Abstract

Intracellular Delivery of Drug & DNA with α-Synuclein-coated Gold-Nanoparticles

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α-Synuclein, the protein related with Parkinson' s disease, has been reported that it can penetrate the cell membrane. Herein, we achieved successful intracellular delivery of drugs and gene using this membrane penetrating characteristic of α-synuclein. First, α-synuclein was immobilized on the gold-nanoparticles (AuNPs) by the cysteine mutant at the C-terminal and this binding orientation showed the highest delivery efficiency to the nucleus. Also, this α-synuclein-coated AuNP formed complexes with DNA and mesoporous silica nanoparticle (MSN) at acidic pH and our in vitro studies showed high intracellular uptake of these complexes into the mammalian cells.

The MSN-AuNPαsyn complex was constructed in which the pores of MSN were blocked by AuNPαsyn thus preventing the leaking of the loaded drugs. We called this complex as Particles-on-a-particle (PoP) and the cells treated with Doxorubicin (DOX) loaded PoP showed severe cytotoxicity level implicating that the drugs were successfully delivered.

Another complex constructed was the DNA-AuNPαsyn complex for the purpose

of gene delivery. A complex of GFP expressing plasmid and AuNPαsyn was treated to the cells and by measuring the fluorescence intensity we determined the gene transfection efficiency. In comparison to lipofectamine, this AuNPαsyn system showed significantly faster transfection rate.

We suggest that AuNPαsyn as the novel cell internalization agent for delivering drugs and gene. In future studies, we expect to evaluate the nucleus localization signal within the α-synuclein sequence.

Keywords : α-Synuclein, nanoparticle complex, drug delivery, gene delivery, Intracellular delivery of nanoparticles

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Ⅰ. Introduction

1.1 α-Synuclein and AuNPαsyn

1.1.1. α-Synuclein

α-Synuclein relating with Parkinson's disease is consisted of 140 amino acids. α-synuclein is notable amyloidogenic protein developing β-sheet-enriched protein nanofibrils through a specific self-assembly process. And it performs a key role in the pathology of Parkinson's disease.[1] Recent studies reported that Lewy bodies are consisted of α-synuclein. [2-4] α-Synuclein is widely known that it is associate with membranous compartments in cultured cells and brain tissue[5]. Also α-synuclein interacts with lipid layers[6] and the 11-amino acid imperfect repeat which is located in N-terminal region carry out the key role to transverse the plasma membrane.[7]

1.1.2. AuNPαsyn

When gold-nanoparticles (AuNP) coated with α-synuclein (AuNPαsyn) is made, thiol group was obtained to make covalent bond α-synuclein with AuNP.[8] As known, wild-type of α-synuclein doesn't have cysteine functional group.[9] Therefore cysteine mutant of α-synuclein was fabricated and three type of mutants were prepared such as S9C, A53C, and Y136C. This mutants were used to make AuNPαsyn. (Figure 1) AuNPs9c represent the gold-nanoparticles coated with S9C cysteine mutant α-synuclein. In this gold-nanoparticle, C-terminal of α-synuclein towards out. Similarly, both end of α-synuclein towards out in AuNPa53c and N-terminal of α-synuclein towards outside in AuNPy136c.

Self-assembly of α-synuclein can be controlled by changing the pH of the reaction buffer.[10] For this purpose, the pH of reaction buffer was lowered to pH 4.2. This pH value is lightly lower than the isoelectric point of α-synuclein (pI = 4.7). Thus, when the pH is lowered to 4.2, the formation of chain, 2-D array and PoP is observed. (Figure 2) By this method, we can make MSN-AuNPαsyn and DNA-AuNPαsyn complexes and also applicate to cell study.

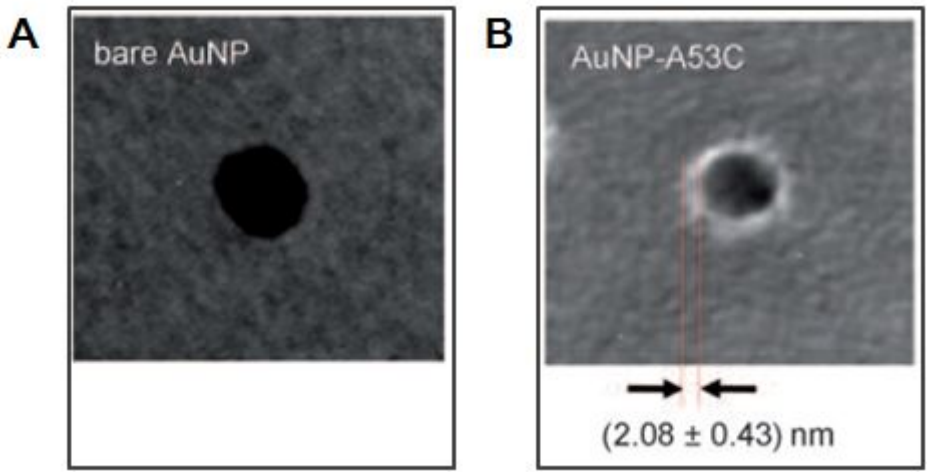


Figure 1. α-Synuclein-coated AuNPs (A) TEM image of the bare gold-nanoparticle (B) α-Synuclein layer on AuNP is shown in negative stain with 2 % uranyl acetate.
*Lee, D. et al. Angew. Chem. Int. Ed. 2011, 50, 1332-1337.

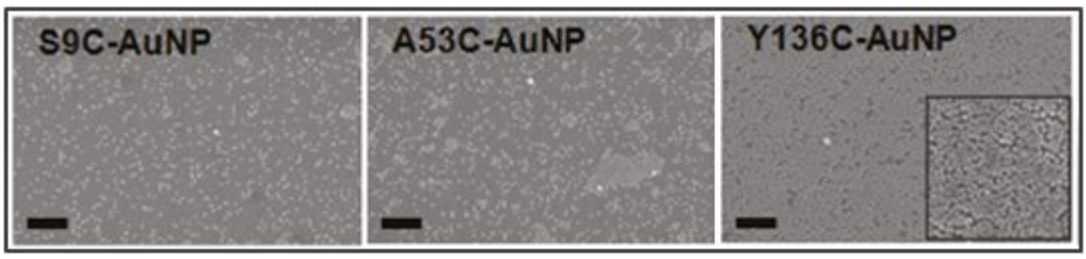


Figure 2. 2-D array of AuNPαsyn on a glass surface FE-SEM images of the 2-D array of AuNPs coated with three mutant α-synucleins (S9C, A53C, Y136C)
*Lee, D. et al. Langmuir. 2011, 27, 12782-12787.

1.2. Mesoporous silica nanoparticles and drug delivery

1.2.1. Drug delivery system (DDS) using nanoparticles

Drug delivery system using nanotechnology was started with liposome developed in 1960s.[11] Other molecules such as organic, inorganic, polymer system was developed sequentially for drug release. Controlled-release polymer system was introduced for delivering macromolecules in 1976.[12] More complicated drug delivery system such as pH control or cell-specific targeting was developed in 1980.[13,14] As the usage of polyethylene glycol (PEG), circulation of time increase was reported; liposome[15] and polymeric nanoparticles[16].

Now a days research and applications of nanotechnology was grew. And application of nanotechnology to medicine will show meaningful advances in the diagnosis and curing of disease.

There are a lot of advantages when nanotechnology is used, for example, (1) enhancement delivering efficiency of water-soluble drug (2) targeting (3) transcytosis of drug through epithelial and endothelial wall (4) delivering of macromolecules (5) complicated therapy can be carried out (6) Zero permature release (7) imaging[17] (8) real-time observation of drug release on a living body.

1.2.2. Drug delivery system (DDS) using Mesoporous silica nanoparticles (MSNs)

The essential factor of carrier material to make efficient DDS is biocompatible. Also suitable drug controlled release It is necessary that high loading of drug molecule, encapsulation, cell-specific ability, and controlled drug release.

Now, mesoporous silica nanoparticle has been adopted in drug delivery system because of its biocompatibility and porous structure that can entrap drug. [18-20] To achieve controlled recluse, gate-keeping was performed by stimuli-responsive system based on MSN. This type of DDS can conduct zero permature release and it is valuable when the cargo has the toxicity. Nanoparticles organic molecules and supramolecules are used in gatekeeping. V.S.Y. Lin group developed redox DDS by blocking MSN with cadmium sulfide nanoparticles.[20] Site-directing, site-specific DDS was reported that use magnetic motor effect.[21] In addition to nanoparticle, organic molecules can be used to gatekeeping. Tanaka and co-workers also showed that

photo-triggered controlled release system can work in organic solvent.[22] By using MSN, we can prepare more efficient vehicle and this vehicle performed ligand-responsive drug delivery.

1.3. Gene delivery

Gene therapy has gained serious attention over the past two decades as a promising method for curing genetic disorders such as severe combined immunodeficiency[23], cystic fibrosis, and Parkinson's disease[24], as well as an different wat to traditional chemotherapy used in curing cancer.[25] The curing of disease at the genetic level has been developed consecutively. Gene therapy to cure genetic deficiencies has performed as evidenced by the 600 clinical trials.[26] Usually, there are two methods to deliver genes. Using viruses is the first one. These viruses are quite efficient but strong problems associated with viral systems, including toxicity, immunogenecity, and limitation with respect to scale-up procedures.[27] The second one is nonviral method such as, DNA alone or DNA complexes that accomplished with carrier molecules; cationic lipids, polymers, dendrimers, peptides. This method have advantage over viral compartments because of the convenient of controlling the complex. Unfortunately, the efficiency of gene delivery is less than viral systems. Although a lot of improvements have been conducted in efficiency of nonviral gene delivery. And biocompatibility and potential for large-scale production make these compounds increasingly attractive for gene therapy.[28]

Gold nanopartices are able to delivery large biomolecules, without inhibiting themselves as carriers of only small molecular drugs.[29, 30] Tunable size and functionality make them a useful scaffold for profitable recognition and delivery of biomolecules.[31] Peptide, proteins, or nucleic acids like DNA or RNA can be delivered by gold nanoparticles. Gold-nanoparticles can be used as nonviral method. Several attempts have been conducted to use AuNP for gene delivery.[32-36] For example, Klibanov and Thomas have validated that the polyethylenimine (PEI)—conjugated AuNPs deliver plasmid DNA more efficiently.[24] Mirkin et al. developed gold nanoparticle oligonucleotide conjugates as intracellular gene regulation agents.[31] By Using the gold nanoparticles, we can create fast and biocompatible drug delivery system that can work in mammalian cells.

Ⅱ. Materials and Methods

2.1. Materials

Gold nanoparticles in 5nm, 10nm size were purchased from Sigma Aldrich. MSNs for drug delivery experiment were offered by prof. Hyeon's laboratory. Carbon coated 200-mesh copper grid was purchased from Ted Pella Inc.. α-Synuclein antibody and Alexa fluor 488 conjugated secondary antibody were purchased from Santa Cruz. DAPI for nucleic acid staining was purchased from Sigma-Aldrich. DMEM/High glucose and Trysin/EDTA for mammalian cell culture was purchased from Thermo Scientific. Lipofectamine 2000 for transfection was purchased by life technologies. Celltiter 96 AQueous for cell proliferation assay was purchased from Promega. BAPTA for intracellular Ca2+ regulator was purchased by Sigma-Aldrich. Doxorubicin hydrochloride for chemotherapeutic agent was purchase from Sigma-Aldrich. Plasmid midi kit for plasmid amplification, gel extraction kit and PCR purification kit for DNA fragment purification were purchased by QIAGEN. pET303/CT-His Vector for purification of truncated α-synuclein was purchased from Invitrogen. Premix Taq for PCR reaction was purchased from Takara. Restriction Enzyme Xho I and Xba I were purchased from Promega. EZ cloning kit for DNA ligation was purchased by Enzynomics.

2.2. Purification of α-Synuclein

Recombinant α-synuclein DNA inserted in pRK172 and it was transformed in BL21 competent E.coli. Plate the cells on LB agar plate containing 0.1 mg/ml ampicillin and incubate a plate 37℃ for 12 hours. Pick a single colony from a plate and transfer it into 100 ml of LB medium containing 0.1 mg/ml ampicillin. Pre-culture the cell for 8 hours at 37℃ with 200 rpm shaking. For a large scale culture, transfer 1 ml of the pre-culture into 1 L of LB medium containing 0.1mg/ml ampicillin. Incubate the culture at 37℃ with 200 rpm shaking. When optical density (O.D.) at 600 nm of the culture reaches 0.6-0.8, add isopropanol-b-D-thiogalactopyranoside (IPTG) and continue to incubate until reaching the absorbance of 1.2 at the wavelength 600 nm. Harvest cells by centrifugation at 3600rpm (Kontron) for 20 min at 4℃ and discard supernatant. Freeze the pellet at -20℃ overnight. Pellet was thawed at 37℃

and resuspended the cell in 200 ml of lysis buffer (20 mM Tris/Cl, pH 7.5, containing 0.1 M NaCl, 0.1 mM PMSF, 1 μg/ml Leupeptin, 2 mM EDTA, 0.1 mg/ml lysozyme and 10 units/ml DNase). Centrifuge the cell lysate at 7000rpm for 20 min at 4℃. Supernatant was collected into a flask and heat at 100℃ for 20 min and cool down on ice. Supernatant was re-centrifuged at 7000rpm for 20 min at 4℃ and then filtered it through 0.22 μm syringe filter in order to remove any pellet. The protein solution was purified continuously with DEAE Sephacyl, S-200 Sephacyl, S-Sepharose. the purified protein was dialysed with 20 mM MES (pH 6.5) at 4℃. After dialysis, the protein solution was stored at -80℃.

2.3. Cell culture

HeLa human epithelial carcinoma cell line was stored in liquid nitrogen. The frozen cell was thawed quickly within 1 min at 37℃ water bath. It was transferred to a culture dish containing medium (DMEM including 10 % Fetal bovine serum). The dish was incubated at 37℃ in 5 % CO_2 atmosphere for 3 hours. When cells attache the dish after 3 hours, replace the medium containing trace amount of freeze medium with fresh medium. Medium was replaced every 2-3 days. When the confluence of the cell reaches over 80 %, the cell were sub-cultured.

2.4. Preparation of the protein coated gold nanoparticles

Gold nanoparticles sized in 5, 10 nm of 200 μl were carried on 1.5 ml conical tube. The sample was mixed with 50μl of 1 mg/ml α-synuclein (1mg/ml) and incubated at 4℃overnight. The sample was centrifuged at 13200rpm for 30 minutes to change the buffer. The supernatant was discarded and added 200 μl of MES buffer (pH 6.5). The sample was re-centrifuged and this washing step was repeated 3 times. The final washed sample was stored in 4℃.

2.5. Transmission electron microscope (TEM)

10 μl of sample was dropped on to a carbon-coated copper grid and kept for 5 minutes. Remained sample was absorbed in filter paper and dropped 10 μl of 2 % uranyl acetate. After 5 minutes, the sample was washed with distilled water instantly.

The samples are observed with transmission electron microscopy (JEM 1010, JEOL, Jaoan) at 80 kV modes.

2.6. Confocal laser scanning microscope(CLSM)

Cells are grown on coverslips. The cells were washed briefly with PBS buffer after treatment of nanoparticles into cells. Then, cells were fixed with 3 % paraformaldehyde for 10 minutes and rinse with PBS. 0.5 % Triton X-100 were adjusted to permeabilizing cells for 5 minutes. The cells were rinsed again with PBS and covered with blocking buffer (1 % BSA in PBS) for 30 minutes at 37℃ to minimize mom-specific adsorption of the antibodies to the coverslips. Dilute primary antibody to 10 μg/ml in blocking buffer. After 30 minutes, Blocking buffer was removed and 200 μl of the primary antibody solution was distributed on each coverslip and incubate at 4℃ overnight. The antibody solution was decanted. Coverslips were washed three times with PBS (5 minutes each wash). Dilute the secondary antibody to the 1:50 in blocking buffer. Secondary antibody solution was added each coverslip. coverslips were washed three times with PBS (5 minutes each wash). Dilute the 4',6-Diamidino-2-phenylindole (DAPI) stock to 300nM in PBS. 300 μl of this dilute DAPI staining solution was added approximately to the coverslip and incubate for 5 minutes. The sample was rinsed 3 times in PBS. 10μl of mounting media was mounted on glass slide. The coverslips were inverted onto glass slide. The edge of coverslide was sealed with manicure. The sample was inspected under the confocal laser scanning microscope(CLSM, Carl Zeiss LSM710).

Ⅲ. Results and Discussion

3.1. Drug delivery into the cell

3.1.1. Intracellular delivery of PoP

To ensure that the applicable into the cell of PoP(particles-on-a-particle) and drug delivery, the experiment was performed. Here, PoP is abbreviation of Particles-on-a-Particle and it signify MSN-AuNPαsyn complex. The PoP was made as described in the previous report.[34] MSN (Mesoporous silica Nanoparticle, 20μg) and α-synuclein-coated gold-nanoparticles (AuNPαsyn, 3.6 pmole) was incubated in 100 μl of 10mM citrate at pH 4.4. AuNP was used in particle size of 5, 10, 20 nm. (Figure 3) And AuNPαsyn was used AuNPs9c, AuNPa53c, AuNPy136c. (Figure 4) When PoPs were prepared, AuNP 5nm and AuNPy136c was used. The buffer was changed citrate buffer (pH 4.4) to PBS before treating to HeLa cell. PoP was treated 50 μg to HeLa cell for 1 hour in CO_2 incubator. The cellular uptake of PoPs was analyzed by Confocal laser scanning microscopy (CLSM). (Figure 5) Immuno-fluorescent staining with anti-α-synuclein antibody (LB509) showed a lot of green fluorescent spot inside the HeLa cells. This data indicate that PoPs can penetrate in intracellular and PoPs can use as drug delivery nanoparticle into the cell when we entrapped drug in MSN. Also we noticed that PoPs can enter the nucleus through that sky blue color in nucleus. After, we can applicate this phenomenon in DNA delivery.

3.1.2. Comparison of drug delivery with PoP_{DOX} and MSN_{DOX}

To compare the drug delivery of PoPs, MSN_{DOX} and PoP_{DOX} was prepared. MSN_{DOX} and PoP_{DOX} each represent doxorubicin that doxorubicin, chemotherapeutic agent, were loaded in MSN and PoP. (Figure 6) Drug delivery into the cell was compared with MSN and PoP which blocked the pore of MSN. MSN and PoP was treated to HeLa cell. After 1 hour, cell media was changed with clean media that dose not contain any particles and incubate 18 hours in addition. This is the process for observing drug delivery of nanoparticles that remain in cytoplasm. Drug delivery was examined by cell viability. (Figure 7)

First, cell viability in MSN was lower 30 % than control. Here, no treatment was carried out in control. The meaning of lowered cell viability is the influence of

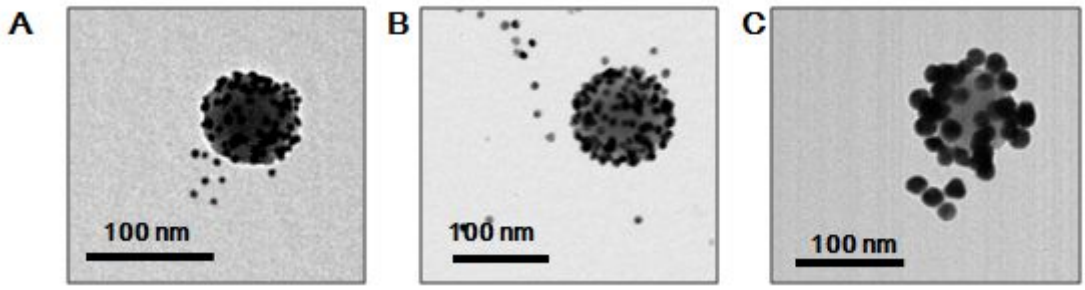
doxorubicin in MSN and doxorubicin killed the cell. However, the cell viability was lowered 50 % when PoP was treated. It is because drug delivery was lowered by loosing the drug in MSN when it is entering into the cell. Thus, PoP_{DOX} can deliver the drug more efficiently than MSN_{DOX}.

3.1.3. Ligand-responsive gate control system

α-Synuclein is known that it is interact with cation and accelerated amyloidogenesis by specific molecular interaction.[37, 38] We use this phenomenon in drug delivery system and Ca^{2+} was chosed as cation that stimulate the amyloidogenesis. Ca^{2+} is important cation in our body system. Because it act as the second messenger and which is stored in endoplasm reticulum.

And BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N0,N0-tetra-acetic acid, acetoxy methyl ester) was used to control Ca^{2+} level in cytoplasm. BAPTA-AM is the membrane-permeable Ca^{2+} chelator that decrease cytosolic Ca^{2+} level.

To figure out ligand-related gate control system, BAPTA-AM was used. BAPTA-AM (50 μg) was incubated with HeLa cell during 1 hour. After changing fresh media, PoP_{DOX} was applied to the cell. And cell viability test was carried out to analyze drug release by Ca^{2+} ligand. (Figure 8)As previous data, 40 % decrease in cell viability was observed in presence of PoP_{DOX}. In comparison, cell viability was 80 % which was increased value in case of treating PoP_{DOX} (60 %). This result suggest that PoPs can response to the change of intracellular Ca^{2+}level by controling the release entrapped drug molecules.



F**iguere 3. PoPs made by a variety of the AuNP sizes** MSN-AuNPαsyn was made by (A) 5nm, (B) 10nm, and (C) 20nm sized AuNPs.

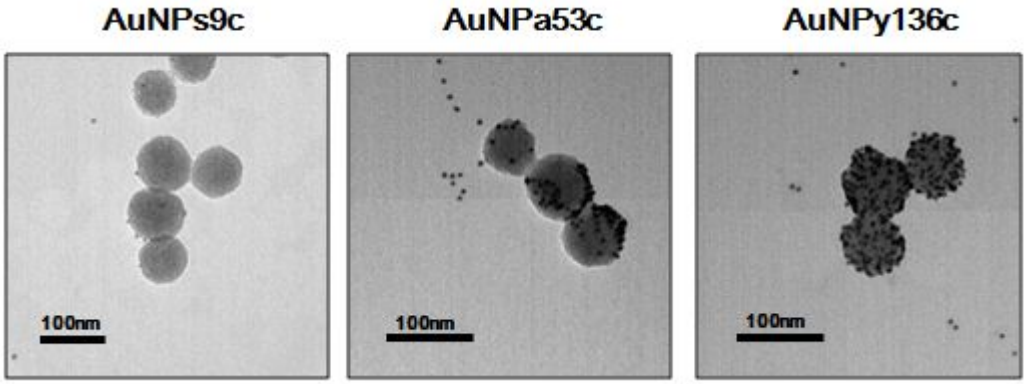


Figure 4. MSN-AuNPαsyn complex α-Synuclein cysteine mutant, S9C, A53C, and Y136C, was mixed with MSN in 10mM citrate buffer (pH 4.4).

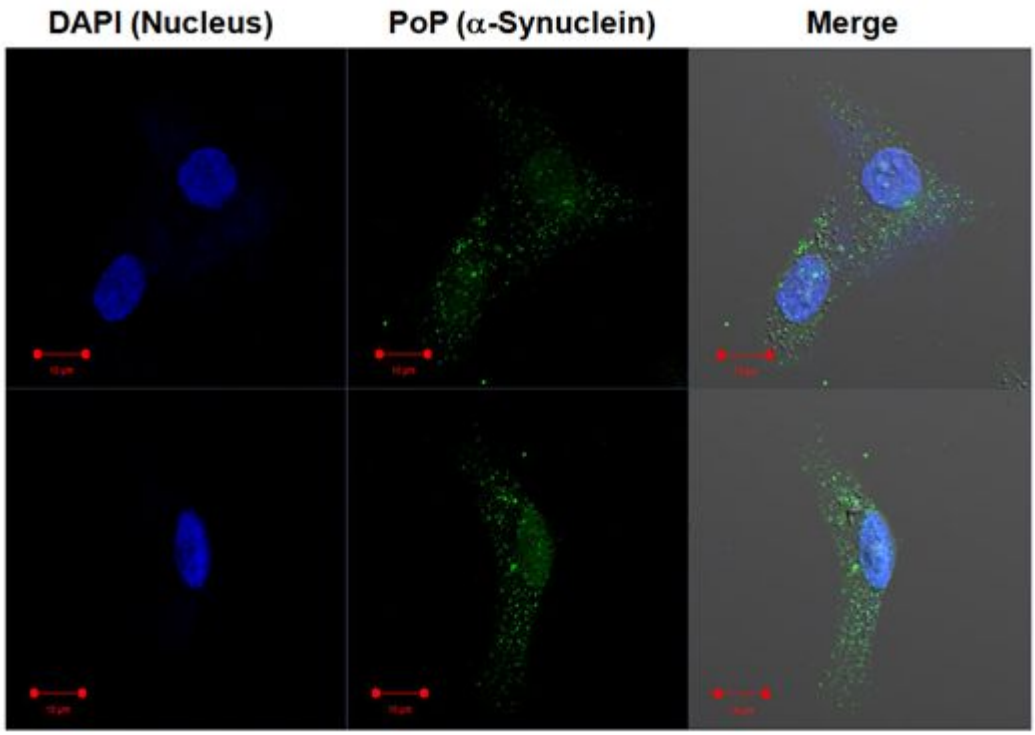


Figure 5. Intracellular delivery of MSN-AuNPαsyn To verify internalization of PoP into the cell, the cells were analyzed by CLSM. Cells were stained with DAPI (blue) and anti-α-synuclein antibody (green). Both fluorescent images were merged with bright-field image.

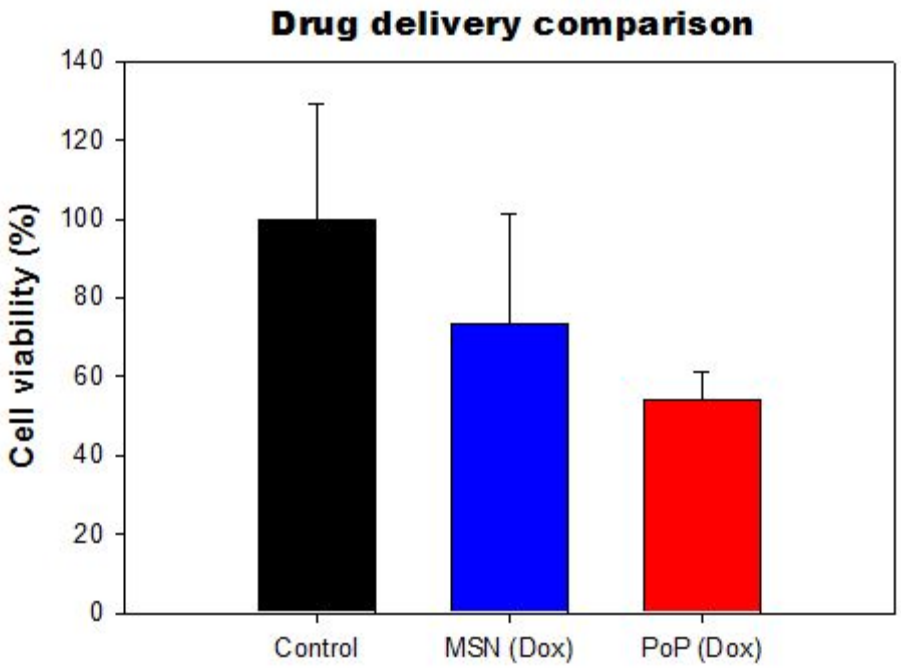


Figure 6. Comparison of drug delivery with MSN and PoP In vitro cytotoxicity of PoP_{DOX}. Any treatment was carried out in control. Compared with control, the cell viability treated with MSN (DOX) and PoP (DOX) was lowered 70% and 50 %, respectively.

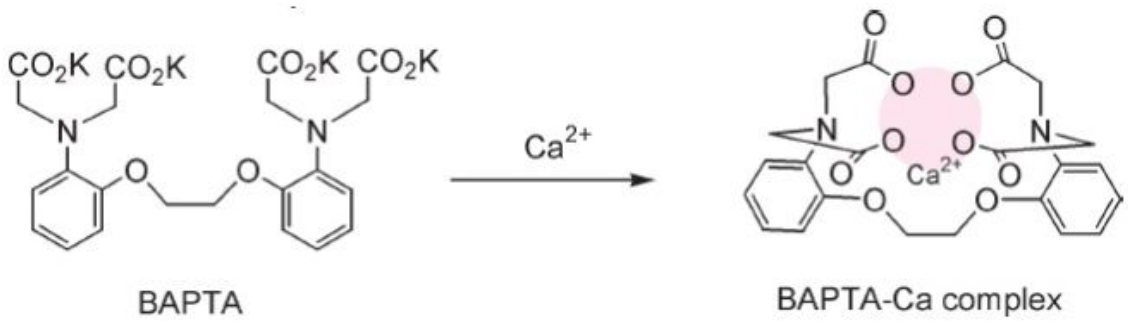


Figure 7. Structure of BAPTA-AM BAPTA-AM is a calcium-selective chelator that binds intracellular calcium ions. And it is useful for controlling the intracellular calcium concentration.

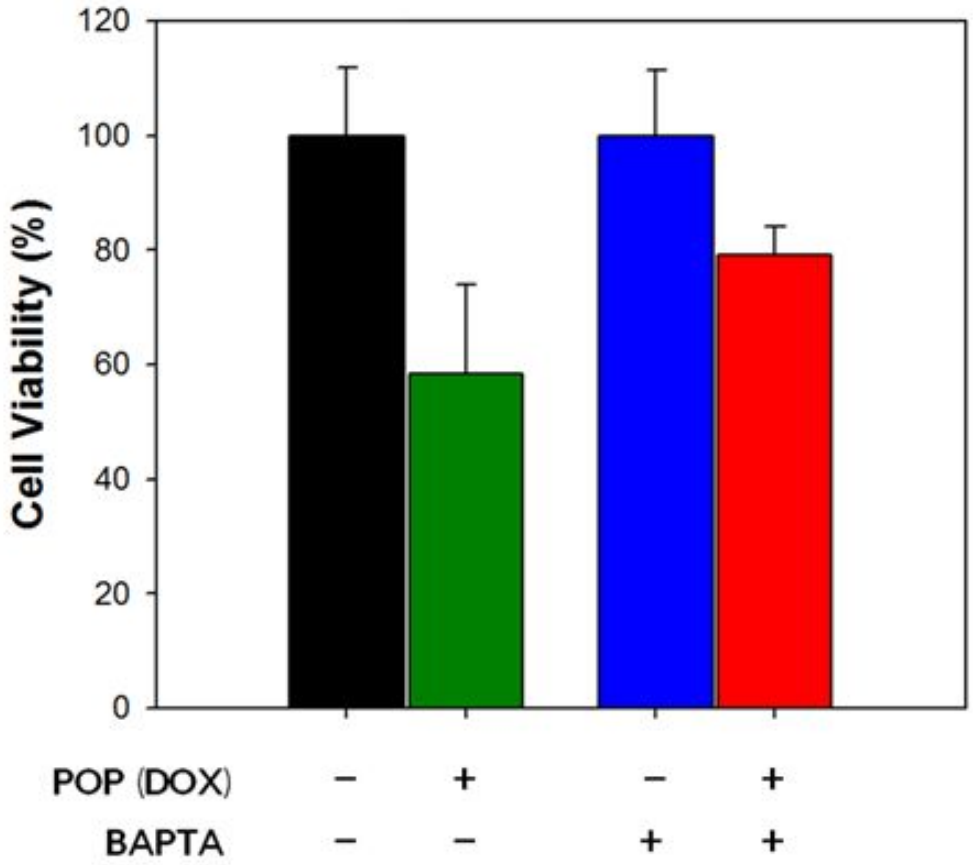


Figure 8. Ca2+-dependent cytotoxic effect of PoP_{DOX} HeLa cells were incubated with PoP_{DOX}. in the absence (left two bars) and in presence (right two bars) of BAPTA. Cell counting was performed following incubation for 18 hours.

3.2. DNA delivery into the cell

3.2.1. Nucleus transportation of α-synuclein and AuNPαsyn

PoP was observed that it can internalize cytosol and nucleus and it was very unique phenomenon. But α-synuclein is known that it remain cytosol. We suspected this event might be related with α-synuclein that coated on AuNP and experiment was conducted.

α-Synuclein (30 μM) and AuNPs9c (0.78 pmole), AuNPa53c (0.78 pmole), AuNPy136c (0.78 pmole) was treated to HeLa cell and incubated 24 hours in CO_2 incubator. (Figure 9) This experiment was analysed by Confocal Laser Scanning Microscopy. α-Synuclein was observed in cytosol only. On the other hand, when α-synuclein was attached on AuNP towards the beginning, middle, and the end part, it can deliver not only in cytoplasm but also in the nucleus. In other words, nucleus penetration can be carried out when α-synuclein is oriented in AuNP. Besides, in case of N-terminal was oriented outside on AuNP, AuNPy136c, nucleus localization was observed. This characteristics of AuNPy136c was applicated in DNA delivery system and AuNPy136c was chose as DNA carrier. Hereafter, AuNPαsyn stand for AuNPy136c in DNA delivery study.

3.2.2. Formation of DNA-AuNPαsyn complex

To apply AuNPαsyn in DNA delivery system, the characteristic of accumulating in nucleus of AuNPαsyn was adopted. And DNA-AuNPαsyn complex was prepared in the first stage of DNA delivery. AuNPαsyn was mixed with DNA in pH 4.4, 10mM citrate buffer and it was incubated at room temperature during 30 minutes. (Figure 10) Formation of DNA-AuNPαsyn complex was observed by transmission electron microscopy (TEM). AuNPαsyn was attached to DNA and it was certain by observing DNA and AuNP incubation. (Figure 10, A) and by agarose gel (Figure 11).

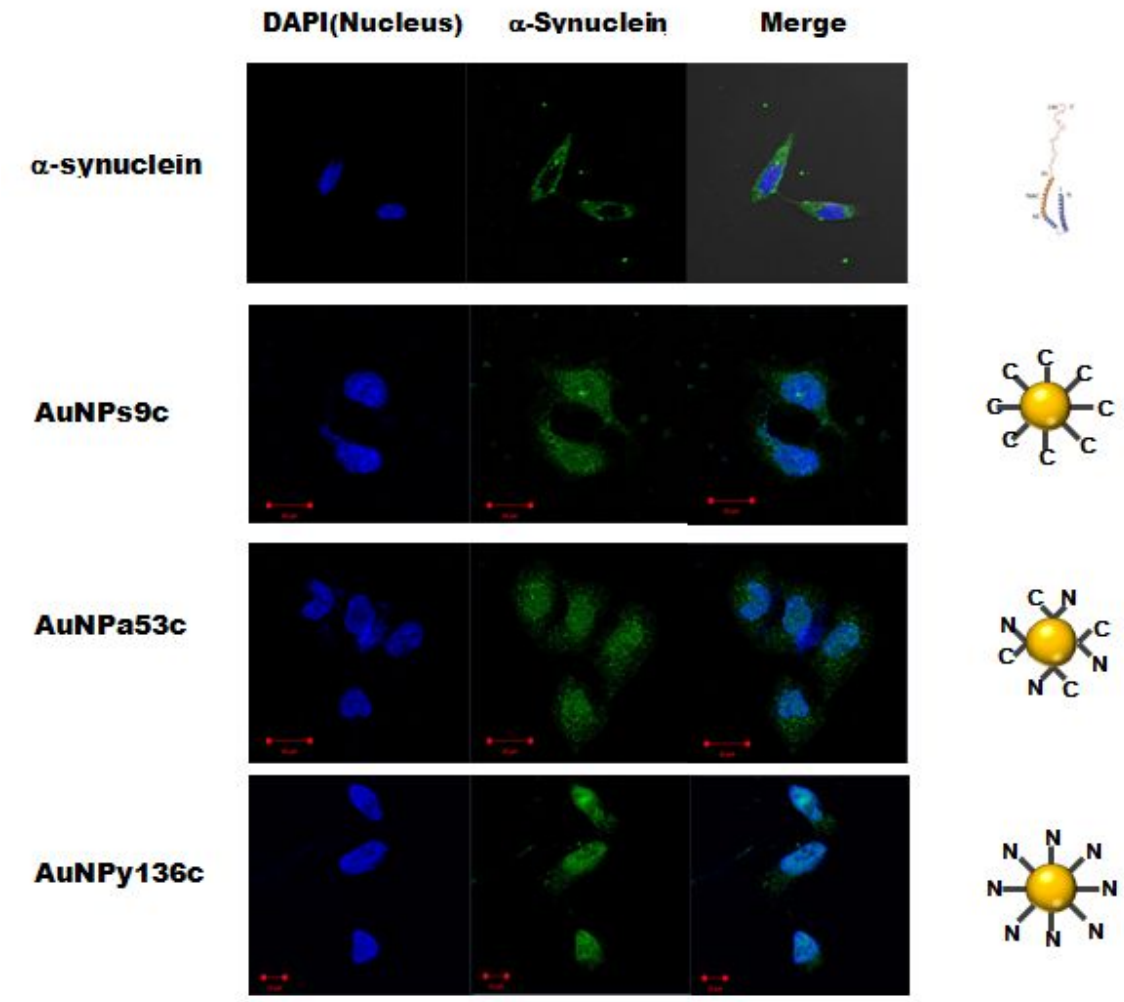


Figure 9. Intracellular delivery α-synuclein and AuNPαsyn Confocal images of HeLa cells treating α-synuclein and AuNPαsyn. Cells were stained with DAPI (blue) and anti-α-synuclein antibody (green) Both fluorescent images were merged with bright-field image.

3.2.3. Confirmation and comparison of transfection

To figure out internalizing into the cell of DNA-AuNPαsyn and complementing the role of transfection, protein expression by gene deliver was verified over time. (Figure 12) In order to compare the degree of gene delivery, lipofectamine was used. Lipofectamine was transfection reagent widely used and it can penetrate cell membrane by wrapping the DNA.

Protein expression level was measured over time by transfecting GFP plasmid respectively. In DNA-AuNPαsyn system, AuNPαsyn (0.0592 pmole) was mixed with GFP plasmid (0.8 μg). In lipofectamine system, lipofectamine (2 μl) and GFP plasmid (0.8 μg) was used. Each the mixtures were practiced on HeLa cell and incubated 6, 12 and 36 hours. (Figure 13)

When compared 6, 12, 36 hours, DNA-AuNPαsyn and lipofectamine can express protein with a similar level in 36 hours. Especially, DNA-AuNPαsyn can express the GFP protein in 6 hours. This result suggests that the faster transfecting and expressing of the protein in the case of DNA-AuNPαsyn compared of lipofectamine.

3.2.4. Comparison of cell cytotoxicity

There are couple of reports of the cytotoxicity when newly engineered nanomaterials are adopted into the cell.[10] So cell cytotoxicity is important factor in nanoparticles application.

In order to observe the bio-compatibility, cell cytotoxicity test was performed. The cells were spreaded in 96-well dish. AuNPαsyn, lipofectamine were added into the cell and incubated. After 24 hours, the cell was incubate with tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] solution for 2 hours. This light red solution changed to dark red color if there are live cell. Because NADH in alive cell can change MTS to Formazan. (Figure 14)

In the case of lipofectamine, its cytotoxicity was very high. So the color of cell media didn't’t change much from light red. (Figure 15) And when cel viability test performed each concentration, the same phenomenon was observed. (Figure 16) The

cells are killed by lipofectamine when the amount of lipofectamine has increased little. On the other hand, AuNPαsyn did't killed the cell much even though the amount is relatively increased a lot.

This new gene delivery system, DNA-AuNPαsyn, has allowed very fast protein expression and observed the less cell cytotoxicity. In order to find out that AuNPαsyn enables delivery of DNA, the next study was performed.

3.3. Mechanism of accumulating in the nucleus of AuNPαsyn

This study was performed to find out that AuNPαsyn enables delivery of DNA. α-Synuclein is known to stay only in the cytoplasm. When α-synuclein was oriented on AuNP, especially N-terminal is oriented out, Truncated α-synuclein was prepared to learn how the phenomenon of accumulation progressed. Especially, N-terminal of α-synuclein was truncated because this part was thought to be critical part.

α-Synuclein consisting of 140 amino acids was cut 20 amino acids each and made 21-140, 41-140 truncated α-synuclein. The reason for this accumulating phenomenon in the nucleus is related with structure or sequence of protein which coated on AuNP. First, pET303/CT-His was digested with restriction enzyme of Xho I and Xba I so it could be liberalized vector. This liberalized vector was found in agarose gel. (Figure 17, A) Also, the insert was amplified by PCR with the forward primer TTAAGAAGGAGGTCTAGAATGAAAACCAAACAGGGTGTGGC, TTAAGAAG GAGGTCTAGAATGGTGGCTGAGAAGACCAAAGA each for 21-140, 41-140 truncated α-synuclein and the reverse primer GTGGTGGTGGTGCTCGAGGGCTTCAGGTTCACA GTCTT containing the restriction enzyme sites underlined above, respectively. The amplified insert DNAs were purified and inset DNA band could find in agarose gel. (Figure 17, B) The insert DNA was ligated into digested pET303/CT-His vector in site-specific recombination manner. All constructs were confirmed by DNA sequencing. Assembled DNA was transformed into the BL21 competent cells. Even though the further study couldn't performed, novel nucleus localization signal obtaining is expected through making AuNPαsyn(21-140, 41-140).

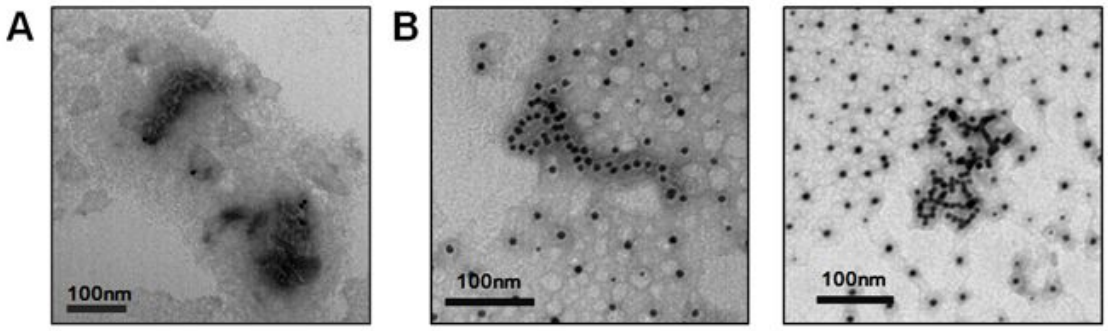
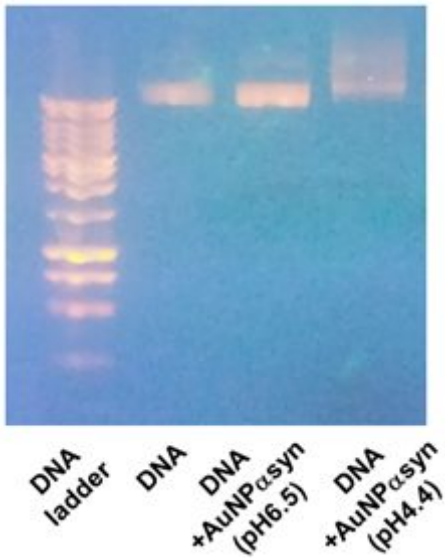


Figure 10. Formation of DNA-AuNPαsyn (A) No complex was observed when DNA and AuNPαsyn were incubated in pH 4.4. (B) DNA-AuNPαsyn complexes were observed by transmission electron microscopy (TEM).



F**igure 11. pH dependnet formation of DNA-AuNPαsyn** DNA-AuNPαsyn complex was made at pH 4.4. It can be sure by smearing DNA band in agarose gel. On the other hand, the comples was not made at pH 6.5 with DNA and AuNPαsyn.

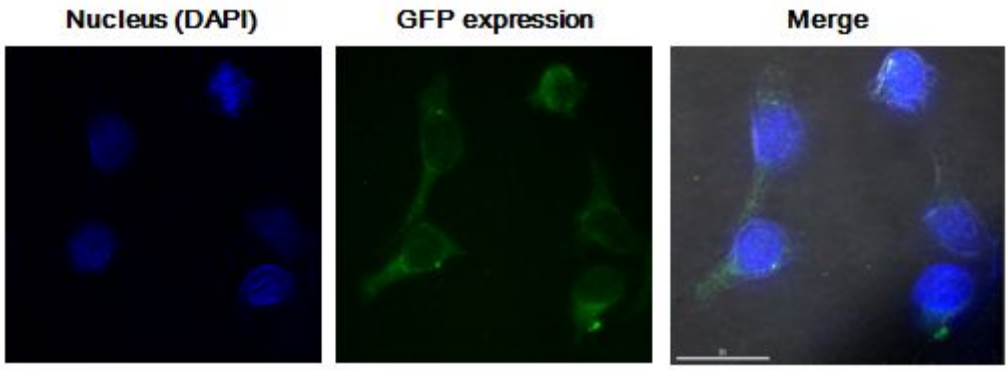


Figure 12. Confirmation of Gene delivery DNA-AuNPαsyn were incubated with HeLa cell. It was This image was detected by Image restoration microscopy. Cell was stained with DAPI (blue) and GFP expression ca be certified.

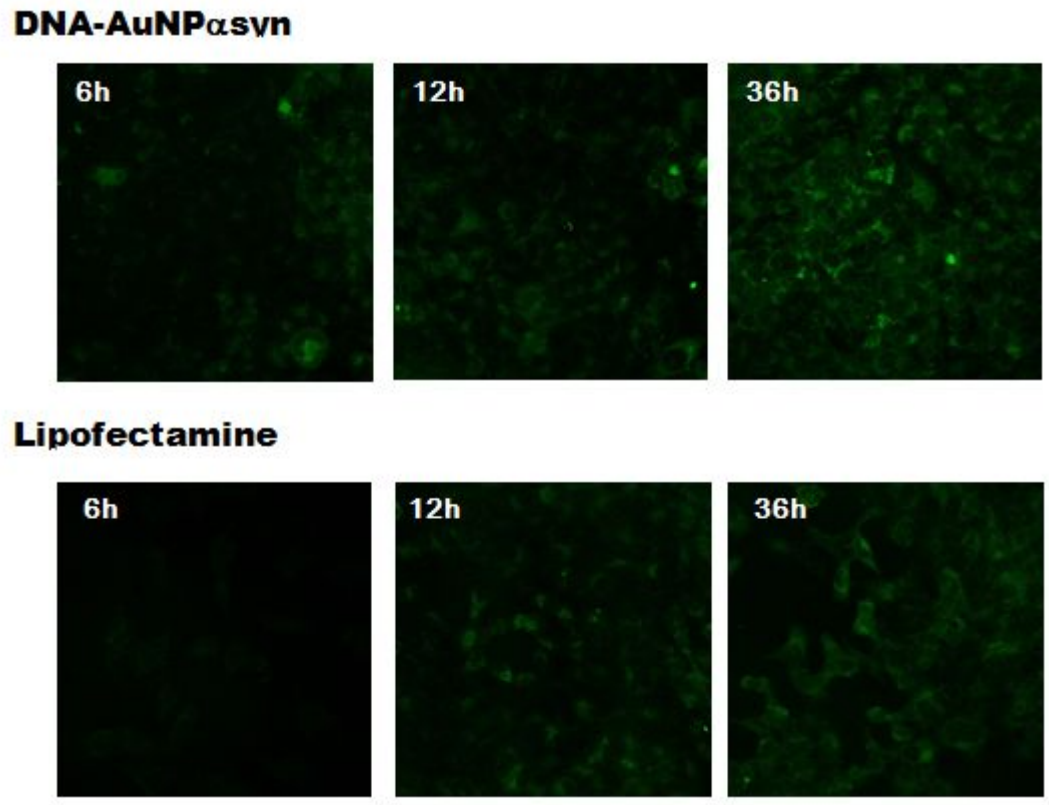


Figure 13. Time dependent GFP expression Plasmid containing GFP sequence were mixed with AuNPαsyn and lipofectamine and treated to HeLa cell. After 6, 12, 36 hours, the cells were observed by confocal laser scanning microscopy.

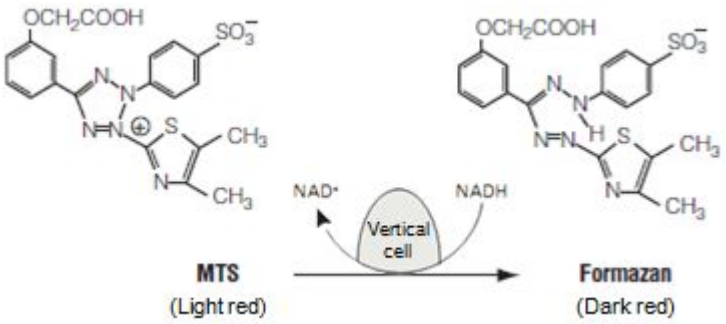


Figure 14. Structure of MTS tetrazolium and its formazan product MTS Light red colored MTS solution change color in dark red by NADH in the vertical cells.

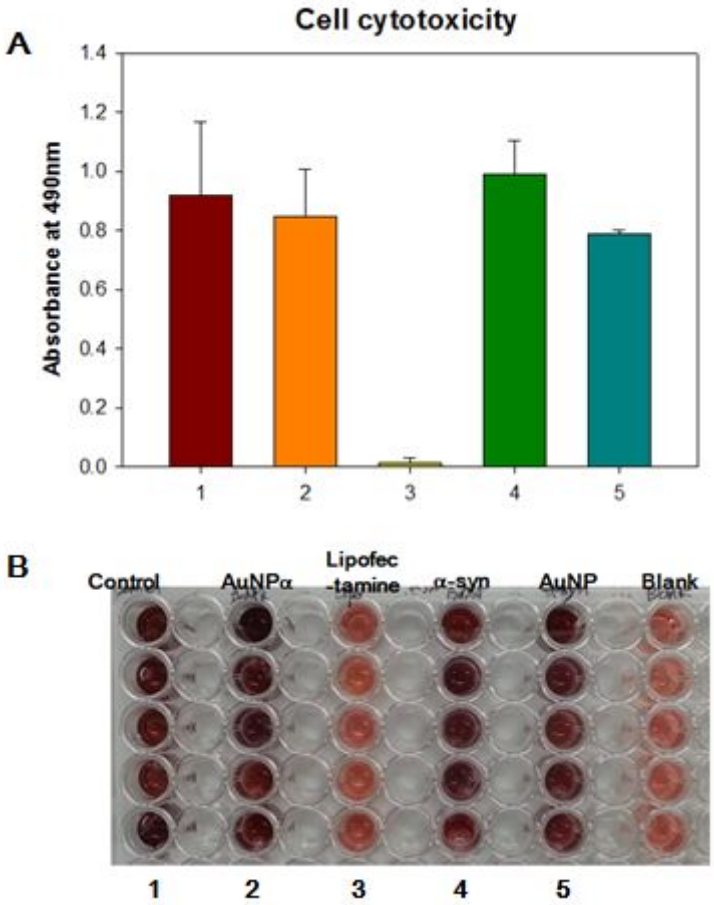


Figure 15. Comparison of the cell cytotoxicity Cell cytotoxicity was measured by MTS solution. (A) Each solution, control (1), AuNPαsyn (2), Lipofectamine (3), α-synuclein (4), AuNP (5), was treated to HeLa cell. The absorbance at 490 nm of each treatment was detected. The result was plotted with graph. (B) The result color of MTS solution is shown.

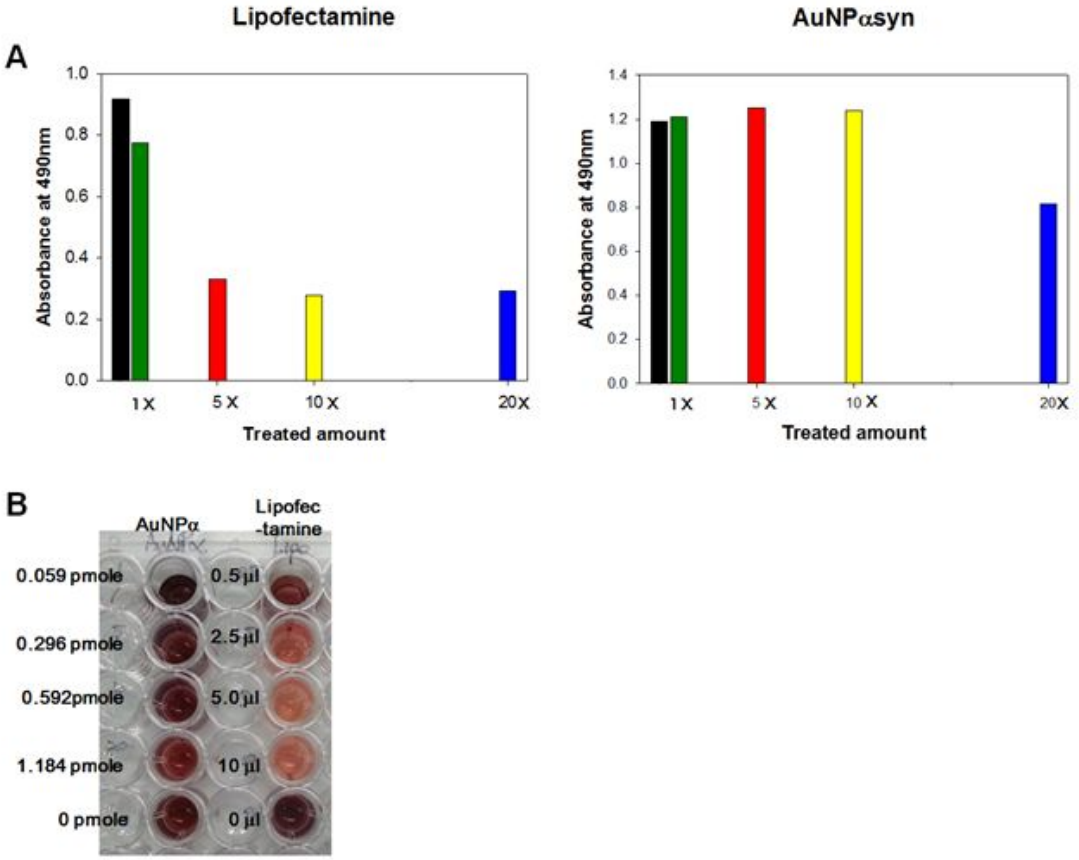


Figure 16. Comparison of the cytotoxicity in terms of concentration (A) Cell proliferation assay, MTS assay, was conducted. Lipofectamine and AuNPαsyn. Lipofectamine and AuNPαsyn was incubated with HeLa cell 24 hours and add MTS solution. The absorbance of samples was measured by plate reader at 490 nm. (B) The sample that incubated each concentration of lipofectamine and AuNPαsyn in 96-well plate.

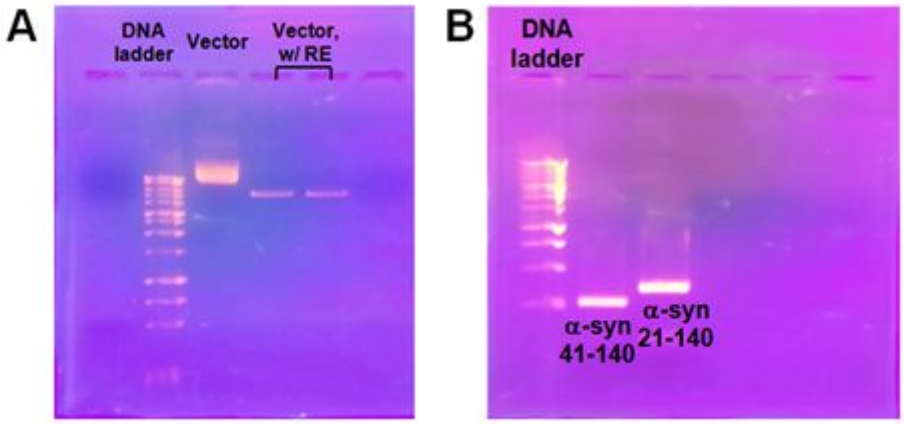


Figure 17. Confirmation the cloning data by agarose gel (A) Purified vector and digested vector was compared by agarose gel. (B) Insert 41-140, 21-140 that amplified by PCR.

Ⅳ. Conclusion

By utilizing the property of α-synuclein and AuNPαsyn, applications of the nanocomplexes of PoP and DNA-AuNPαsyn were performed in the cell. (Figure 18)

First, the drug delivery system was demonstrated into the cell. When PoPs that constructed with AuNPαsyn and MSN were treated to the HeLa cell, PoP can internalize in cytosol. To examine drug delivery of PoPs, PoP_{DOX} and MSN_{DOX} was used and confirmed that PoP_{DOX} can deliver the drugs well. It is indicated that PoP_{DOX} can retain drugs while moving to the cell. Ligand-related gate-control system was showed by using Ca2+ regulator, BAPTA-AM. It was demonstrated by cell cytotoxicity test regulating Ca2+ level.

Second, DNA delivery method was created with DNA-AuNPαsyn. In drug delivery study, we observed that PoPs can internalize in nucleus. It can be used in DNA delivery study. α-Synuclein is the protein that remained in cytosol. But when α-synuclein was oriented respectively on AuNP, AuNPαsyn can go through the nucleus. To applicate this phenomenon in gene delivery system, DNA-AuNPαsyn complex was prepared. The gene delivery system was verified by GFP protein expression using DNA-AuNPαsyn. GFP protein expression and cell cytotoxicity of DNA-AuNPαsyn was compared with lipofectamine. And it was observed that DNA-AuNPαsyn can achieve faster protein expression and lower cell cytotoxicity than lipofectamine.

Third, the mechanism study of accumulating AuNPαsyn in the nucleus was performed. Truncated α-synuclein of 21-140 and 41-140 was prepared on the basis of the assumption that accumulating in the nucleus is related with N-terminal of α-synuclein.

These findings suggest that α-synuclein plays key role in penetrating cytosol and nucleus. Therefore, efforts to elucidate the internalizing in cell of α-synuclein may lead to findings of novel nucleus localization signal for further bionanothechnology.

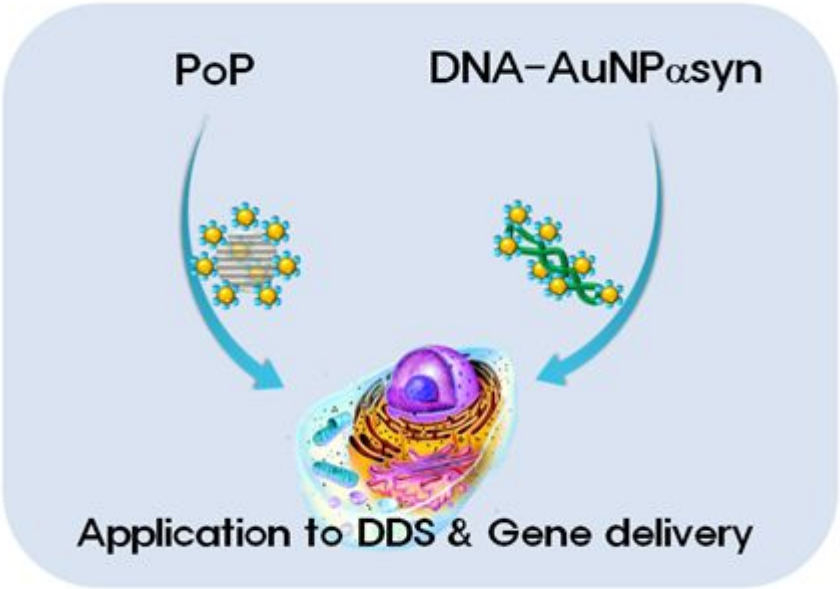


Figure 18. Application of AuNPαsyn to drug delivery and gene delivery

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국문 초록

α-Synuclein은 파킨슨씨 병과 관련 있는 단백질이며, 이것은 세포막을 통과할 수 있다고 보고되어져왔다. 여기서 우리는 α-Synuclein의 이 특성을 이용하여 세포 내로 약물과 유전자 수송을 가능하게 하였다. 첫 번째로, α-Synuclein 변이체를 사용하여, α-Synuclein의 C-terminal 쪽을 금 나노 입자에 붙였고 (AuNPαsyn), 이렇게 만든 나노입자는 핵으로 수송이 잘 일어나는 특성을 보였다. 그리고 AuNPαsyn의 pH를 낮췄을 때 다공성 나노 입자 (MSN)와 유전자가 복합체를 형성하는 것을 관찰할 수 있었다. 이렇게 만들어진 복합체들은 in vitro 실험에서 동물 세포 안으로 들어갈 수 있었다.

MSN의 구멍을 AuNPαsyn으로 막음으로써 MSN-AuNPαsyn을 만들었고, MSN 안에 탑재한 약물의 소실을 막을 수 있었다. 우리는 이 나노입자, MSN-AuNPαsyn을 PoP이라고 명명하였다. PoP 안에 항암 화학 치료제인 doxorubicin을 넣음으로써 극명한 세포 독성을 확인할 수 있었고 이를 통해 PoP안에 탑재된 약물이 세포 안까지 전달됨을 증명할 수 있었다.

DNA-AuNPαsyn은 세포 내 유전자 전달을 위해 사용되었다. GFP 플라스미드와 AuNPαsyn을 복합체로 만들고 세포에 처리하였고 형광 강도의 비교를 통해 시간에 따른 단백질이 발현을 알 수 있었다. 통상적으로 사용되는 형질 주입 시약인 Lipofectamine과의 비교하였을 때, DNA-AuNPαsyn은 더 빠른 형질 주입이 가능하고 더 낮은 세포 독성을 갖는 것으로 이해할 수 있었다.

우리는 AuNPαsyn이 나노입자들이 세포를 통과할 수 있도록 도와주는 시약으로 사용될 수 있음을 제안한다. 따라서 AuNPαsyn을 이용하여 세포에 약물과 유전자의 수송을 가능하게 할 수 있을 것이다. 앞으로의 연구를 통해 우리는 α-Synuclein 내에 존재하는 핵 국소화 신호 서열을 찾아낼 수 있을 것이라 기대한다.

주요 단어 : α-synuclein, 나노 입자 복합체, 약물 수송, 유전자 수송, 나노 입자의 세포 내 수송

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