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Insertion of Inorganic-Biomolecular Nanohybrid into Eucaryotic Cell

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ABSTRACT

It has been clearly demonstrated that ATP could be intercalated into inorganic layered double hydroxide (LDH), giving rise to a biomolecular-inorganic nanohybrid with preserving its physico-chemical and biological integrity. It shows a remarkable transfer efficiency of ATP into target cells by alleviating an electrical repulsion at the cell walls due to the neutralization of negative charge of phosphates by positive hydroxide layers. From cellular uptake experiment with laser scanning confocal fluorescence microscopy, it is revealed that the FITC-LDH hybrid is effectively transferred into 293 cells. Such an unique feature of biomolecule-LDH hybrid will open a new field of reserving and delivering genes, drugs and other functional biomolecules.

INTRODUCTION

Drug delivery system is gaining attention for life threatening diseases. For the efficient introduction of foreign gene-based therapeutics or drugs into target organ or cells, a carrier system is required. Both viral and non-viral vectors are presently under investigation. However, the conventional approaches have many problems, such as cytotoxicity, immunoreaction, low transfection, etc. Thus, there remains a need for less toxic and more efficient delivery vehicles for drug and other gene-based therapeutics.

Generally, the layered double hydroxide (LDH) consisting of magnesium and aluminum has already been used as medicine due to its applicability as an agent for treatment of peptic ulcers. An effective method for treating gastric ulcer is to inhibit the action of hydrochloric acid and pepsin in the gastric juice. $Mg_2Al(OH)_6(CO_3^{2-})$-LDH meets various requirements imposed on an antiacid, and its excellence may be attributed to its structural features. The rate of reaction of $Mg_2Al(OH)_6(CO_3^{2-})$-LDH with gastric acid is similar to that of $Mg(OH)_2$, while the buffering pH is rate-controlled by dissolution of $Al(OH)_3$ monomer, and reaches a pH value around 4, which is slightly higher than the one at which $Al(OH)_3$ is dissolved. The high antipeptic activity of LDH can be attributed both to the adsorption of negatively charged pepsin onto positively charged surface of LDH, and to buffering of the pH at about 4 for a long time. Therefore, we believe that the present LDH is quite biocompatible.

In the present study, adenosine 5-triphosphate (ATP) is selected as a guest molecule to be inserted into LDH, since it has been well known as energy carrier in biological system. But negatively charged ATP cannot be incorporated effectively into cell due to the negative cell wall. It is, therefore, thought that the encapsulated and charge neutralized ATP with LDH could enter the cell more effectively.

In this work, we were able to confirm the effective insertion of γ-^{32}P labeled ATP by hybridizing with LDH. We made an effort to visualize the insertion of bio-LDH nanohybrid. The cellular uptake of fluorescein 5-isothiocyanate (FITC)-LDH hybrid was observed with laser

confocal fluorescence microscopy. From the cytotoxicity test of $Mg_2Al(OH)_6(NO_3^-)$-LDH, our primary attention was focused on verifying the possible application of bio-LDH nanohybrids as a non-viral vector by a systematic experimental approach.

EXPERIMENTAL DETAILS

The pristine $Mg_2Al(NO_3)$–LDH was prepared by the coprecipitation under N_2 atmosphere following the conventional route. A mixed aqueous solution containing Mg^{2+} (0.024 M, from $Mg(NO_3)_2$) and Al^{3+} (0.012 M, from $Al(NO_3)_3$) was titrated dropwisely into a NaOH solution with vigorous stirring. During the titration, the solution pH was adjusted to 10 ± 0.2 at the room temperature. The resulting white precipitate was collected by the centrifugation and washed with decarbonated water thoroughly. The biomolecule–LDH hybrids were then prepared by ion-exchanging the interlayer nitrate ions in the pristine LDH with adenosine–5'–triphosphate (ATP, containing 40 μCi of [γ-^{32}P] ATP, DuPont) and fluorescein 5-isothiocyanate (FITC) at pH =7, for 48 h with a constant stirring. The reaction products were then isolated and washed as described above.[1,2]

The isotope labeled ATP-LDH hybrid and the [γ-^{32}P] ATP only were added to 4×10^6 HL-60 cells, human promyelocytic leukemia cells, in 20 ml of RPMI-1640 with 10 % heat-inactivated fetal bovine serum, and then incubated in a 5 % CO_2 incubator at 37 °C for 1, 2, 4, 6, 20, and 24 hours.[3] For each reaction time, 1 ml of sample was taken, centrifuged, then the separated supernatant was saved and the cell pellet was washed once with 1 ml of phosphate buffer (10 mM Na_2HPO_4, pH 7.4, 150 ml NaCl) and followed by sedimentation. The supernatant was again separated and saved, and the cell pellet was lysed in 200 μl of lysis buffer (10 mM Tris/Cl, pH 7.4, 150 ml NaCl, 1 % sodium dodecyl sulfate) and then extracted with 200 μl of phenol. After separating the aqueous phase, the phenol phase was extracted again with 200 μl of water. Aliquots of the combined aqueous extracts, cell walls, and culture-medium supernatant were analyzed by liquid scintillation counting. The percent of hybrid and [γ-^{32}P] ATP taken up by the cells was calculated by dividing the counts in the combined aqueous phases of the cell pellet extract by the total counts in the cell pellet, cell wash, and culture-medium supernatant. All the procedures were repeated 3 times to check the reproducibility.

The 2×10^5 293 cells were grown on round cover-slips in a 24-well culture plate and cultured for a day. The 1 μM and 5 μM of FITC-LDH hybrids were added to the cells and incubated for 1, 2, 4, 6, and 8 hrs, respectively. In order to compare, control experiment was performed with only 5 μM of FITC itself under the same condition. All of the samples were washed with PBS buffer three times and fixed with 3.7 % formaldehyde in PBS. After washing again with PBS, the samples were observed with a laser scanning confocal fluorescence microscope (Carl Zeiss LSM 410). The samples were excited by a 488 nm argon laser, and the images were filtered by a longpass 515 nm filter.[4-6]

The HL-60 cells were cultured in a 24-well culture plate at an initial concentration of 5×10^4 /ml. Cells were exposed to various amounts of LDH from 1 μg/ml to 1000 μg/ml. A control experiment was performed without LDH under the same conditions. Cell viability was determined daily until the fourth day by MTT assay.[3,7]

DISCUSSION

In a previous report,[8] we have demonstrated that the LDH could successfully encapsulate

Time (hours)	1	2	4	6	20	24
Relavite uptake by cells (fold)	21.0	24.0	11.5	9.3	4.5	4.0
Standard deviation	1.0	0.8	0.5	1.0	0.5	0.3

Table I. Relative uptake efficiency of [γ-32P] radioactive isotope labeled ATP-LDH hybrid into HL-60 cells. The uptake efficiency of ATP-LDH hybrids was normalized to that of ATP only.

biomolecules via intercalation route. According to the XRD and IR analysis, it has been concluded that the biomolecules stabilized in the interlayer space of LDH retain their chemical and biological integrities.

To elucidate the transfer efficiency, isotope-labeled [γ-^{32}P] ATP-LDH hybrid was prepared by ion exchange method and the uptake of such hybrids by eucaryotic cell was monitored with respect to incubation time.[3] Table 1 clearly demonstrates that the exogenously introduced ATP-LDH hybrid can enter into HL-60 cells effectively within a relatively short time. The transfer efficiency was found to be much higher, up to about 25-fold after 2 hours incubation, than that of ATP only. Whereas after 4 hours incubation, the uptake amount of the hybrids becomes lower, below 12-fold. The triphosphate group of [γ-^{32}P] ATP is negatively charged, which inhibits [γ-^{32}P] ATP to be taken up by the cell through the negatively charged cell walls. While the hybridization between ATP and LDH neutralizes the surface charge of anionic phosphate groups in ATP due to the cationic charge of LDH, which becomes favorable for endocytosis of cells, and eventually results in enhanced transfer efficiency. The longer the incubation time is in a CO_2 atmosphere, the more the ATP will be released from the interlayer space of hydroxide lattice. In spite of that, the transfer efficiency of the hybrid remains higher than that of ATP only, up to about 4-fold after 24 hours incubation. This result reflects that the hybridization between cationic layers and anionic biomolecules would greatly enhance the transfer efficiency of biomolecules to mammalian cells or organs. The charge neutralization through hybridization between LDH and ATP would facilitate the penetration of hybrids into cells, *via* so-called endocytosis,[9,10] because it greatly reduces the electrostatic repulsive interaction between negatively charged cell membranes and anionic biomolecules during endocytosis.

Further evidence on cellular uptake of the FITC-LDH hybrid was obtained directly from laser scanning confocal fluorescence microscopy experiments.[4-6] the 1 μM and 5 μM FITC-LDH hybrids were added to 293 cells and then incubated for 1, 2, 4, 6, and 8 hrs. Then the cells were washed with PBS, fixed with 3.7 % formaldehyde, prior to measurements. Figure 1 shows the cellular localization of the fluorophore obtained after a fixed incubation time. The fluorophores are detected in cells within an hour of incubation, and the fluorescence intensities are increased continuously up to 8 hrs. The fluorophores in the cells are distributed primarily in peripheral and cytosol parts with some in the nucleus. Moreover, the cells treated with 5 μM of FITC-LDH hybrids show more intense fluorescence than those with the 1 μM. In contrast, the cells treated with 5 μM FITC only remain dark regardless of incubation time, because cells could not take up FITC itself even with high concentration. It is obvious that LDH plays an important role in mediating the cellular uptake of FITC. All the cells can engulf the neutralized nanoparticles

through phagocytosis or endocytosis. Therefore, we conclude that the intracellular fluorescence has been created by deintercalated FITC or some by FITC-LDH hybrids in the cell.

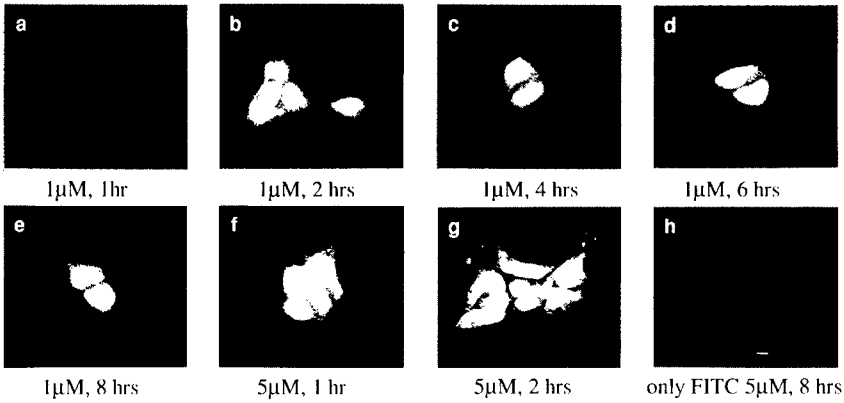

Figure 1. Laser confocal fluorescence microscopy of fluorophore in 293 cells. 2×10^5cells/well were incubated with 1 μM FITC-LDH for 1, 2, 4, 6, and 8 hrs and 5 μM FITC-LDH for 1 hrs, respectively. The other fluorescence microphotograph was obtained with 5 μM FITC only. The bar is 10 μm.

It is necessary to test cytotoxicity of LDHs themselves in the cells for use as gene or drug delivery carrier. In fact, one critical element for the overall transfection efficacy of gene delivery system is cytotoxicity. Cell damage resulting from a cytotoxic delivery system is deleterious because the following delivery in the cell must be capable of supporting translation and transcription. As shown in Figure 2, LDHs themselves have no effect on the viability of HL-60 cells, when administered at levels below 1000 μg/ml for up to 4 days. However, many cationic lipid complexes previously examined were bound to be toxic to cells at concentrations near their effective doses if exposure times were extended to several hours,[11] suggesting that the molecules could not easily be metabolized. For example, polylysine was the first polymer used to mediate the transfection of cells,[12] and polyethyleneimine(PEI) was of the new "proton sponge" category and was hypothesized to mediate the escape of plasmid DNA from endosomal pathway.[13,14] Though they demonstrated the aptitude of polycations to mediate transfection, those polymers are associated with a considerable degree of cytotoxicity.[15,16] When COS-7 cells (immortalized African green monkey kidney fibroblasts) were incubated with polylysine and PEI, their cell viability was ~50 % at 20 μg/ml and ~2 % at 10 μg/ml after 48 hrs, respectively.[17] In addition, when Vero cells (African green monkey kidney) were exposed to the various concentrations of DOTMA/DOPE (Lipofectin™, a 1:1 (w/w) formulation of the cationic lipid, DOTMA, and at 100 μg/ml there were no viable cells left after 4 days of continuous treatment.[18] Thus, it is needed to develop less toxic and more efficient delivery vehicles for gene or drug based therapeutics. In contrast, LDHs themselves show no discernible cytotoxic effect on HL-60 cells, when administered at levels below 1000 μg/ml for up to 4 days.

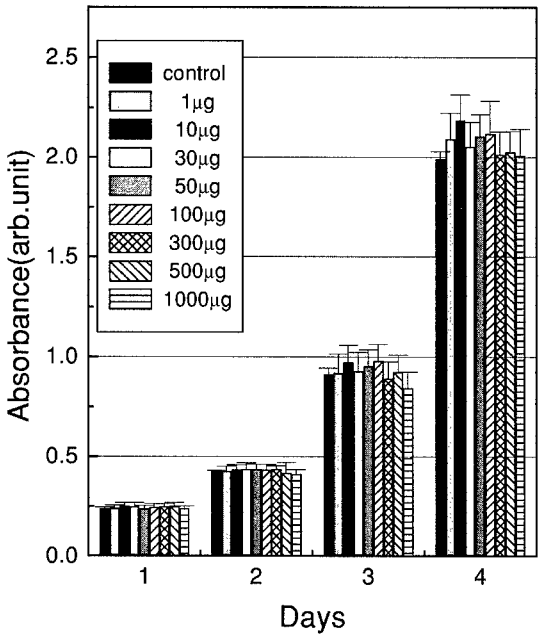

Figure 2. Cytotoxicity experiment of LDH in HL-60 cells. Various amounts of LDH were added to cells at final concentrations from 1 μ g/ml to 1000 μg/ml.

CONCLUSION

This study has shown that inorganic-biomolecular nanohybrids can enter into cell through the cellular uptake experiment using the isotope labeled ATP and the fluorescent FITC. In addition, from the cytotoxicity test, we demonstrate clearly that the inorganic-biomolecular nanohybrids are a safe way of delivering genes or drugs. And we are now developing new inorganic delivery carrier, which can be improvement over conventional delivery carrier in vivo.

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REFERENCES

1. J. H. Choy, S. Y. Kwak, J. S. Park, and Y. J. Jeong, *J. Mater. Chem.*, **11(6)**, 1671-1674 (2001)
2. J. H. Choy, S. Y. Kwak, Y. J. Jeong, and J. S. Park, *Angew. Chem. Int. Ed. as a highlight comm.* **39(22)**, 4042-4045 (2000)
3. E. L. Wickstrom, T. A. Bacon, A. Gonzalez, D. L. Freeman, G. H. Lyman, E. Wickstrom, *Proc. Natl. Acad. Sci. USA* **85**, 1028 (1988).
4. R. J. Lee and P. S. Low, *J. Biol. Chem.* **269**, 3198-3204 (1997).
5. Z. W. Lee *et al. Ibid.* **273**, 12710-12715 (1998).
6. K. Vogel, S. Wang, R. J. Lee, J. Chmielewski, P. S. Low, *J. Am. Chem. Soc.* **118**, 1581-1586 (1996).
7. M. B. Hansen, S. E. Nielsen, K. Berg, *J. Immunol. Methods* **119**, 201-203 (1989).
8. J. H. Choy, S. Y. Kwak, J. S. Park, Y. J. Jeong, and J. Portier, *J. Am. Chem. Soc.*, **121**, (1999)1399-1400.
9. S. S. Davis, *Trends Biotechnol.* **15,** 217 (1997).
10. I. M. Verma, N. Somia, *Nature* **389,** 239 (1997).
11. C. F. Bennet, M. Y. Chiang, H. Chan, J. E. E. Shoemaker, C. K. Mirabelli, *Pharmacol.* **41,** 1023 (1992).
12. G. Y. Wu, C. H. Wu, *J. Biol. Chem.* **11**, 1050 (1987).
13. O. Boussif, F. Lesoulac'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. emeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **92,** 7297 (1995).
14. M. A. Zanta, O. Boussif, A. Adib, J. P. Behr, *Bioconjugate Chem.* **8,** 839 (1997).
15. S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, M. Hashida, *J. Controlled Release* **34,** 233 (1995).
16. G. A. Brazeau, S. Attia, S. Poxon, J. A. Hughes, *Pharm. Res.* **15,** 680 (1998).
17. D. Putnam, R. Langer, *Macromolecules* **32,** 3658 (1999).
18. K. G. C. Judith, V. Bodepudi, J. S. Bishop, K. Jayaraman, N. Chaudhary, *J. Biol. Chem.* **270,** 31391 (1995).