Receptor-Mediated Gene Delivery Using Chitosan Derivatives *in Vitro* and *in Vivo*

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Summary: The development of an efficient targeted gene delivery system into cells is an important strategy for the advancement of gene therapy. The targeted gene delivery system is especially important in non-viral gene transfer which shows the relative low transfection efficiency. And it also opens the possibility of selective delivery of therapeutic plasmids to specific tissues. Chitosan has been investigated as a non-viral vector because it has several advantages such as biocompatibility, biodegradability and low toxicity with high cationic potential. However, low specificity and low transfection efficiency of chitosan need to be overcome prior to clinical trial. In this review, we focused on the specific ligand modification of chitosan for enhancement of cell specificity and transfection efficiency via receptor-mediated endocytosis *in vitro* and *in vivo*.

Keywords: chitosan; ligand modification; non-viral vector; receptor-mediated gene delivery; transfection efficiency

Introduction

Gene therapy is a powerful treatment for inborn and acquired diseases. The development of safe and effective gene delivery systems is a great challenge to make the human gene therapy a reality. Viral vectors have been commonly employed due to the high transfection efficiency, however, their application to the human body is often frustrated by immunogenicity, potential infectivity, complicated production, and inflammation.[1] Non-viral vectors have been widely proposed as safer alternatives to viral vectors by reason of unique advantages such as less immune reaction against repeated administration, ease of synthesis, cell/tissue targeting, unrestricted plasmid size, and low cost.[2] Among non-viral vectors, chitosan has been considered to be a good candidate as one of gene carriers, since it is already known as a biocompatible, biodegradable, and low toxic material with high cationic potential.[3] and it can spontaneously form microspheres via complex coacervation.[4] However, low specificity and low transfection efficiency of chitosan need to be overcome for clinical trial. It has been reported that several factors such as degree of deacetylation[5] and molecular weight of the chitosan[6,7], pH[8,9], serum[8,10], charge ratio of chitosan to DNA[11] and cell type[12,13] affected transfection efficiency. In this paper, specific ligand modification of chitosan for enhancement of cell specificity and transfection efficiency via receptor-mediated endocytosis *in vitro* and *in vivo* were reviewed.

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Chemical Modification of Chitosan for Hepatocyte-Targeting

The liver is an attractive target tissue for gene therapy due to its large size, metabolic

capacity and rich blood supply that can be useful for the delivery of genes to the liver as well as for the distribution of gene products from the liver to the systemic circulation. Above all, mammalian hepatocytes are the only cells that possess large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins (ASGP).[14] The asialoglycoprotein receptors (ASGP-R) are known to be present only on hepatocytes at a high density of 500,000 receptors per cell, and retained on several human hepatoma cell lines.[15] This receptor system can not only bind galactose-containing ligands, but can internalize them within membrane-bound vesicles or endosomes.[16]

Firstly, trimethylated chitosan bearing antennary galactose residues as the chitosan derivatives was checked for specific targeting to HepG2 cells after forming complexes with DNA.[17] The conjugates had an inclination to increase the cellular recognition ability with an increase of galactose residues in themselves, indicating that the conjugates were specifically internalized via the ASGP-R on the HepG2 cells. Gao *et al.* reported that galactosylated low molecular weight chitosan vectors transfected gene into HepG2 selectively via the receptor-mediated endocytosis pathway and the transfection efficiency was influenced by the galactosylation degree and N/P ratio in addition to the modified chitosan had relatively low cytotoxicity.[18] Kim *et al.* coupled lactobionic acid bearing galactose to water-soluble chitosan (WSC) for hepatocytes specificity as shown in Figure 1 because WSC increase the stability of chitosan in water and decrease the cytotoxicity induced by acetic acid.[19] As shown in Figure 2, GC/DNA complex showed much higher transfection efficiency compared with WSC/DNA complex on HepG2 whereas the luciferase activity of WSC and GC/DNA complex did not show any difference on HeLa cells that have no ASGP-R, indicating that galactose ligand attached on GC played a great role to recognize the ASGP-R. Also, as shown in Figure 3, the transfection of GC was highly inhibited in the presence of the galactose, indicating that GC/DNA complexes were transfected by receptor-mediated endocytosis.[19]

However, the transfection efficiency of GC was not good enough for the further application. So to enhance the gene transfer

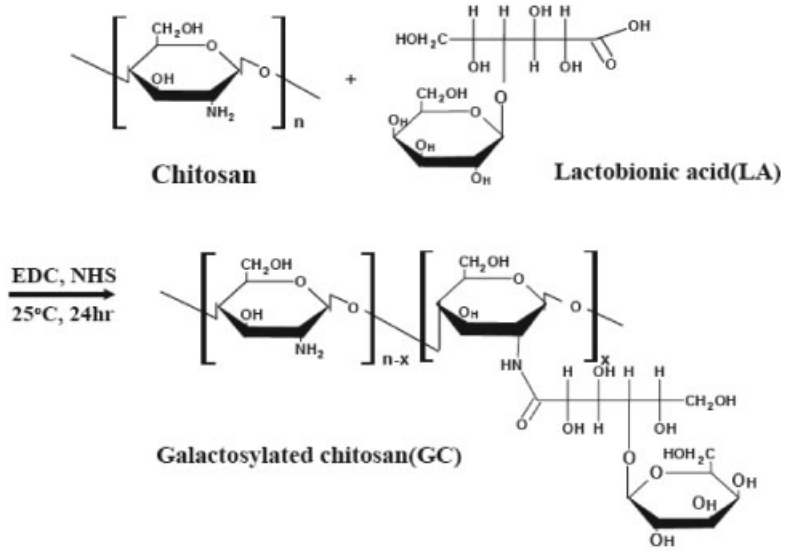

Figure 1.
Synthesis scheme of GC. Source: From Ref. [19].

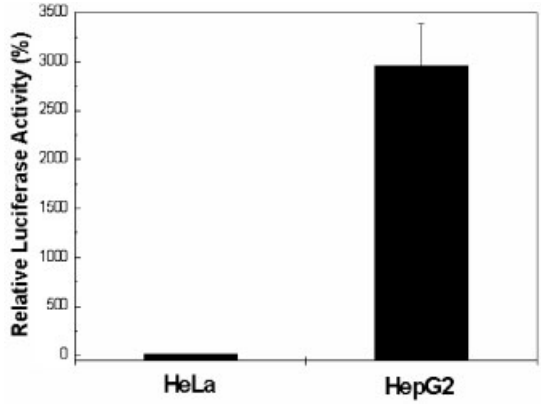

Figure 2.
Luciferase activity of GC/DNA complex at charge ratio of 10 normalized by that of WSCS/DNA complex on HepG2 and HeLa cells (n = 3). Source: From Ref. [19].

ability of GC, polyethylenimine (PEI) known as the most efficient gene carrier among the polymeric non-viral vectors was combined to GC/DNA complex.[20] PEI 10K which has the less cytotoxic effect compared with PEI 25K was combined to GC/DNA complex before transfecting into cells. The synergism appeared when the plasmid DNA was partially complexed but not fully condensed with GC before adding PEI. And we found that the luciferase activity of GC was markedly enhanced when PEI was combined to the GC/DNA complexes according to the increase of amount of added PEI. As shown in Figure 4A, GC/DNA complex showed much higher luciferase activity than WSC/DNA complexes in HepG2 even after addition of PEI. Also, there was not much difference in transfection efficiency between GC/DNA complex and WSC/DNA one in HeLa cells which have no ASGP-R, indicating that galactose ligand attached on GC played a great role to recognize the HepG2 (Figure 4B). To support these results, the blocking experiments

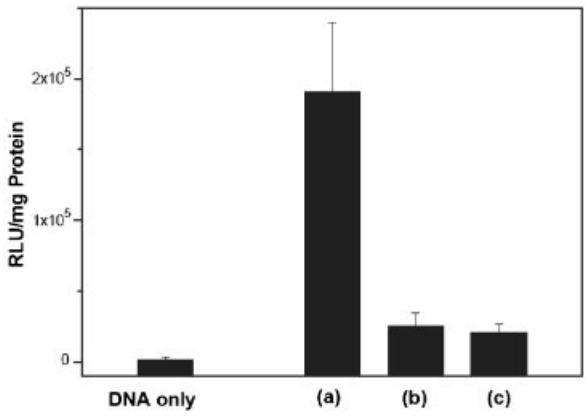

Figure 3.
Competition assay of GC/DNA complexes prepared at charge ratio of 10 by adding galactose [(a) 0 mM, (b) 5 mM and (c) 20 mM] as a competitor of galactose in GC (n = 3). Source: From Ref. [19].

were carried out by preincubation of galactose before treating with both WSC/DNA and GC/DNA complex combined with PEI. And then, we found that only GC/DNA complex was highly inhibited by an addition of galactose, indicating that GC/DNA complex still had the behaviour of galactose-mediated gene transfer even after combination with PEI (Figure 4C). In other words, it can be said that the uptake of GC/DNA complex combined with PEI was achieved via ASGP-R, and the PEI facilitated DNA release from the endosomal compartment via proton sponge effect.

Also, GC/DNA complex combined with PEI showed much decrease of cytotoxicity compared with PEI/DNA complex even though the same amount of PEI was used in both experiments as shown in Figure 5.

Chemical Modification of Chitosan for Antigen Presenting Cell-Targeting

The regulation of immunity is a major goal in treatment and prevention of viral infections, cancer and autoimmune disease.[21] For a successful modulation of immunity, antigens have to be presented by antigen presenting cells (APCs) that can activate the effector cells of the immune defense, the naïve T-cells and the B-cells.[22] These specialized cells include macrophages, B lymphocytes, and dendritic cells (DCs), and the most powerful of which is the DCs that can stimulate the primary immune responses and subsequently establish the immunologic memory.[23] Also, DCs have several innate functions, such as the production of large amount of protective cytokines and the activation of natural

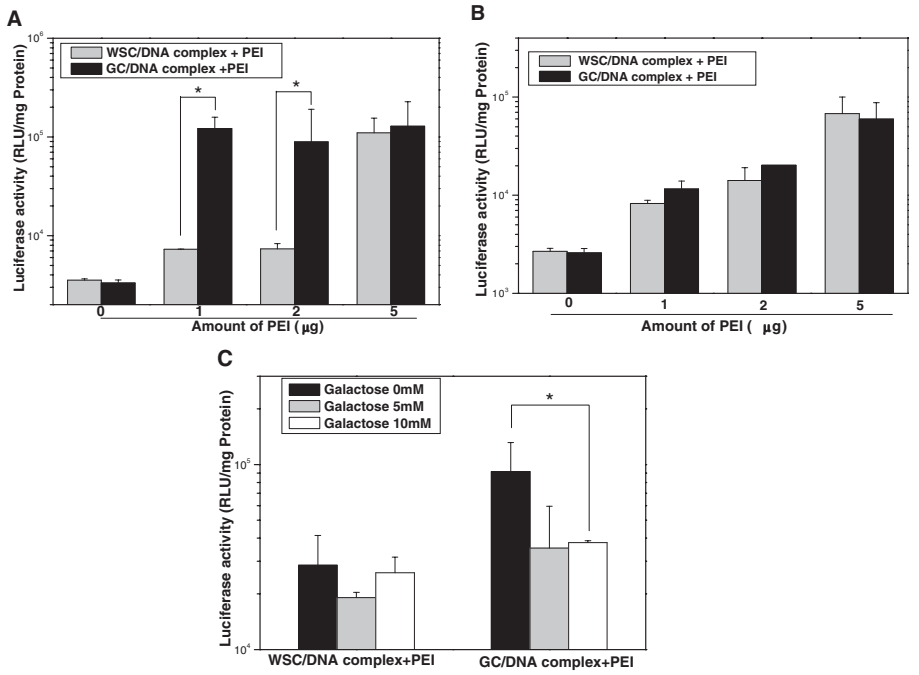

Figure 4.

Effect of PEI on transfection by WSC/DNA and GC/DNA complex.Luciferase activities of WSC/DNA and GC/DNA complexes on HepG2 (A) and HeLa cells (B) were studied at various amount of PEI added. Also, blocking experiment with addition of various amounts of galactose for inhibition was performed (C). Complexes were prepared at charge ratio 10. Transfection was performed with 1 μg of pGL3-control plasmid, and the amount of PEI was used as weight per 1 μg of DNA. In blocking experiments, the amount of added PEI was 2 μg. These data are expressed as relative light unit (RLU) of luciferase activities per mg of total cell protein (mean ± SD, $n=3$, $^{*}P < 0.05$) Source: From Ref. [20].

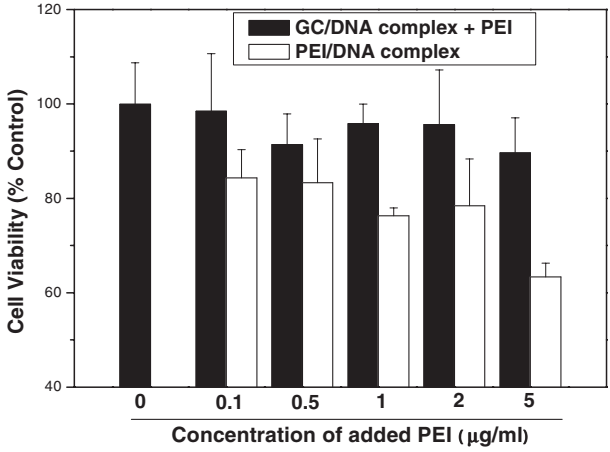

Figure 5.
Cytotoxicity of GC/DNA complexes combined with PEI and PEI/DNA complexes. HepG2 was seeded at a density of 1.5×10^4cells/well in 96-well plate. Complexes were added and cells were incubated with polymers for 48 h and 20 μl of CellTiter 96 AQ_{ueous} One Solution Reagent was added to each well. After further incubation period of 4 h, the absorbance was then read at 540 nm using a microplate reader ($n = 5$). Source: From Ref. [20].

killer (NK) cells, in addition, DCs provide a direct link between innate and adaptive immunity.[24] Strategies to genetically manipulate macrophages and DCs have been investigated in animal models and in human clinical trials for tumor vaccination, protective immunity against infection, subversion of autoimmune responses, and transplant tolerance.[25,26] It is well known that APCs such as macrophages and immature DCs express high levels of mannose receptor that are used for endocytosis and phagocytosis of a variety of antigens that expose mannose.[27,28] Therefore, the attachment of mannose residues to vectors for cell-selective gene transfection can induce the efficient gene delivery to APCs such as macrophages and DCs.

Kim *et al.* prepared mannosylated chitosan (MC) as shown in Figure 6 to induce the receptor-mediated endocytosis for targeting into APCs.[29]

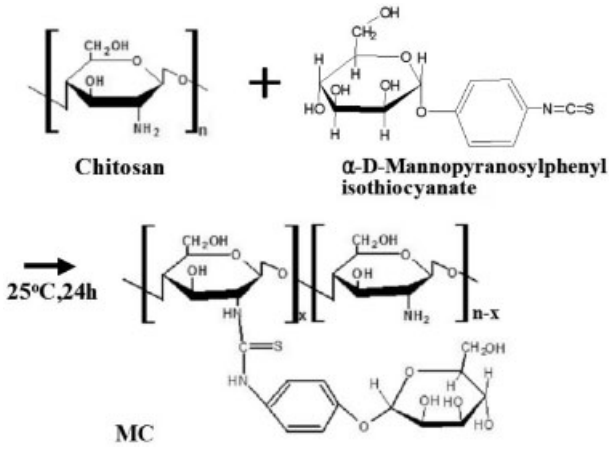

Figure 6.
Synthetic scheme of mannosylated chitosan. Source: From Ref. [29].

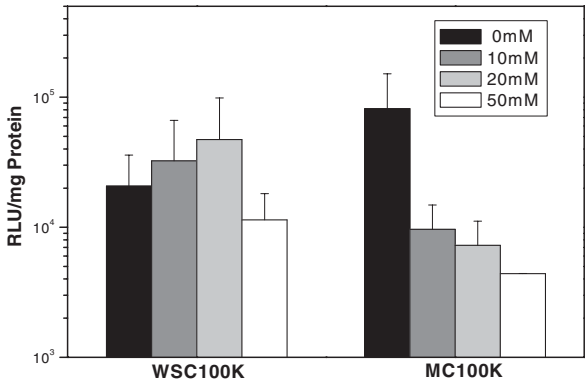

Figure 7.
Luciferase activities of WSC/DNA and MC/DNA complexes on Raw 264.7. Blocking experiment with addition of various amounts of mannose for inhibition was performed ($n = 3$). Source: From Ref. [29].

MC showed great ability to form complexes with DNA and showed suitable physicochemical properties for gene delivery system. MC/DNA complexes showed well-formed spherical shape and compact structure, and the particle sizes were less than 150nm. MC transfection represents a targeted delivery system that capitalizes on gene transfer by receptor-mediated endocytosis via surface-bound mannose receptor that is highly expressed on macrophages and DCs. Our results clearly demonstrated that gene transfer by MC was mannose receptor dependent because mannose could block the binding in a concentration-dependent manner in Raw 264.7 macrophage cell line as shown in Figure 7. Also, we compared the function of DCs to produce cytokines after transferring IL-12 gene by using chitosan and MC. IL-12 is well known to stimulate the proliferation of NK and T cells as well as augmenting their cytolytic activity, and induce the production of IFN-γ.[30] MC/DNA complex was found to be more efficient in induction of INF-γ from DCs compared to chitosan complex after delivering IL-12 gene into DCs as shown in Figure 8. Hashimoto *et al.* also coupled formylmethyl mannoside prepared by reductive ozonolyzation of allyl

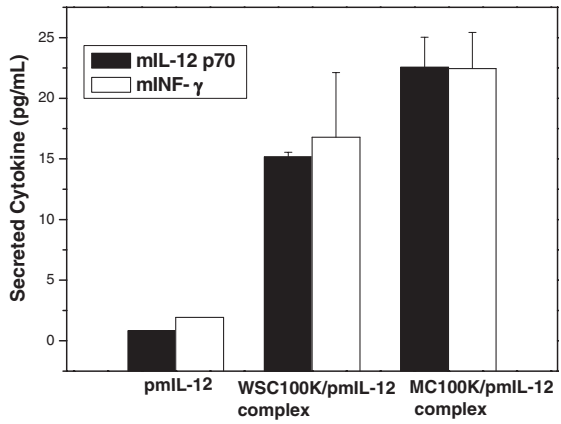

Figure 8.
ELISA for mIL-12 and mINF-γ levels in DCs after transfection by WSC/pmIL-12 and MC/pmIL-12 ($n = 3$). Source: From Ref. [29].

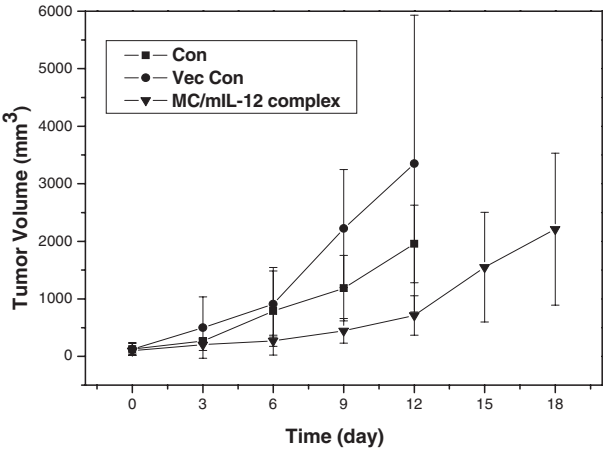

Figure 9.
Suppression of tumor growth by MC/pmIL-12. The tumor volume in BALB/c mice bearing CT-26 carcinoma cells was recorded every 3 days. MC/pmIL-12 complex suppressed the tumor growth effectively. Source: From Ref. [32].

mannoside with chitosan by reductive alkylation in the presence of sodium cyanoborohydride.[31] The results indicated that mannose-modified chitosan/DNA complexes showed much higher transfection efficiency than chitosan/DNA ones although recognition of the man-chitosan/DNA complexes depended on the substitution degree of mannose residues in chitosan.

Furthermore, Kim *et al.* performed cytokine gene delivery after intratumoral injection of MC/plasmid encoding murine interleukin 12 (pmIL-12) complex into BALB/c mice bearing tumor at the injected sites. As shown in Figure 9, intratumoral delivery of MC/pmIL-12 complex into BALB/c mice bearing tumor clearly suppressed tumor growth compared with control and vector itself due to the higher production of IL-12 p70 and INF-γ compared to control.[32] Also, as shown in Figure 10, IL-12 expression by MC/pmIL-12 complex was significantly higher than control and vector control. And the production of mIFN-γ determined by Western blot analysis was increased for the complex compared to control and vector control because IL-12 primarily exerts in its anti-tumor effect via indirect

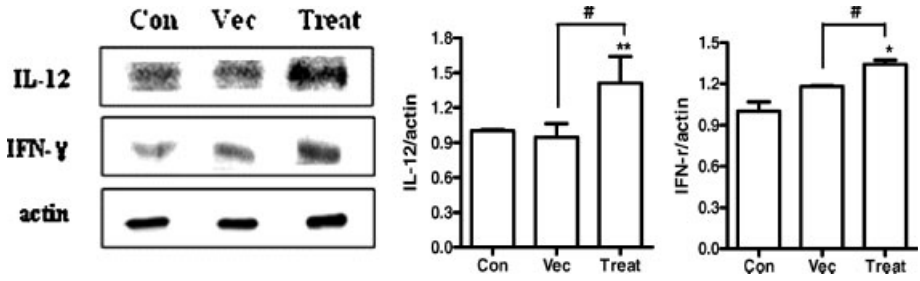

Figure 10.
Western blot analysis and densitometric anaylsis of mIL-12 p70 and mIFN-γ in tumor tissues of BALB/c mice bearing CT-26 carcinoma cells treated with MC/pmIL-12. MC/pmIL-12 increased the level of mIL-12 p70 and mIFN-γ significantly compared to control and vector control ($^{*}p < 0.05$, $^{**}p < 0.01$ compared to control, $\#p < 0.05$ compared to vector control, $n = 5$.) Source: From Ref. [32].

interaction with tumor cells by stimulating potent cytokines such as IFN-γ.[33]

Conclusions

As described in this review, among non-viral vectors, galactose- or mannose-modified chitosan has been developed to enhance transfection efficiency and cell specificity for *in vitro* and *in vivo* gene delivery system. The transfection efficiency of the chitosan was increased by the specific ligand modification of the chitosan owing to receptor-mediated endocytosis. However, more *in vivo* studies need to be carried out for clinical trials although majority of studies carried out so far is only *in vitro* and in animal models.

Acknowledgements: This work is supported by grants from Ministry of Science and Technology in Korea (M10414030002-05N1403-00210).

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