection. According to Köping-Höggård *et al*,[32] for the preparation of stable complexes capable of cell transfection, the DD of chitosan should be $>65\%$.

MacLaughlin *et al.*[38] reported that with decreasing MW, the polyplex particle size also decreases. These results were unexpected, because earlier it had been considered that chitosans with high MW provide more efficient packing of plasmids and, hence, complexes of smaller size are formed in this case.

In several publications,[25, 36, 39] the increase in the MW of chitosan was found to be beneficial for the transfection efficiency of complexes, which is attributable to enhanced stability of the obtained nanoparticles and better protection of nucleic acids from nuclease-induced degradation. However, these complexes have high polydispersity and tend to form aggregates.[40] Furthermore, chitosan with high molecular weight ($MW > 1.5\times10^5$) is less soluble than the low-MW chitosan.

Conversely, Weecharangsan *et al.*[41] found that lower-MW chitosans (2×10^4 , 4.5×10^4 and 20×10^4) transfect the CHO-K1 cell culture (Chinese hamster ovary cells) more efficiently, whereas the transfection efficiency of high-MW chitosan ($MW = 4.6\times10^5$) is comparable with that of free DNA. The authors attributed this to the fact that nucleic acids are faster released to the cytoplasm if the complexes are formed by low-MW chitosan.

It follows from the above that in the development of a vector, it is necessary to maintain a balance in the selection of the polymer molecular weight. The high-MW chitosan ($MW > 1\times10^5$) forms more stable complexes with nucleic acids, better protects them from enzymatic degradation and suppresses fast release of the genetic material. However, at the site of potential action, the nucleic acids should dissociate from the complexes and, in this case, low-MW chitosans show better results.

However, high-MW chitosan has a number of drawbacks such as low solubility at a physiological pH and the slow release of the nucleic acid from the complex.[32, 33, 42] Bloomfield[43] and Strand *et al.*[44] found that low-MW chitosan facilitates the formation of stable soluble polyplexes without aggregation, and the polymer solution has a low viscosity.

Akbuğa *et al.*[45] prepared DNA – chitosan nanoparticles by precipitation for the transfection into MAT-LyLu rat prostate carcinoma cells. The use of low-MW chitosan in this experiment gave better results than the use of high-MW chitosan.

Yang *et al.*[46] investigated the capacity of chitosan for the delivery of DNA vaccine *via* mucosa. The results they obtained demonstrate that low-MW chitosan has a lower binding affinity to DNA, but provides higher transfection efficiency than the high-MW chitosan, and that an intranasal vaccination with the low-MW chitosan – DNA complex elicits significant systemic immune responses.

Köping-Höggård *et al.*[40] demonstrated that chitosan with MW = 3000 – 4000 forms stable complexes and induces higher gene expression than high-MW chitosan. The researchers[32, 40, 42] attempted to identify the optimal MW of oligochitosans for which the protective properties inherent in high-MW chitosans are retained. By means of gel electrophoresis, it was shown that the oligochitosan with the degree of polymerization of 24 completely binds nucleic acids. The transfection efficiency of the resulting complexes into the HEK293 cell culture was higher than that for chitosan with $MW = 4\times10^5$ and comparable with that of polyethyleneimine (PEI).

It was also found that the transfection stability and efficiency of chitosan polyplexes depend not only on MW and DD but also on the pH of the transfection medium[29, 40, 42, 47 – 50] and nucleic acid concentration.[29, 44, 47, 51] At a physiological pH, polyplexes are shaped like coils, while in an acidic medium they occur as globules. The number of globular polyplexes increases as the pH decreases from 6.5 to 3.5, which results in high transfection efficiency. According to a number of studies, transfection of chitosan-DNA complexes is more efficient for pH of the culture medium <7 than at the physiological pH. These results are attributable to low stability of the chitosan – DNA complexes at physiological pH and aggregation of the complexes with subsequent positive charge neutralization, which decreases the interaction with the negatively charged cell membranes.[52, 53] Guliyeva *et al.*[49] reported that DNA is released from chitosan nanoparticles much faster in an acid medium than at pH 6.5, while the released amount is greater at pH 4.5. Moreover, it was found that the stability and efficiency of transfection of chitosan polyplexes is affected by not only MW and DD, but also by the pH of the transfection medium.[29, 40, 42, 47 – 50]

Chitosan and its derivatives are employed for transfection of various cell cultures, with the transfection efficiency being strongly dependent on the chosen type of cells. This may be related not only to the differences in the composition and preparation procedure of the chitosan – DNA interpolymer complexes, but also to different

natures of the cells (receptor types, cell membrane charge, enzymatic composition and so on). The HEK293 cell line is used most often for chitosan-mediated transfection. It is for these cells that the best results on DNA uptake were achieved.[29, 32, 44, 54, 55]

Leong *et al.*[56] demonstrated that the HEK293 cancer cell culture is transfected by chitosan complexes more efficiently than HeLa. Corsi *et al.*[57] also reported a good ability of chitosan to transfect HEK293 cells — the transfection efficiency of polyplexes was higher in HEK293 than in MSC (mesenchymal stromal cells) or MG63 (human bone osteosarcoma cells). Dastan and Turan[58] ascertained that HEK293 cell culture is transfected by chitosan, unlike HeLa and Swiss3T3 (mouse embryonic fibroblasts). Mao *et al.*[59] reported transfection by chitosan into the cell cultures HEK293, HeLa, IB-3-1 (bronchial epithelial cells) and 9HTEo (human epithelial cells). The highest transfection efficiency was observed for HEK293 and a somewhat lower efficiency was found for IB-3-1 cells. The transfection of the 9HTEo cells was twice lower than that of HEK293 cells, and the HeLa cells showed the lowest transfection level.

Small interfering RNAs (siRNAs) are being currently studied as a new tool for the therapy of diseases related to gene expression.[60] However, siRNA tends to be highly prone to degradation and to be poorly uptaken by cells.[61, 62] Thus, the optimal system for the delivery of siRNA is still an open issue. Howard *et al.*[63] reported that chitosan – siRNA nanoparticles suppress the expression of the green fluorescent protein in the H1299 human non-small cell lung carcinoma cells and in the mouse peritoneal macrophages and that the same results are obtained upon intranasal administration to transgenic mice. Malmo *et al.*[64] used flow cytometry and transfection of the H1299 cells to demonstrate that the gene expression is suppressed most efficiently when completely deacetylated chitosan with the average degree of polymerization of 100 – 300 is used.

IV. Non-covalent modification of chitosan

In order to increase the solubility, the transfection efficiency and the stability of chitosan complexes in blood serum, non-covalent modification of chitosan is used. The modification consists of ionic gelation or electrostatic interaction with polyanions. In particular, chitosan complexes with non-toxic tripolyphosphate (TPP), which easily forms gels, were obtained in the presence of DNA; the complexes showed high transfection efficiency.[65 – 67] The siRNA delivery systems were developed based on chitosan gels in the form of TPP hydrochloride and glutamate; the transfection efficiency increased with increasing chitosan MW up to $(2.7 – 4.7) \times 10^5$. Subsequently, effective delivery systems for other types of RNA were also developed, *e.g.*, for small hairpin RNA.[68]

Natural polyanions — hyaluronic and poly(γ-glutamic) acids — have also been used as complexing agents. The complexes based on hyaluronic acid showed high transfection efficiency, low toxicity, easy biodegradability and high biocompatibility.[69] Later, efficient nanoplatforms for nucleic acid delivery containing polyethylene glycol (PEG)-modified chitosan and hyaluronic acid were developed.[70] Effective systems for the delivery of siRNA[61,71,72] and DNA[73] were fabricated based on chitosan complexes with poly(γ-glutamic acid).

V. Covalent modification of chitosan

V.1. Introduction of cationic substituents

Most shortcomings of chitosan in the nucleic acid delivery application are associated with its rather low cationic density.[74 – 76] For example, the increase in the cationic density of chitosan is the key factor affecting endosomal rupture, which markedly increases the transfection efficiency. A number of polymer modification routes have been proposed to overcome this problem. In particular, moieties of urocanic acid,[77, 78] imidazole,[79, 80] triethylamine,[81] spermine and PEI[82 – 87] were introduced into chitosan to increase the charge.

It was shown[77, 88, 89] that chitosan modified with urocanic acid (Scheme 1, pathway *a*) efficiently delivers the gene encoding the p53 protein, which inhibits the growth of human hepatoblastoma and induces apoptosis. High transfection efficiency of urocanyl-chitosan into the HEK293T cells was also found; it was shown that urocanic acid groups form complexes with DNA, thus providing efficient protection of nucleic acids from the action of enzymes.[78]

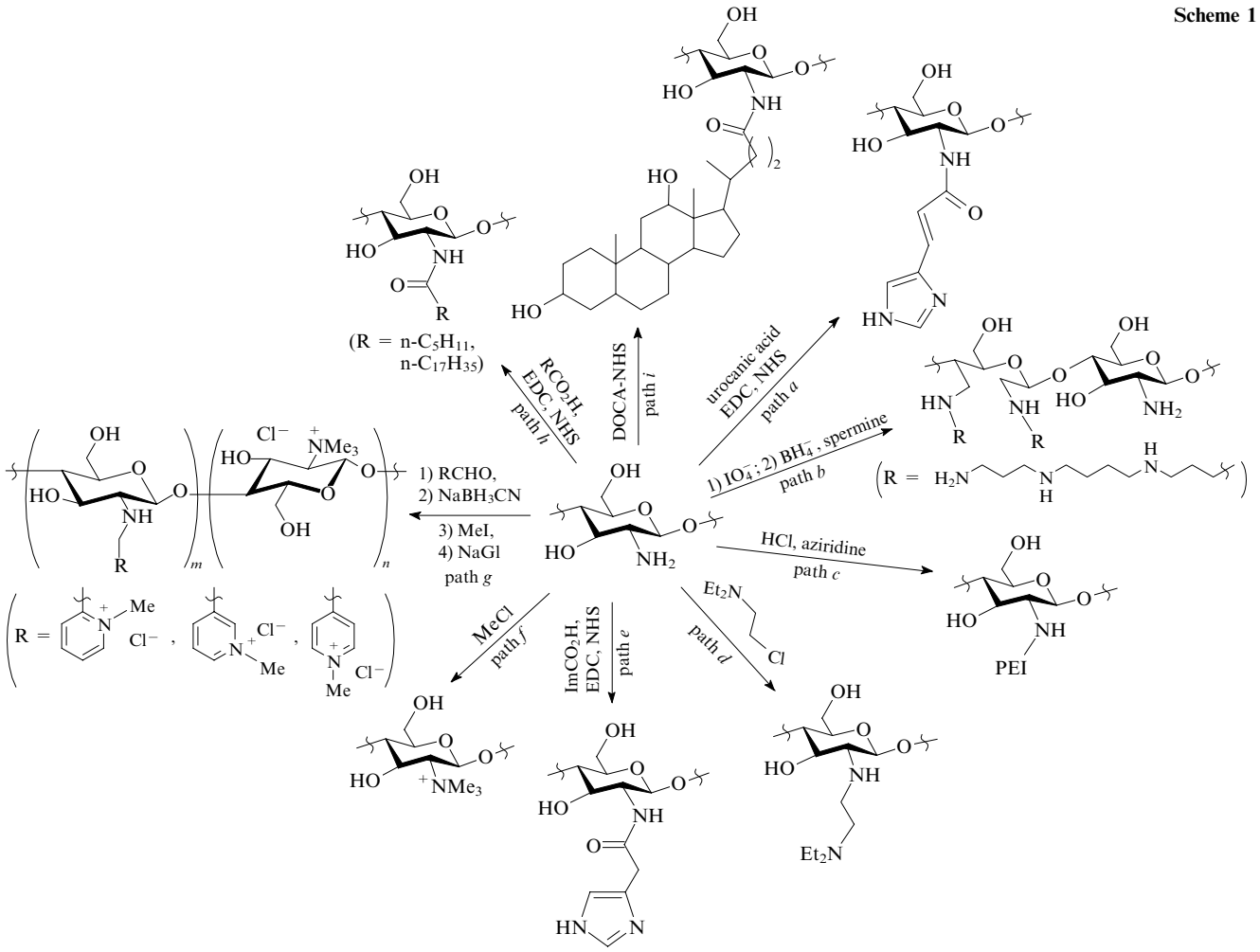
Chitosan contains numerous primary amino groups. During endosome maturation, the amino groups are gradually protonated, which in turn induces an influx of negatively charged chloride ions through the vesicle membrane and increases the ionic strength of the endosome contents (proton sponge effect). The above-listed processes induce the osmotic swelling and rupture of endosomes. This effect was used for the design of nucleic acid delivery systems based on urocanic acid-modified chitosan. It was shown that the imidazole ring of urocanic acid present in the modified chitosan facilitates the efficient endosome rupture by the proton sponge mechanism.[79, 80]

For increasing the cationic density, a spermine chitosan derivative was obtained by the reaction of periodate-oxidized chitosan with spermine (see Scheme 1, pathway *b*). This derivative showed high transfection efficiency and low toxicity *in vitro* and *in vivo*.[90]

Polyethyleneimine is the most efficient non-viral vector based on cationic polymers, as it is readily soluble and has high cationic density;[91] however, it is toxic. Furthermore, PEI is not biodegradable, which also restricts its use.[86] For increasing the transfection efficiency and decreasing the toxicity, PEI was conjugated with chitosan. In particular, a chitosan-PEI conjugate synthesized by cationic polymerization of aziridine with water-soluble oligochitosan (MW = 3400) (see Scheme 1, pathway *c*) possesses a high capacity for complex formation with DNA, low toxicity and high transfection efficiency *in vitro* and *in vivo*.[82]

Lu *et al.*[83] synthesized a chitosan copolymer with low-MW PEI by the Michael reaction with succinyl- and maleyl-chitosans. The resulting polymers were readily soluble in water and exhibited higher efficiency of transfection of the HEK293T and HeLa cells than was observed with the non-modified PEI. The approach based on a combination of PEI with chitosan was further developed. Zhang *et al.*[92] modified the chitosan – PEI graft copolymer with arginine. This strategy was substantiated by the fact that owing to the positive charge, arginine promotes the penetration of complexes through the cell membrane. This modification improved the transfection efficiency as compared with the chitosan – PEI copolymer and decreased the toxicity to the level of non-modified chitosan. Grafting of PEI to chitosan was also accomplished by means of 1,1′-carbonyldiimida-

Scheme 1



EDC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS is *N*-hydroxysuccinimide, DOCA-NHS is the NHS ester of deoxycholic acid, $ImCO_2H$ is imidazol-4-ylacetic acid.

zole.[86] The chitosan – PEI graft copolymer obtained by the imime reaction was successfully used for transfection of siRNA into the A549 human lung cancer cells.[93] Zhao *et al.*[94] attached PEI to chitosan through a disulfide bond, which increased the transfection efficiency to a level comparable with that of the commercial Lipofectamine agent.

The modification with diethylamine (see Scheme 1, pathway *d*) or imidazole derivatives (pathway *e*) substantially increases the cationic density of chitosan; furthermore, polyplexes efficiently escape from endosomes and the transfection efficiency increases.[70, 80]

V.2. Hydrophilic modification of chitosan

The major strategy used to increase the chitosan solubility is the hydrophilic modification, which includes quaternization (see Scheme 1, pathway *f*) and PEG modification. Quaternization of chitosan increases the positive charge density of the polymer, which leads to more efficient binding of nucleic acids to the polymer[95 – 102] and increases the paracellular permeability.[103]

The major method for chitosan quaternization is complete alkylation (for example, trimethylation) of the amino group (see Scheme 1, pathway *f*). Polyplexes based on trimethylated chitosan derivatives possess high solubility and, as a rule, show higher transfection efficiency than non-modified chitosan. High transfection efficiency was observed for the polyplexes based on trimethylated chitosan with grafted poly(ε-caprolactone).[104] The transfection efficiency of the trimethylated chitosan oligomer (TCO) and the trimethylated chitosan polymer (TCP) with a lower MW compared with that of PEI was also investigated. The TCO and TCP with different degrees of quaternization showed higher transfection efficiency than PEI into MCF-7 breast cancer cells and monkey kidney fibroblasts;[98] a high degree of quaternization of chitosan entailed a decrease in the toxicity.[103, 105, 106] Germershaus *et al.*[105] additionally modified TCO with PEG, which resulted in even further increase in the solubility and a decrease in the toxicity.

Quaternized derivatives can also be obtained by introduction of groups containing a quaternary nitrogen atom into chitosan. For example, the treatment of chitosan with trimethyl(2,3-epoxypropyl)ammonium chloride yields *N*-(2-hydroxypropyltrimethylammonio)chitosan chloride.[107] This derivative possesses high solubility in water and gives a much higher transfection efficiency into the MSC cells than non-modified chitosan.[108] The introduction of *N*-methylpyridine moieties in a similar way also enhances the solubility and transfection efficiency of chitosan derivatives. The transfection efficiency was found to depend on the position of the side chain connecting the *N*-methylpyr-

idine moiety with the chitosan backbone. The best results were observed when the pyridine ring was attached *via* position 4 (see Scheme 1, pathway *g*).[109]

V.3. Hydrophobic modification of chitosan

The introduction of hydrophobic substituents into the polymeric vector gives certain benefits in comparison with non-modified chitosan polyplexes, for example, facilitates the DNA dissociation inside the cell and provides efficient protection from enzymatic degradation and enhanced penetration across the cell membrane.[110 – 112] Grafting of hydrophobic moieties to chitosan results in the formation of an amphiphilic polymer.[113] The compounds used to introduce hydrophobic moieties include stearic and caproic acids, alkyl bromides,[74, 75, 110, 114, 115] bile and deoxycholic acids[116, 117] and 5β-cholanic acid.[76] For example, nanoparticles based on linolenic or oleic acid-modified chitosan form polyplexes characterized by high transfection efficiency and fast release of DNA.[118]

Fatty acid moieties are usually grafted to the chitosan backbone by the carbodiimide method (see Scheme 1, pathway *h*). These chitosan derivatives tend to form micelles in aqueous solutions; the transfection efficiency of these micelles proved to be 15% higher.[75] A high transfection efficiency was found for micelles based on chitosan with grafted stearic acid and poly(ethylene glycol)-β-poly(γ-glutamic acid) residues and DNA[119] and for micelles based on *cis*-aconitate-modified chitosan with stearic acid residues.[120] Dodecylated chitosan – DNA nanoparticles with an average diameter of 90 – 180 nm were prepared for the local gene delivery by coating endovascular stents.[115] A high level of green fluorescent protein expression was detected in the cells grown on the stent surface.

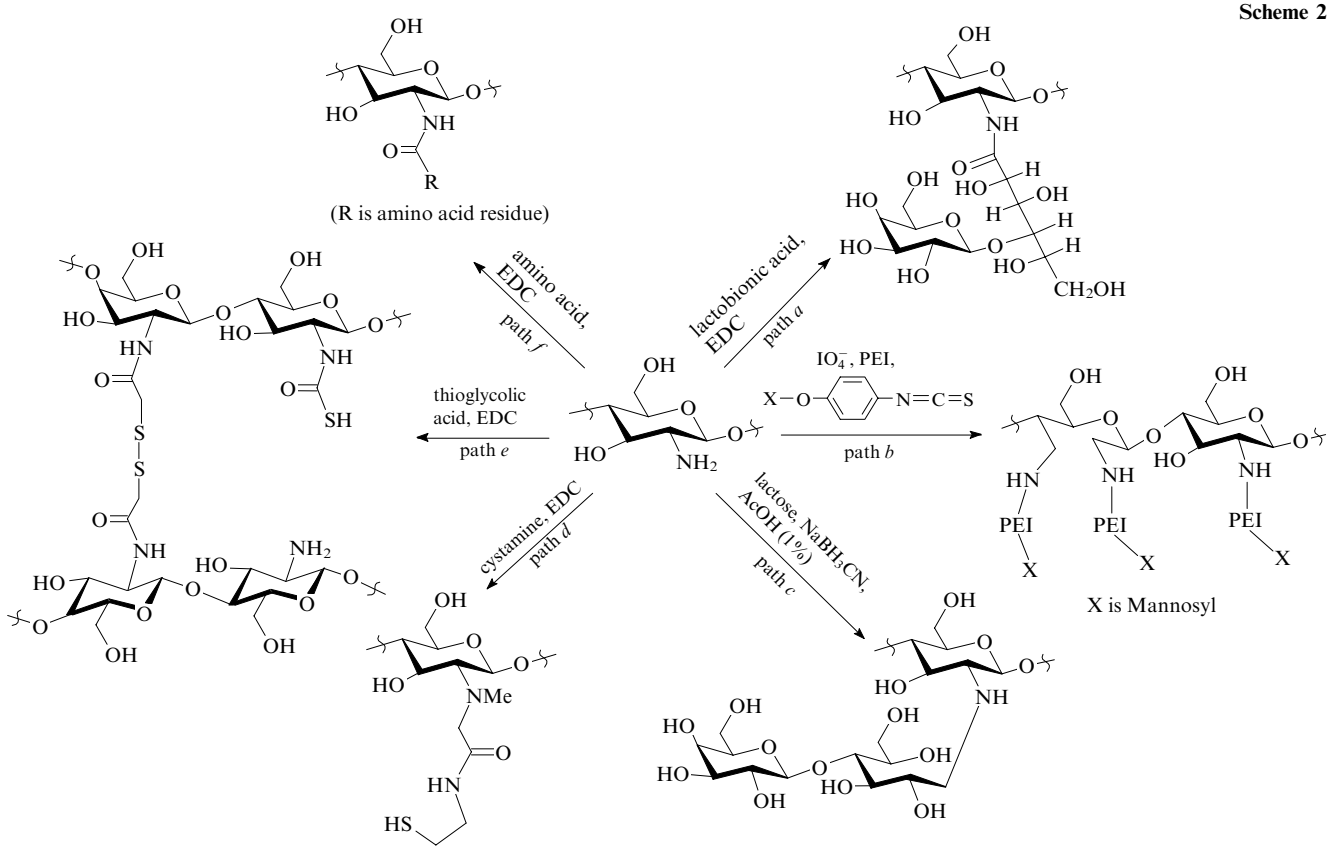
Chitosan derivatives with grafted caproic acid were synthesized to study the effect of the degree of substitution by hydrophobic groups on the capacity for DNA binding, cellular uptake, transfection efficiency and biocompatibility.[110] The transfection efficiency of these nanoparticles proved to be high, the highest efficiency observed for a degree of substitution ranging from 5% to 25%.[110] The polyplexes based on hydrophobic chitosan derivatives obtained by *N*-alkylation of butyl, octyl, dodecyl and hexadecyl bromides demonstrated high transfection efficiency.[114] Chae *et al.*[116] reported that chitosan nanoparticles with grafted deoxycholic acid with a degree of substitution of 5% showed a high level of gene transfection efficiency. A considerable enhancement of *in vivo* transfection was also found for chitosan nanoparticles with grafted 5β-cholanic acid (see Scheme 1, pathway *i*).

V.4. Modification of chitosan by ligands that confer targeted delivery capability

Modification of chitosan by ligands that confer a capability for targeted delivery can substantially enhance the transfection efficiency. Compounds used as these ligands include galactose,[121 – 125] mannose,[126 – 129] lactose,[130] folates,[131 – 133] transferrin[134] and arginyl-glycyl-aspartic acid (RGD).[135]

The hepatocyte surface bears asialoglycoprotein receptors (ASGPR) for galactose.[136, 137] Polyplexes based on water-soluble galactosylated chitosan (Scheme 2, pathway *a*) possess high transfection efficiency[124] and induce a high level of luciferase expression in human hepatocytes (HepG2).[122] In order to increase water solubility and transfection efficiency into HepG2 cells, a chitosan – PEI copolymer with grafted galactose has been prepared.[138]

Scheme 2



Macrophages are provided with mannose receptors, which can be used for mannose-mediated endocytosis of polyplexes. The DNA complexes with chitosan bearing grafted mannose residues (see Scheme 2, pathway *b*) showed high transfection efficiency in CT-26 carcinoma cells.[129]

Various cancer cells (ovarian, lung, intestinal and kidney cancer) have rather large numbers of folate receptors expressed on the surface. Polyplexes based on chitosan conjugated with folic acid showed a high transfection efficiency into the HEK293 cells.[131 – 133]

The surface of proliferating malignant cells bears large numbers of transferrin receptors. Therefore, transferrin is used as a ligand for the targeted delivery of anticancer drugs.[139, 140] This approach was utilized to prepare polyplexes based on chitosan conjugated with transferrin. The presence of the transferrin moiety markedly increased the transfection efficiency of polyplexes into the Caco-2 cells (human colon adenocarcinoma).[134]

The tripeptide RGD was also used as a ligand for targeted nucleic acid delivery, as it interacts with the anb3 receptor expressed in large numbers in cancer cells. In particular, high transfection efficiency and strong suppression of gene expression were observed for nanoparticles of chitosan conjugated with RGD.[135]

A considerable enhancement of transfection was attained by using lactose as a ligand for targeted delivery into the HepG2 cells (see Scheme 2, pathway *c*).[130]

V.5. Miscellaneous methods for the covalent modification of chitosan

The introduction of thiol groups serves, as a rule, for increasing the stability of polyplexes before penetration into the cell, improving the release of nucleic acids inside the cell and increasing mucoadhesiveness through the formation of disulfide bridges between the polymer thiol group and mucin glycoproteins.[141 – 145] For example, the introduction of thiol groups into trimethylated chitosan *via* cystamine modification (see Scheme 2, pathway *d*) considerably enhances the extracellular stability of the nucleic acid – polymer complex and facilitates the RNA release inside the cell. These polyplexes show high transfection an efficiency into H1299 cells (human lung carcinoma) and enhanced stability in the presence of anionic macromolecules such as hyaluronic acid.[141] The introduction of thioglycolic acid into chitosan (see Scheme 2, pathway *e*)[146, 147] or 2-iminothiolate[148] improves the transfection behaviour and increases the intracellular release.

The modification of chitosan with peptides and/or amino acids (see Scheme 2, pathway *f*) is also utilized to provide the targeted delivery. In particular, conjugation with positively charged amino acids facilitates penetration into cells. The conjugation with cysteine is a method for introduction of thiol groups, conjugation with arginine increases the hydrophilicity and conjugation with nonpolar amino acids (alanine, valine, leucine and isoleucine) results in increasing hydrophobicity, which finally boosts the intracellular penetration and release and enhances the transfection.[142, 149 – 151]

The material covered in this review demonstrates that the possibility of fine chemical modification of chitosan opens up the way for the design of new, safe, non-toxic nonviral nanoplatforms with high transfection efficiency for nucleic acid delivery into the cells and that studies of the biological activity and mechanisms of action of these structures would undoubtedly contribute to the progress in medicinal chemistry, pharmacology and related sciences.

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