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An *in vitro* comparative study of layered-double hydroxide nanoconjugate in the delivery of small interference and short-hairpin ribonucleic acid

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Abstract. Alzheimer's disease is a disease which cannot be cured completely. In this aspect ribonucleic acid interference (RNAi) therapy is a prospective therapeutic mechanism which can be used for identifying a future curative procedure. RNAi therapy comprises small interfering RNA (siRNA), short hairpin (shRNA) and micro-RNA therapeutics. Within these three mechanisms we have identified two of them as an effective method of combating this genetic incurable disease. siRNAs and shRNAs are very much effective *in vitro* that is already proved in many research work. In our study we have used a very potent, biocompatible nanoparticle-layered double hydroxide for delivering these macromolecules. However, the intercalation and cellular internalization of these macromolecules demonstrated significant differences. As siRNAs have low-molecular weight than shRNAs they demonstrated different characteristics in the case of internalization within layered-double hydroxide and while cellular internalization. At the end of this study it has been found that both of these macromolecules may be used as a therapeutic approach of Alzheimer's disease after studying it in future in animal and human subjects.

Keywords. siRNA; shRNA; layered-double hydroxide; Alzheimer's disease; RNAi therapy.

1. Introduction

Layered-double hydroxide (LDH) is currently well-known anionic clay for multiple uses [1–5]. The general formula of this nanoparticle is $[M^{2+}_{(1-x)}\ M^{3+}_{x}(OH)]A^{n-}_{(x/n)} \cdot mH_2O$, in which M^{2+} is a bivalent cation, M^{3+} is a trivalent cation and A is a anion with valence of n. Mg Al–LDH has a close resemblance with brucite. Brucites have the octahedral positively charged layers and in between these layers there are negatively charged anions. These anions can be readily exchanged by any other anions in the media. The process is known as the ion-exchange method. There are several types of inorganic and biomolecules which are used for this ion-exchange method, among them different types of drugs [6–8] and genetic materials [7,9–11] are included.

High biocompatibility [12], easily available precursors for synthesis, cationic in nature [13], high-loading capacity [14] and low cost [15] make LDH an excellent delivery vehicle. Moreover, the mechanism of intercalation within the layers provides a protective shield [16] and controls the release mechanism [17] of the loaded particle. Although LDH with its intercalation produces a stable configuration of the host molecule, however, in the presence of more electronegative ions in the environment it can lead to ion exchange which on the other hand helps to improve targeting and release within the cell [18].

The biomolecules which can be intercalated within the LDH layers include deoxyribonucleic acids (DNAs) and ribonucleic acids (RNAs). The major difference between a DNA and RNA is at the 2′-hydroxyl (2′-OH) group which plays the pivotal role in the RNA structure and function. Similar to A, B or Z DNA, RNA also comprises diverse structures like hairpin-loop RNA, linear-duplex RNA, etc. [19,20]. The RNA world hypothesis discussed about the RNA molecules which can work as a catalyst in addition to carry genetic information [21]. The deep hydrothermal vents are considered to be the source of primitive RNA molecules though it remains a question that at that high temperature and pressure how these molecules remained intact. The possible explanation of this query is that the clay-like particles similar to LDH may have protected the RNA molecules which are highly fragile.

Small-interfering RNAs (siRNAs) are the double-stranded RNAs which have a significant role in RNA-interference (RNAi) therapy. As from the name it can be stated that it is smaller in structure compared to other types of RNAs. Only 20–25 base pairs are enough for preparing a synthetic siRNA [22]. Another type of RNA is short-hairpin RNAs (shRNAs) comprises hairpin loops in their structure. Expression of this type of RNA can be accomplished by the delivery of a plasmid or viral or bacterial vector [23]. These types of RNAs are not naturally available in the body of an organism rather they are synthesized externally and can be used for several therapeutic

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procedures. However, the main drawback of this therapeutic measure is bare siRNAs or shRNAs which cannot be used in the body of an organism as they can be easily degraded in the blood flow due to the presence of several RNase enzymes in the blood. When the RNAs are solely inserted, it is impermeable through the cell membrane and it can produce immunogenic reactions in the blood vascular system. To overcome this problem nanoparticles help to a large extent.

The most used RNAi therapy can be mediated by both chemically synthesized siRNA and plasmid-based shRNA. Although both of these molecules produce similar functional outcomes but they have some basic differences. The first drawback of siRNAs is their short life within the target cells. It has been found that siRNAs accumulate within the cell for 4 h and then they become plateau [24]. The kinetics of siRNA interference peaks were observed at around 24–48 h. As opposed to siRNA, shRNAs have a long-lasting effect as the delivery plasmids of these RNAs are integrated in the host genome and in the nucleus of the cell and transported to the cytoplasm [25]. The fluorescent-tagged siRNA study demonstrated high degradability and turnover of this type of RNA. On the other hand, shRNAs can be continuously synthesized within the host cell so much more durable than the former.

The next important difference between these two types of RNAs is that siRNAs are chemically synthesized and requirement of complex structures makes them expensive. On the other hand, in the case of shRNAs making modifications is difficult as the same relies on the host machinery of the expression vector [26–28].

However, due to their structural and functional implications the shRNAs are found to be more potent in gene silencing than siRNAs. Although in the case of dosage manipulation siRNAs are found to be better than shRNAs.

Both siRNA and shRNAs have several advantages and disadvantages from mechanical point of view. In this study, we are intended to find out the differences between these two most important RNAs when they are intercalated within the LDH nanoparticle.

2. Experimental

2.1 *siRNA intercalation in LDH*

The amyloid precursor protein (APP) siRNA sense strand sequence corresponds to 5′-AAGUGAAGAUGGAUGCAGA AUUC-3′ (Dharmacon). The siRNA and $Mg_2Al(OH)_6NO_3$–LDH nanoparticles were mixed in various mass ratios ranging from 1:10 to 1:60 and deionized water was added to a final volume of 10 µl, maintaining the final siRNA concentration constant at 0.1 µg µl^{-1}. Samples were incubated at 37°C for 30 min after which an appropriate amount of RNA-loading buffer was added to each sample. Gel electrophoresis of samples was carried out on 3% agarose gels (TBE buffer, 1% ethidium bromide) at 80 V for 45 min and gels were subsequently imaged using a Biorad Gel Documentation System.

2.2 *Isolation of plasmid from Escherichia coli*

Following the autoclaving of 2.5% LB broth medium, ampicillin was added to a final concentration of 100 µg ml^{-1}, and then *E. coli* transfected with the TRIPZ Inducible Lentiviral shRNA plasmid (M/S Dharmacon™ GIPZ™ Lentiviral shRNA GE Healthcare, UK) was added and cultured overnight at 37°C with mild shaking. The plasmid was isolated using a Geneaid High-Speed Plasmid Mini Kit, Geneaid Biotech, Taiwan, according to the manufacturer's protocol, followed by the quantification of its amount by measuring absorption of the solution at 260 nm, and confirmation of the identity, purity and amount using the gel retardation assay.

2.3 *Intercalation of shRNA-plasmid in LDH nanoparticles*

A suspension of the LDH nanoparticles was prepared in DNase-free water at a final concentration of 10 mg ml^{-1}, and mixed with the shRNA-plasmid (200 ng µl^{-1}) with the shRNA-plasmid:LDH mass ratios of 1:12.5, 1:25, 1:50 and 1:75, followed by incubation for different periods at 37°C for inducing the intercalation to form a shRNA-plasmid–LDH nanoconjugate. 0.7% agarose gel containing ethidium bromide was prepared, and the loaded samples containing 0.1 µg of the shRNA plasmid and DNA loading buffer were electrophoresed in TBE buffer containing ethidium bromide at 80 V for 45 h. The bare plasmid was used as the control and the gel images were captured using a UV transilluminator (M/S Bio-Rad, Philadelphia, PA, USA).

2.4 *Cell culture*

The SH-SY5Y cell line (neuroblastoma) procured from NCCS, Pune was cultured under laboratory conditions. RPMI-1640 media (AL-162S, HIMEDIA) supplemented with 10% fetal bovine serum and 1% antibiotic were used for the culture of the cells. The cells were kept at 37°C and 5% CO_2.

2.4a *Fluorescence-activated cell sorting (FACS) analysis*: For flowcytometry, cells were seeded in a six well plate at 1×10^6 cells per well. The cells were treated with 10 and 20 µg of intercalated-fluorescein isothiocyanate (FITC) tagged siRNAs or green fluorescent protein (GFP)-tagged shRNAs per 2.5 ml media. The cells were incubated for 24 h. After 24 h, the cells were centrifuged and the pellet was washed three times with phosphate-buffered saline (PBS) and subjected to flowcytometry in FACS VERSE (BD biosciences, US). For the time-dependent study the cells were plated as before and treated with 10 µg of intercalated-FITC tagged siRNAs or GFP-tagged shRNAs per 2.5 ml of media for 6, 12 and 18 h. After incubation the cells were centrifuged and washed with PBS as before and subjected to flowcytometry analysis in FACS VERSE (BD biosciences, US).

2.5 *Enzyme-linked immunosorbent assay (ELISA)*

(a) SH-SY5Y cells were stably transfected by Lipofectamine 2000 with the vector containing the full-length APP_{695} isoform as a wild-type (APP wild-type). After this the APP siRNA–LDH or shRNA–LDH conjugate was used for the treatment of the cell line at the previous optimized concentration for 24 h.

(b) Secreted Aβ42 was measured in cell culture medium samples using a human Aβ42 ELISA kit (Thermo Fisher Scientific, Cat. #KHB3441) in cells plated at an equal density in triplicates. A standard curve was generated by linear regression analysis and used to calculate the amount of Aβ42 in each sample.

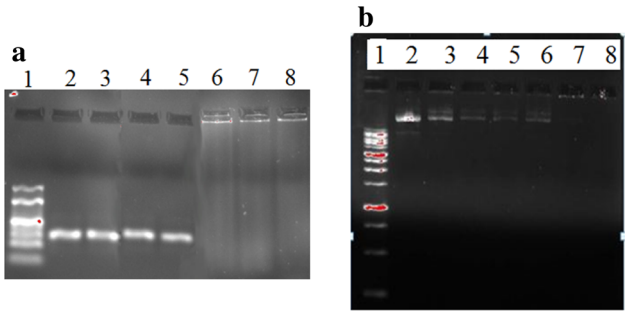

Figure 1. (**a**) Gel retardation assay of siRNA intercalation into LDH nanoparticles. Lane 1: ladder; Lane 2: bare siRNA; Lanes 3–8: siRNA:LDH at the mass ratios of 1:35, 1:40, 1:45, 1:50, 1:55 and 1:60 at 37°C. (**b**) Gel retardation image of shRNA-plasmid intercalation into LDH nanoparticles. Lane 1: 1 Kb ladder; Lane 2: bare plasmid (11.8 Kb); Lane 3: 1:30; Lane 4: 1:40; Lane 5: 1:50; Lane 6: 1:60; Lane 7: 1:70 and Lane 8: 1:80 in a mass ratio of plasmid:LDH, post 2 h incubation at 37°C.

3. Results

3.1 *Intercalation*

siRNA having the complimentary sequence of the APP gene mRNA was used in this study. We have used specifically the siRNA from Dharmacon for our work. Similarly Dharmacon shRNA-plasmid specific for the APP gene was used. For intercalation, different siRNA:LDH mass ratios were used from 1:35 to 1:60 and incubated at 37°C for 1 h and then subjected to agarose gel electrophoresis. The intercalation is more when the presence of LDH is more, in a 1:50 mass ratio the total siRNA was intercalated. A 50-fold increased amount of LDH is sufficient to retain the siRNA in the gel electrophoresis well (figure 1a). Similarly shRNA was incubated with LDH at 37°C using shRNA:LDH mass ratios of 1:30 to 1:80. It was observed that complete intercalation of shRNA was traced at a mass ratio of 1:70 after 2 h of incubation, when the mass of the nanoparticles was 70-fold over the mass of the plasmid, leading to complete conjugation (figure 1b). These data demonstrated a distinct difference between the intercalation of

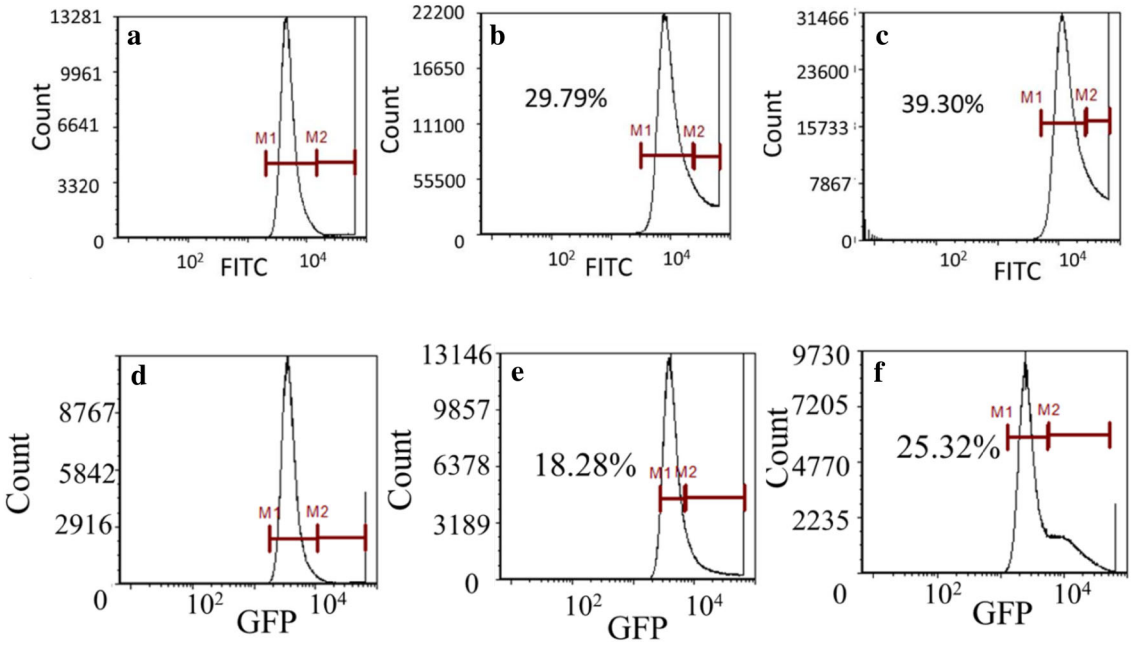

Figure 2. Flowcytometry of cellular internalization of the FITC–siRNA/LDH conjugate at different concentrations by the SH-SY5Y cell line. (**a**) Control, (**b**) 10 μg siRNA–500 μg LDH and (**c**) 20 μg siRNA–1000 μg LDH. Concentration-dependent cellular internalization of shRNA–LDH, (**d**) control, (**e**) 10 μg ml^{-1} and (**f**) 20 μg ml^{-1} indicating the percentage cellular uptake.

siRNA and shRNA of the APP gene. As the molecular weight of siRNA is lower than the molecular weight of the shRNA it is prominent that higher the molecular weight greater the time required for intercalation within the LDH layers.

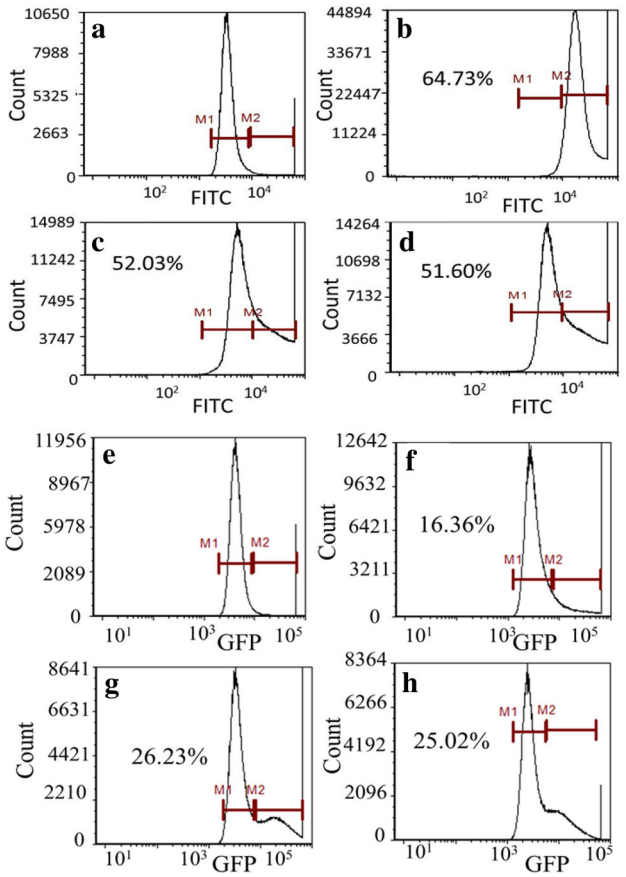

Figure 3. Flowcytometry of cellular internalization of the FITC–siRNA/LDH conjugate at different time points by the SH-SY5Y cell line. (**a**) Control, (**b**) 6 h, (**c**) 12 h and (**d**) 18 h. Time-dependent cellular internalization of shRNA–LDH (**e**) control, (**f**) 12 h, (**g**) 24 h and (**h**) 48 h indicating the percentage cellular uptake.

3.2 *Cellular internalization depending on concentration*

The siRNA/shRNA sequence of the APP gene which we have used does not correspond with any other human gene sequences. So, we have investigated the cellular uptake of the FITC-tagged siRNA/LDH and GFP-tagged shRNA–LDH conjugate in the SH-SY5Y cell line. For this purpose, the SH-SY5Y cells were seeded in a six well plate and treated with the siRNA/LDH or shRNA–LDH conjugate with the previous concentration as mentioned before. After 24 h incubation, the cells were subjected to FACS analysis.

As demonstrated in figure 2, the FITC-APP-siRNA/LDH conjugate was efficiently internalized by the SH-SY5Y cells. A total of 39.3% cells were found to be FITC positive after treatment with the siRNA/LDH conjugate for 24 h. Similarly, 25.32% cellular uptake was found in the case of the GFP-shRNA–LDH nanoconjugate (figure 2).

So, it is demonstrated that as the molecular weight of siRNA is low that is why the cellular internalization is greater in the case of the same, on the other hand as the shRNAs have the higher-molecular weight their cellular uptake is comparatively low.

3.3 *Cellular internalization in a time-dependent manner*

To find out the approximate time required to internalize the APP siRNA/LDH conjugate the next experiment was conducted. For this purpose the SH-SY5Y cells were seeded as before in six well plates and treated with various concentrations of the conjugate at different time points e.g., 6, 12 and 18 h. However, at 6 h the FITC–siRNA/LDH conjugates showed 64.73% uptake which is maximum in 24 h study by the cells (figure 3). It can be said that after 6 h the cell released the fluorescence and demonstrated lesser extent of fluorescence intensity.

Similarly, in the case of shRNA the maximum uptake is found at 24 h and also it is lower than siRNA which is 26.23% after 24 h (figure 3). So, it is confirm that shRNA takes a longer time than siRNA for cellular uptake. Moreover, the

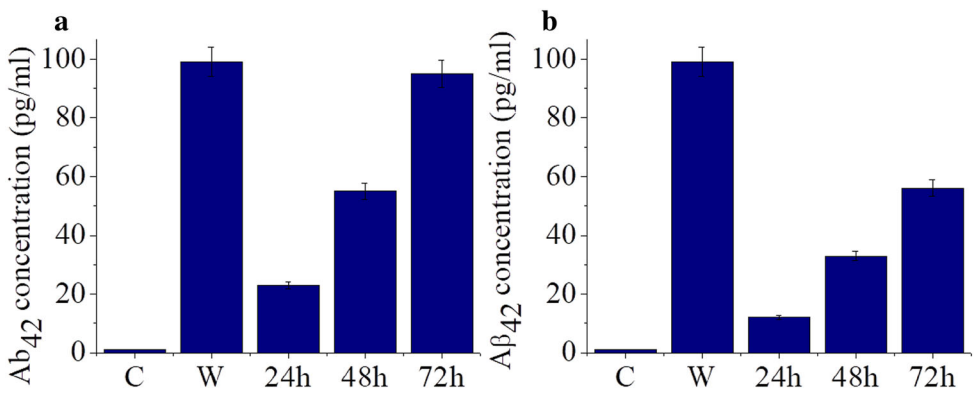

Figure 4. (**a**) APP level after treatment with siRNA and (**b**) APP level after treatment with shRNA. 'C' denotes control, 'W' denotes wild type and others indicate the incubation time.

uptake percentage after 6 h in the case of siRNA is also very higher than the shRNA–LDH nanoconjugate.

3.4 *ELISA for the quantification of extracellular APP*

We measured Aβ42 levels by ELISA in medium collected from cells expressing endogenous APP or overexpressing APP wild-type (figure 4). As expected, cells overexpressing APP wild-type secrete more Aβ42 compared to control cells. Moreover, after treatment with the siRNA–LDH and shRNA–LDH was also checked by ELISA analysis and it also demonstrated a significant amount of reduction in the expression of the APP protein level with a p value <0.05.

4. Discussion and conclusion

In conclusion the basic difference of siRNA and shRNA intercalated within LDH is their molecular weight. Due to these differences the time of intercalation is more, the release kinetics is different, the optimum concentration of internalization within the cells is different and the time for cellular uptake is also greater in the case of shRNA. Moreover, the ELISA results showed that shRNAs have a long standing effect compared to siRNA.

Finally, it can be concluded that shRNA is a more potent therapeutic agent than siRNA although in some cases siRNA can be used. siRNAs are dose specific; short time requirement of gene therapy can be achieved using siRNA but to achieve long term effect shRNAs are the better option.

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