Development of Modified Pectic Galactan Nano-System for Targeted Gene Therapy

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ABSTRACT SUMMARY

A novel targeted gene therapy nano-system for glioma brain tumors was developed and characterized. Since cell's membrane receptor, galectin-3, is found in high levels in glioma, we based our non-viral gene delivery on modified pectic galactan. Pectic galactan is a galactose-rich region of pectin which can serve as potential ligands for interaction with galectin-3. The pectic galactan was successfully modified with quaternary ammonium group and complexed with plasmid DNA. Complexes were found to be suitable for cellular uptake and with minimal cytotoxicity to C6 rat glioma cell line.

INTRODUCTION

Brain tumors represent one of the most malignant forms of human cancers, where the most common and aggressive brain tumor is the glioma. Despite recent progress with both new therapeutics and new delivery approaches, there is a tremendous need for fundamental new biological approaches. Therefore we have explored a novel approach to gene therapy for brain tumors. The major challenge in using gene therapy is finding efficient and stable ways to introduce the required genes into target cells[1,2]. Non-viral gene delivery systems based on natural polysaccharides may be advantageous over the current available synthetic ones, due to several characteristics, such as biodegradability, biocompatibility, low immunogenicity and minimal cytotoxicity. Furthermore, carbohydrate mediated interaction with cell surface lectins play an important role in many biological processes and can be utilized to enhance the binding step and cell uptake in a specific manner (gene targeting).

Galectins is a family of galactose-binding lectins. Galectin-3 is highly expressed in a variety of metastatic cancer cells, such as glioma cell lines, and carries an active carbohydrate recognition domain as shown for C6 rat glioma cells[3]

Since pectin has been proven to be effective in inhibiting or blocking cancer cell's aggregation, adhesion, and metastasis[4], it is studied in our laboratory for its potential as a non-viral gene delivery carrier[5]. The galactose-rich side chains (galactans) of the pectin molecule may be beneficial for targeted gene delivery.

Our hypothesis is based on the highly specific carbohydrate interaction between modified pectic galactan, and galectin-3 receptors on glioma cell membrane that will result in higher concentrations of complexes at given cell surface, and therefore, increase their chances for internalization. In this manner, modified pectic galactan cannot just prevent metastasis, but also might have a preferred incorporation pathway into cells as a DNA carrier.

We intend to deliver p53 tumor suppressor gene in targeted manner, effectively restoring its function. In order to enable the delivery across the blood-brain barrier (BBB), high intensity focused ultrasound would be utilized to selectively and non-invasively open the BBB[6]. This gene therapy approach may become the therapeutically relevant option for the treatment of malignant gliomas.

EXPERIMENTAL METHODS

Quaternized pectic galactan (Q-galactan) was synthesized by quaternizing pectic galactan chains originating from potato. Briefly, 500mg of pectic galactan was added to basic solution and the mixture was stirred for 30min. 9g of the quaternization reagent ((3-chloro-2-hydroxypropyl) trimethylammonium chloride) solution was dissolved in 20mL water and was added into pectic galactan solution and stirred for 20h. The product was then precipitated in an acidic mixture and the solvent was decanted. The product was washed with 80% ethanol solution, dialyzed and lyophilized until dryness[5].

Q-galactan was complexed with plasmid DNA encoding to Green Fluorescence Protein (pGFP). Complexes formation was evaluated as a function of N/P ratio, calculated from the molar ratio between the cationic amine groups on the Q-galactan (N) and the anionic phosphate groups on the pGFP (P). According to the desired ratio, a quantity of Q-galactan solution (in double distilled water) was introduced into a tube containing DNA plasmid solution. The tube was incubated for 1h until complexation was established. Different N/P ratios for complexation were determined by agarose gel electrophoresis. Complexes containing 0.6μg DNA were loaded on 0.8% agarose gel containing ethidium bromide.

Characterization of the complexes was obtained by Cryo-transmission electron microscopy (cryo-TEM) followed by ImageJ statistical analysis software. Complexes at desired N/P ratio were prepared and 3μL of each sample was dripped onto copper grid coated with carbon and was blotted. Samples were mounted and examined in a Tecnai 12 TWIN TEM (FEI) instrument. Further characterization of complexes size was obtained by Nanoparticle tracking analysis (NTA). Complexes at desired N/P ratio were prepared to a final concentration of 3μg/mL and were measured by the NanoSight's NS300 instrument.
C6 rat glioma cells line viability was performed in-vitro by MTT assay. Cells were plated at 75,000 cells/mL and grown to ~90% confluence. For 24 well plates, 1µg DNA was used per well. Complexes were prepared and diluted for a total volume of 250µL with molecular grade water. Complexes were dripped into wells containing 250µL clean DMEM and incubated for 12 h at 37°C under 5% CO2 atmosphere. The media was replaced with fresh media containing serum, and incubated for an additional 3 days under the same conditions. After 72h MTT assays was performed.

RESULTS AND DISCUSSION

Q-galactan was successfully synthesized enabling the formation of complexes with pGFP. The substitution of quaternary amine group was confirmed by 1H NMR, 13C NMR and FT-IR (data not shown). Kjeldahl analysis found the weight percentage of nitrogen to be 3%. Figure 1a demonstrates evaluation of the complexation process by testing the gel migration behavior for different N/P ratios. Until N/P ratio of 1/6, there was not sufficient Q-galactan and some of the DNA migrated in the gel. At N/P ratio of 1/4 and above, complexes were totally formed (i.e., all DNA was complexed with the Q-galactan). In order to evaluate the formation, the shape and the size of the complexes, cryo-TEM was performed, and the images are shown in Figure 1b. Before complete complexation, at N/P 1/6 ratio, uncondensed association of Q-galactan and pGFP was observed. In N/P 1/4, 1/3 and 1/2, small condensed complexes of Q-galactan and pGFP were observed. Supportive results were obtained by atomic force microscopy (AFM) (data not shown).

![Figure 1: (a) Gel electrophoresis for different N/P ratios, lane a is 1Kb DNA ladder, and lane b is free DNA. Lanes 1/96-1/3: duplicates of the marked ratio; (b) Cryo-TEM for different N/P ratios: 1/6-1/2 as marked.](image1)

Statistical analysis of complexes size from cryo-TEM images can be seen in Figure 2a. In all the ratios, complexes' average diameter was under 100nm. Complexes' average size obtained at N/P 1/4 is statistically higher than at N/P 1/3 or 1/2 indicating that the formers are more condensed. Example for statistical analysis of complexes with NTA is shown in Figures 2b and 2c. The obtained size distribution of complexes at N/P 1/2 is 100±40nm and the total concentration of particles is $27.2 \times 10^8 \pm 0.3 \times 10^8$ particles/mL. The corresponding NTA video frame is seen in Figure 2c, in which movement of the complexes under Brownian motion was observed clearly at the recorded videos.

![Figure 2: (a) Complexes' diameter at different N/P ratios as measured in ImageJ software; *p<0.05, **p<0.01 compare to N/P 1/4 (b) Particles concentration versus size distribution from NTA for N/P=1/2; (c) NTA video frame for N/P=1/2.](image2)

Examination of C6 glioma cell line viability (MTT) after exposure to complexes and various materials used in the complex preparation can be seen in Figure 3. In all cases, cells viability was very high, around 90%, compared to the control. However, the viability of the cells that were exposed to Q-galactan at the same concentration used for complexes preparation at N/P= 1/2 was the lowest.

![Figure 3: Cell viability of C6 cells line after exposure to different N/P ratios for 12h.](image3)

CONCLUSION

Q-galactan was successfully synthesized and complexation was clearly observed by gel electrophoresis and cryo-TEM. Q-galactan was able to condense the pGFP to a small size suitable for entering the cell. Moreover, complexes were found to be non-toxic to C6 cell line. Transfection efficiency for the different N/P ratios and intracellular live tracking by microscopic means are now being examined and will be presented.

REFERENCES


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