

Design of Gelatin Nanospheres to Manipulate Mitochondria and Molecular Beacon Functions

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GENERAL INTRODUCTION

Recently, regenerative medicine has attracted a lot of attention as a promising therapy for a wide range of diseases. Regeneration medicine can be classified into regenerative therapy and regenerative research. The core concept of regenerative therapy is to cure diseases by enhancing the natural self-healing ability of potential which is physiologically has the cell ability of proliferation and differentiation. To achieve this goal, two important factors should be considered, such as the extracellular environment of cells and their nutrients. When an appropriate environment is created by biomaterial to positively assist the cell ability, the natural self-healing potential will be promoted for disease treatment. Drug delivery system (DDS) is a biomaterial-based technology which allows drugs to function at the right timing and sites in a suitable concentration. The target drug not only refers to therapeutic drugs, peptides, proteins, and nucleus acids, but also diagnostic and preventive drugs. Since the drug is defined as the substance with a biological function, the cosmetics, functional foods, and health care products are also included in drug. By combining the drugs with biomaterials, drug functions be maximized in several ways, including the controlled release of drugs, the prolongation of drug life-time, the acceleration of drug absorption, and the drug targeting. Therefore, the design of biomaterials and the DDS technology play an essential role in the research field of regenerative therapy¹.

As one of trails to enhance the cell ability, the technology of mitochondria transplantation (MtT) is highly expected. This is because mitochondria (Mt) known as the “power house” in cells, not only participate in oxidative phosphorylation (OXPHOS) to generate energy, but also play an important role in many other cellular processes, such as calcium homeostasis, lipid metabolism, amino acid metabolism, heme biosynthesis, thermogenesis, and apoptosis². The enhanced cell activity will be one of the promising strategies to achieve cell-based regenerative therapy. Mt have a double membrane structure. The outer membrane is smooth and relatively permeable. The inner membrane is highly folded and forms cristae for the reactions of oxidative phosphorylation. The corresponding complex proteins for the electron transport chain as well as adenosine triphosphate (ATP) synthesis, are also located here. The protons are pumped from the mitochondrial matrix to the intermembrane space, leading to a negative charge inside the matrix and a positive charge in the intermembrane space. The established proton gradient, together with the electrical potential, constitutes the

mitochondrial membrane potential, which drives the ATP synthase to synthesize ATP. Therefore, the stability of mitochondrial membrane potential is crucial to maintain the Mt function. The matrix is the reaction site of citric acid cycle (TCA) cycle, where a large number of enzymes as well as the mitochondrial genome, exist. Mt are always under fusion and fission to meet the energy needs in different environments, which is called Mt dynamics³. The fusion refers to the merging of two or more Mt into a whole, which allows the components in the Mt matrix to exchange. The fission is the division of one Mt into two or more smaller Mt, which is important to eliminate the damaged Mt by mitophagy.

MtT is the artificial supplementation or replacement of dysfunctional Mt networks with Mt of respiratory function. This treatment is inspired by the phenomenon of intrinsic intercellular Mt transfer. The intercellular Mt transfer refers to the process by which some Mt are exported from cells, and then delivered to the recipient cells. It can occur through multiple mechanisms including the cell-cell fusion, the exosome-mediated transfer, and the nanotube tunneling⁴. The recipient cells with Mt dysfunction or threatened by the excessive generation of ROS send out distress signals to initiate the Mt transfer. The most common benefit of Mt transfer is to recover the metabolic functions. This benefit varies depending on the recipient cell type. For damaged cells, such as cardiomyocytes, neurons, and alveolar epithelial cells, after receiving healthy mitochondria, their resistance to the oxidative stress enhanced, promoting tissue homeostasis and repair⁵⁻⁶. Mt transfer can also modulate the immune function of immune cells⁶. Some cancer cells even hijack mitochondria within the tumor microenvironment to support their own aerobic respiration and immune evasion⁷. The realization that cells can import Mt from their surroundings or acquire Mt from other cell types, has sparked interest in exploiting this biological property for therapeutic purposes⁸⁻¹⁰. Attempts have been made to artificially isolate Mt from cells and deliver them to recipient cells, leading to the development of Mt transplantation therapy. The internalization of isolated Mt involves an endocytosis pathway of recipient cells¹¹⁻¹². The vast majority of isolated Mt escape endosomally, while a small part of them fail to escape from the endosome and are degraded. The Mt escaped from the endosome will fuse into the original Mt network and share the normal protein, gene, and enzymes to recover the damaged Mt function¹³. Another mechanism is that a minuscule of exogenous Mt triggers the mitophagy and then is rapidly degraded in autophagosomes. The activated mitophagy eliminates the dysfunctional Mt and enhances the Mt biogenesis to produce new Mt, leading to the recovery of Mt functions¹⁴. The MtT has been investigated in clinical trials

for cardiac ischemia-reperfusion injury and has shown encouraging therapeutic effects in animal models of several diseases, including central nervous system diseases, cardiovascular diseases, inflammation, cancer, kidney injury, and lung injury¹⁰.

To promote the MtT technology, the development of biomaterials is essential. The utilization of biomaterials can enhance the MtT efficiency. The common method for the in vivo delivery of M is to co-culture isolated Mt with recipient cells. This method is widely used because it will not change the extracellular environment or produce cytotoxic substances¹⁵. However, it is important to note that the Mt outer membrane has a negative charge same as the cell membrane, which often causes a low efficiency in Mt transplantation. Several methods have been reported to solve this problem. The methods can be categorized into 2 groups. The first group required an additional physical force to assist the isolated Mt penetrate through the cell membrane, such as magnetomitotransfer¹⁶, mitoCeption¹⁷⁻¹⁹, mitoPunch²⁰, and microinjection²¹. The utilization of physical force is highly efficient, but limits the application of in vivo experiments. The second group is the utilization of chemical modification or biomaterial as vectors to deliver isolated Mt. Cell function peptides (Pep-1²²⁻²⁴, transactivator of transcription (TAT) peptide²⁵), cationic polymers (polycation, polyethyleneimine (PEI)²⁶), and cationic lipids (DOTAP and DOPE²⁷, Lipofectamine 2000²⁸), are reported to enhance the efficiency of Mt internalization.

In terms of regenerative research, the main objective is to investigate the mechanism of cell functional maintenance and activation in vivo and discover potential drugs by utilizing energetic cells. In biological experiments, the viability, proliferation, differentiation, and vitality of cells are important indicators to assess and predict the biological function of cells. Environment changes result in the regulation of gene expression which is transcribed to messenger RNA (mRNA), translating to the encoded protein. As the important components of biochemical reactions, mRNA and protein are usually used to determine the cell functions. There have been reported several methods to measure mRNA and protein so far, such as polymerase chain reaction (PCR) and western blotting, respectively²⁹. Because of the high sensitivity, these two methods are widely used in cell research. However, the time-consuming procedures and the destruction of cells often prevent us from evaluating the time course of biological functions for the same cell. As one trail to tackle this problem, molecular imaging (MI) is served as a potential approach. MI is a technique which enables to visualize, characterize, and measure the biologic processes and phenomena at the cellular

or molecular level spatiotemporally³⁰⁻³². In this technique, imaging probes are introduced into the target cell of interest and detected by the diagnostic imaging equipments. There are many types of imaging modality, such as computed tomography (CT), positron emission tomography (PET), and molecular magnetic resonance imaging (mMRI)³⁰. Among them, the optical imaging is one of the most practical modalities because of their high sensitivity, high specificity and non-invasive real-time detection capability³³. Several fluorescent probes have been explored so far. They can be classified into two categories. One is the “always on” probes which are accumulated to the target point of interest and labeled small molecules to emit an enhanced fluorescence signal. However, the high background signal and the requirement of washing step to remove the extra or unbound probes are sometimes their disadvantageous points. The other is activatable probes. They are designed to specifically recognize and conjugate with the target molecule which results in the emission of fluorescence reducing the background signal³⁴⁻³⁵. To conduct the qualitative and quantitative analysis of cell functions in images, it is necessary to design and develop a high specific imaging probe and a high delivery efficiency of imaging probe.

Molecular beacon (MB) is one of the candidates as an activatable fluorescent imaging probe. MB is a nucleic acid derivative modified with a fluorophore at the 5' end and a quencher at the 3' end which enables it to hybridize specifically with the corresponding nucleic acid. Due to the hybridization between the complementary base pairs, MB has a stem-loop structure. Individual MB is in the “fluorescence off” stage. The MB loop hybridization with the specific sequence causes the structure change of MB and results in the fluorescence emission which is the “fluorescence on” stage³⁶. Thus, this high specificity is one advantage of MB. Another advantage of MB is its versatility. By changing the target sequence, varied MB can be readily designed to detect varied targets related to different cell functions, such as proliferation, differentiation, apoptosis, and metabolism. MB is widely used in chemical, biological, biotechnological, and medical science fields³⁷. It is reported that MB is available to detect mRNA³⁸, microRNA³⁹, virus⁴⁰ which show great potential as a useful activate fluorescent probe. In this study, we aim to detect the mRNA expression in the living cell. Therefore, it is necessary to deliver MB to the cell cytosol in the alive condition to allow them hybridize with the target mRNA specifically. However, due to the negative charge of MB itself, it is not always easy to allow MB to internalize into cells at a high efficiency. Thus, the transfection method of high efficiency is required to enhance the efficacy of MB imaging. Several methods of MB transfection have been reported. The physical transfection methods, such as

electroporation⁴¹⁻⁴² and direct injection⁴³⁻⁴⁴, require a special equipment or tool and time-consuming procedures, which are not always acceptable for the visualization of cell functions. On the other hand, transfection carriers including gold nanoparticles⁴⁵⁻⁴⁶, cationized polymer⁴⁷⁻⁴⁹, and liposome⁵⁰⁻⁵¹, have been investigated. However, the materials sometimes show cytotoxicity⁵² and may affect the function of host cells⁵³. In addition, MB is often trapped and degraded in the lysosome due to the inefficient endosomal escape, which sometimes causes a high background signal^{37, 54}. The points should be carefully considered to design the carrier material. Another consideration for the design of MB delivery system is the short remaining time of MB in the cells⁵⁵. It has been reported that unmodified phosphodiester oligonucleotides show half-life as short as 15–20 min in living cells. The nucleic acid derivative of MB will be soon degraded by intracellular nucleases. To resolve this problem, MB is chemically modified with a locked nucleic acid⁵⁶. This modification significantly increased the thermal stability and single-base recognition ability of MB. However, the time duration of detection still limited for 24 hr. As one trail to tackle this duration problem, the intracellular controlled release of MB will be a promising approach. Poly (lactic-co-glycolic) (PLGA) nanoparticles are selected as a carrier for delivering MB into cells to achieve the intracellular controlled release of MB⁵⁷⁻⁵⁸. Based on this finding, it is feasible to chronologically visualize the change of cell functions by the intracellular controlled release of MB.

The objective of this thesis is to design a biomaterial-based delivery system to enhance the delivery of Mt and MB into cells and manipulate their functions. This study focused on the natural polymer of gelatin as the biomaterial. Gelatin is a degraded product of collagen, so-called denatured collagen. It is versatile, biocompatible, and biodegradable, which is widely used in biomedical applications, such as scaffold⁵⁹⁻⁶¹, injectable hydrogel⁶²⁻⁶³, and bio-ink⁶⁴, due to its ability to mimic the extracellular matrix and promote cell adhesion⁶⁵, proliferation and differentiation⁶⁶⁻⁶⁷. Gelatin contains numerous hydrophilic groups and a collagen-like amino acid composition⁶⁸. By the physical encapsulation⁶⁹, chemical bonding⁷⁰, and electrostatic interactions⁷¹, several types of drugs can be incorporated into the hydrogel of gelatin. In the body, gelatin hydrogel incorporating drug is enzymatically degraded, and consequently the drug is released⁷². The profile of drug release from the gelatin hydrogel is regulated by several factors, including the crosslinking density, and the activity of enzymes⁷³.

Another advantage of gelatin is that gelatin is the easiness of chemical modification, because it has multiple functional residues of amine, carboxyl, and hydroxyl groups. For example, to allow gelatin to better interact with substances negatively charged, the cationization is performed to obtain cationized gelatin (cG) by adding amino compounds (for examples, ethylenediamine, putrescine, spermidine, and spermine) to introduce the amino groups. With the positive charge, the hydrogel of cG can release the small interfering RNA (siRNA)⁷⁴ and plasmid DNA (pDNA)⁷⁵⁻⁷⁶ in a controlled manner to achieve the high transfection efficiency. More importantly, the introduction of spermine accelerates the endosomal escape due to the pH buffering capacity of secondary amino groups in spermine⁷⁷⁻⁷⁸. This property is essential in designing the delivery system of Mt and MB because both Mt and MB function in the cell cytosol. Depending on the situation, the gelatin hydrogel can also be prepared into different shapes and sizes, such as films⁷⁹⁻⁸⁰, sponges⁸¹, microspheres⁸², and nanospheres^{74, 83}. In this study, the hydrogel is formulated into nano-size spheres which permit Mt and MB to enhance the cell internalization and the consequent improvemnet of Mt function and the intracellular release of MB to augment their activity, respectively.

In chapter 1, we compared the effect of Mt associated with cG or cationized gelatin nanospheres (cGNS). Isolated Mt were associated with cG (Mt-cG) or cGNS (Mt-cGNS), and then co-cultured with the recipient H9c2 cells. The internalization efficiency significantly increased by utilizing cGNS comparing with cG. There was no significant difference in the internalization efficiency among cGNS with different sizes. Upon incubating with the cells, Mt-cGNS significantly reduced the amount of superoxide generated and up-regulated the ATP amount. It is conceivable that cGNS enhance the cellular internalization of Mt, leading to an improve mitochondrial functions in the recipient cells.

After confirming the feasibility of cGNS to enhance the MtT efficiency to normal cells, we further investigate the fate of exogenous Mt in a Mt-dysfunctional model. In chapter 2, we first optimized the ratio of Mt to cGNS in terms of the Mt activity, and the internalization efficiency. Basing on the optimized condition, we investigated the internalization pathway of Mt-cGNs by treating with different endocytosis inhibitors, and evaluated the endosomal escape to demonstrate that the Mt-cGNS were not degraded. The fusion of exogenous and endogenous Mt was also configured experimentally. It is conceivable that an exchange of Mt content between different Mt through fusion enhanced the Mt activity in the Mt-dysfunctional model.

In Chapters 1 and 2, cGNS were developed as an effective delivery platform to enhance MtT in

terms of an improved cellular internalization, endosomal escape, and mitochondrial function in both normal and Mt-dysfunctional cells. Because these intracellular barriers are also critical for nucleic acid probes, the following chapters apply the same cGNS platform to a different cargo, molecular beacons (MB), which requires an efficient cytosolic delivery and protection from lysosomal degradation to visualize mRNA expression in living cells. By shifting the cargo from therapeutic Mt to diagnostic MB while keeping the gelatin-based carrier, this study evaluates the versatility of the cGNS system as a common biomaterial platform for both regenerative therapy and molecular imaging.

In chapter 3, a two-dimensional (2D) MB direct transfection system was designed by using cGNS. Upon incubating cGNS_{MB} with cells in the opti-MEM, the cGNS_{MB} are transfected in the cells at a high efficiency. After that, the autophagy of cells was induced by culturing in the medium without amino acid and serum. The time profiles of fluorescence intensity of MB well corresponded with the related gene expression during autophagy induction. This indicates that cGNS_{MB} provide a promising method to visualize the autophagy of cells without destroying the cells.

As one model to the body environment, a three-dimensional (3D) cell culture technology was designed. With the combination of biomaterials, the 3D cell culture products, such as multi-layered cell sheets, and cell aggregates, are able to mimic the in vivo environment and survive cells for a long time, which is a strong tool for innovations in cell researches and drug discovery. To understand what exactly happens inside the 3D cell structure, it is important to develop an effective detective method. So far, to visualize the 3D cell aggregate, the section preparation is required to allow the imaging probes to interact with every site of cell aggregates. The way to visualize the 3D cell aggregates without disturbing the cell structure remains a problem. We designed a direct transfection system of cGNS_{MB} to detect the cell function effectively. However, there is still the point to be improved in detecting the cell functions of 3D cell aggregates due to the poor permeability of cGNS_{MB}. In chapter 4, we designed a reverse transfection system of cGNS_{MB} to detect the cell function in 3D cell aggregates at a high homogeneity. The reverse transfection system was achieved by using a gelatin microspheres (GMS) as a scaffold to allow the cGNS_{MB} to be immobilized on GMS. In addition, this system is also effective in internalizing cGNS_{MB} into cells attaching on the surface of GMS. The cell aggregates formed with the GMS associated with cGNS_{MB} (GMS-cGNS_{MB}) achieved the homogeneous transfection of cGNS_{MB} in cell aggregates.

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Chapter 1

Association with Cationized Gelatin Nanospheres Enhances Cell Internalization of Mitochondria Efficiency

Introduction

Mitochondria (Mt) are known as the “powerhouse” of cells. They are involved in multiple important cell functions, such as respiration, biosynthesis, apoptosis, reactive oxygen species (ROS) homeostasis, and metabolism. It has been reported that the mitochondrial dysfunction often results in many diseases, including diabetes, neurodegenerative diseases, cardiovascular diseases, ischemia, spinal cord injury, cancer, and senescence¹⁻⁴. Based on this finding, it is clinically important to find an effective therapeutic way to rescue the mitochondrial dysfunction.

Recently, the intracellular mitochondrial transfer is observed under physiological conditions⁵⁻⁷. The intact healthy mitochondria are transferred from donated cells to recipient cells through four routes, tunneling nanotubes (TNT), extracellular vesicles (EV), gap junction channels (GJCs), and cell fusion^{6, 8}. It is well recognized that the mitochondria transferred in the recipient cells not only contribute to damaged tissue repair, but also the inflammation regulation. This phenomenon gives an idea to treat the mitochondrial dysfunction through the mitochondrial internalization, which means the transplantation of healthy exogenous mitochondria to the mitochondria-defected cells.

As one simple way of the mitochondria internalization, isolated Mt are incubated with the recipient cells to allow them to internalize to the cells. However, due to the electrostatic repulsion between the cell and mitochondria membranes, it is technically difficult to internalize Mt at a high efficiency. Several methods have been reported to solve this problem. For example, a simple method named Mito-Ception is reported⁹. The mixture of cells and isolated Mt is centrifuged, leading to an improved cellular internalization of Mt in vitro, irrespective of the cell type. A Mito-Punch system using a pressure-driven way to achieve highly efficient and quick cellular internalization of Mt is also reported¹⁰. However, there still remain some problems that need to be improved. The methods based on the physical forces often cause the cell membrane opening which threatens the cell viability. In addition, the methods are technically challenging for the Mt internalization in vivo.

From the viewpoint of drug delivery system (DDS) technology, several methods to internalize

nuclear acids, proteins, and molecular probes into cells at a high efficiency, have been developed. The electroporation can assist the cellular internalization of drugs by puncturing a cell membrane with electrical stimulation¹¹. Viral vectors are used for the highly efficient transfection of pDNA¹². Lipid or polymer nanoparticles incorporating drugs have been developed for safe pDNA delivery¹³⁻¹⁴. However, Mt are quite different from the drugs used in terms of the size. In this connection, it is difficult to apply the conventional methods for the cellular internalization of Mt without modification. Therefore, it is necessary to develop techniques suitable for the efficient cellular internalization of Mt.

Modification of Mt surface seems to be essential to increase the cellular internalization of Mt. Mt modification with cell penetrating peptides (CPP) is one of the trials to enhance the Mt internalization. After 48 hr incubation, the CPP-modified Mt were delivered to recipient cells, resulting in the recovery of respiration¹⁵. On the other hand, cationic lipids are utilized for the Mt surface modification. The coating with an artificial DOTAP/DOPE membrane permitted Mt to improve the cellular internalization, leading to an enhanced neuroprotection¹⁶.

Gelatin is a well-known biocompatible and biodegradable material. It is derived from collagen and has been widely applied for food, medicine, and clinical uses, which demonstrates the potential to be a safety carrier material. Cationized gelatin (cG) was successfully synthesized by chemically introducing spermine to the amino groups of gelatin¹⁷. With the positive charge, the hydrogel of cationized gelatin can incorporate the nucleic acids of siRNA¹⁸ and pDNA¹⁷, to achieve the high efficiency of intracellular delivery.

The objective of this study is to evaluate whether or not cGNS are effective in enhancing the cellular internalization of Mt. When the cGNS were mixed with Mt for their simple association and cultured with H9c2 cells for 6 hr, the cellular interaction was evaluated to compare with that of Mt alone and Mt associated with cG. The Mt functions of scavenging ROS and producing ATP were assessed before and after the cells cultured with Mt for internalization.

Materials and Methods

1. Preparation of cationized gelatin (cG) and cationized gelatin nanospheres (cGNS)

According to the preparation procedure previously reported¹⁹ the carboxyl groups of gelatin were chemically converted by introducing amino groups to allow gelatin to cationize. Spermine was added at a molar ratio of 50 to the carboxyl groups of gelatin into 50 ml of double-distilled water (DDW) containing 2.0 g of gelatin (isoelectric point (pI) = 9.0, weight-averaged molecular weight = 99000, derived from pig skin, Nitta Gelatin Inc., Osaka, Japan.). Immediately after that, the solution pH was adjusted to 5.0 by adding 11 M HCl aqueous solution. Next, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was added at a molar ratio of 3 to the carboxyl groups of gelatin. The reaction mixture was agitated at 37 °C for 18 hr, and then dialyzed against DDW for 3 days at room temperature. The dialyzed solution was freeze-dried to obtain cationized gelatin (cG). To determine the percentage of amino groups introduced into gelatin, the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) method was performed. The percentage was 44.5 mole% per the carboxyl groups of gelatin.

cGNS were prepared by the conventional coacervation method¹⁸. In brief, 1.25 ml of cationized gelatin aqueous solution (2.5, 3.25, 6.25, and 10 wt%) was warmed up to 40 °C. Next, 5 ml of acetone was added to the solution, and the coacervate was formed. Then, glutaraldehyde (25 wt%, 20 µl) was added to chemically crosslink cGNS for 6 hr. For the blocking of aldehyde groups unreacted, 2 ml of glycine aqueous solution (0.5 M) was added. The resulting solution was agitated overnight at 40 °C and the residual acetone was evaporated. cGNS were collected by the centrifugation of 250,000 g for 30 min at 25 °C and resuspended in DDW. cGNS were resuspended in 10 mM phosphate buffered-saline solution (PBS, pH7.4), and the apparent size of nanospheres was measured by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). On the other hand, cGNS were resuspended in 10 mM phosphate buffer solution (PB, pH7.4), and the zeta potential was measured by electrophoresis light scattering (ELS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK).

2. Cell culture

H9c2 cardiomyoblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The H9c2 cells were maintained in Dulbecco's Modified Eagle Medium (Thermo

Fisher Scientific, Inc., Massachusetts) supplemented with 10 vol% fetal bovine serum and 1 vol% penicillin/streptomycin mixture. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. Stably Green fluorescent protein (GFP) expressing H9c2 cells (GFP-H9c2) were generated to visualize the mitochondria isolated. A plasmid Su9-EGFP was kindly provided by Dr. Ishihara²⁰ (Department of Biological Sciences, Graduate School of Science, Osaka University). According to the manufacturer's protocol, the pDNA was transfected by using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc., Massachusetts). After the transfection, cells were cultured in the medium containing a selective agent, G418, for 3 weeks. Finally, GFP-expressing cells were selected and collected by Cell Sorter MA900 (Sony Inc., Tokyo, Japan). The Su9-EGFP-expressing H9c2 cells were stained with the mitochondria-specific dye MitoBright LT Deep Red (Dojindo, Kumamoto, Japan) or Hoechst 33258 (Thermo Fisher Scientific, Inc., Massachusetts). The expression of GFP in H9c2 cells was observed by a fluorescent microscopy BZ-X700 (KEYENCE Co., Ltd., Osaka, Japan).

3. Isolation of mitochondria (Mt)

Mitochondria were isolated from H9c2 or GFP-H9c2 cells. After disrupting cells through a 27-gauge needle, the isolation was conducted based on the research procedure of Maeda et al²¹. Briefly, cells were ruptured by 20 strokes using a syringe with 27-gauge needle in homogenization buffer [HB; 20 mM HEPES-KOH (pH 7.4), 220 mM mannitol 70 mM sucrose 100 μM PMSF (Phenylmethylsulfonyl fluoride)]. The homogenate was centrifuged at 400 g at 4 °C for 5 min to remove unbroken cells, and the supernatant was further centrifuged at 6,000 g for 5 min to pellet the isolated Mt-enriched fraction (Mt fraction). The mitochondria isolated from H9c2 cells are defined as Mt, while the mitochondria isolated from GFP-H9c2 are defined as Mt-GFP. The supernatant was collected as a cytosolic fraction. The amount of protein in each fraction was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Massachusetts). The purity of mitochondria obtained was confirmed by detecting a mitochondrial-specific protein, COXIV, and a cytosol specific protein, α-tubulin as comparison.

4. Preparation of Mt associated with cG and cGNS

The cG and cGNS (5 μl, 1 mg/ml) were added to 95 μl suspension of Mt (15 μg) and gently mixed, followed by incubation for 10 min on ice. The zeta potential was measured by ELS

(Zetasizer NanoZS, Malvern Instruments Ltd., Worcestershire, UK). In addition, Mt labeled with MitoBright LT DeepRed and cGNS-labeled with FITC were mixed to allow to associate to each other. The co-localization was observed by a fluorescent microscopy BZ-X700 (KEYENCE Co., Ltd., Osaka, Japan).

5. Confocal microscopy to evaluate cellular internalization of Mt

H9c2 cells were seeded in a glass bottom dishes (diameter= 35mm, Matsunami Glass Industries Ltd., Tokyo, Japan) (1×10^5 cells/well). After cultured overnight, cells were stained with 100 nM of MitoTracker™ Red CMRos (Thermo Fisher Scientific, Inc., Massachusetts) for 30 min. And then, the stained cells were incubated with Mt-GFP-cGNS for 6 hr. The z-stack images were taken by a confocal laser scanning microscope (FV1000D, Olympus, Japan).

6. Western blotting to evaluate cellular internalization of Mt

H9c2 cells were seeded in each well of 6 multi-well culture plates (1×10^5 cells/well). After cultured overnight, cells were incubated with Mt-GFP, Mt-GFP-cG, and Mt-GFP-cGNS for 6 hr. And then, cells were trypsinized and centrifuged (400 g, 3 min, 25°C) to collect the cells. The pellets were mixed with the Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), to prepare the cell lysate. The amount of GFP in the cell lysates was quantified by the western blotting. In brief, samples were loaded on Any kD™ Mini-PROTEAN TGX™ Precast Gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and electrophoretically run at 0.02 A for 15 min and 0.04 A for 40 min. Then, the samples were transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with the blocking solution (Immuno Block™, KAC Co. Ltd., Kyoto, Japan) for 1 hr and washed 3 times with Tris-buffered saline solution containing 1 vol% Tween-20 (TBST). For detection, the primary antibodies were added and the membranes were incubated overnight at 4 °C. The blots were washed 3 times with TBST before incubation with the HRP-conjugated secondary antibody for 1 hr at room temperature. Finally, membranes were washed 3 times with TBST and detected using a Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Inc., Waltham, MA, USA) with a chemiluminescence imaging system LAS-4000 (FUJIFILM Co., Tokyo, Japan). The efficiency was calculated as the ratio of the amount of GFP contained in each sample to that of GFP in the sample cultured with Mt-GFP. The antibodies used in this study are summarized in **Table 3**.

7. Cytochrome c reduction Assay

H9c2 cells (5×10^3 cells/well) were seeded in each well of 96 multi-well culture plate (Corning Inc., Corning, NY, USA) and incubated overnight. After incubation with Mt, Mt-cGNS (50, 100, 200, and 400) for 6 hr, cells were treated with 5 μ M menadione and 0.5 mg/ml cytochrome c in Hank's Balanced Salt Solution (HBSS) for 1 hr. The absorption at 550 nm was measured by a multi-mode microplate reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan).

8. ATP Assay

To determine the activity of isolated Mt, isolated Mt were separated into three group. One group was associated with cGNS to prepare Mt-cGNS, while another group was treated with 10 μ M of oligomycin for 20 min. The triplicate samples of Mt, Mt-cGNS, and oligomycin-treated Mt were added to each well of 96 multi-well white wall plate (SUMITOMO BAKELITE Co. Ltd. Japan) followed by adding same volume of CellTiter-Glo® 2.0 reagent (Promega Corporation, Madison, USA). After mixing on the shaker for 5 min covered with a aluminum foil, the luminescent signal was stabilized for 10 min and record by Multi-mode Microplate Reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan).

H9c2 cells (5×10^3 cells/well) were seeded in each well of 96 multi-well plate (Corning Inc., Corning, NY, USA) and incubated overnight. After incubation with Mt, Mt-cGNS (50, 100, 200, and 400) for 6 hr, the cells were washed with PBS two times. Then, 50 μ l PBS was added to each well, followed by adding 50 μ l of CellTiter-Glo® 2.0 reagent into each well. After mixing on the shaker for 5 min covered with a aluminum foil, 70 μ l solution in each well was removed to each well of 96 multi-well white wall plate to decrease the crosstalk of the luminescent signal during detection. After stabilization of the luminescent signal at room temperature for 10 min, the luminescence was recorded by Multi-mode Microplate Reader.

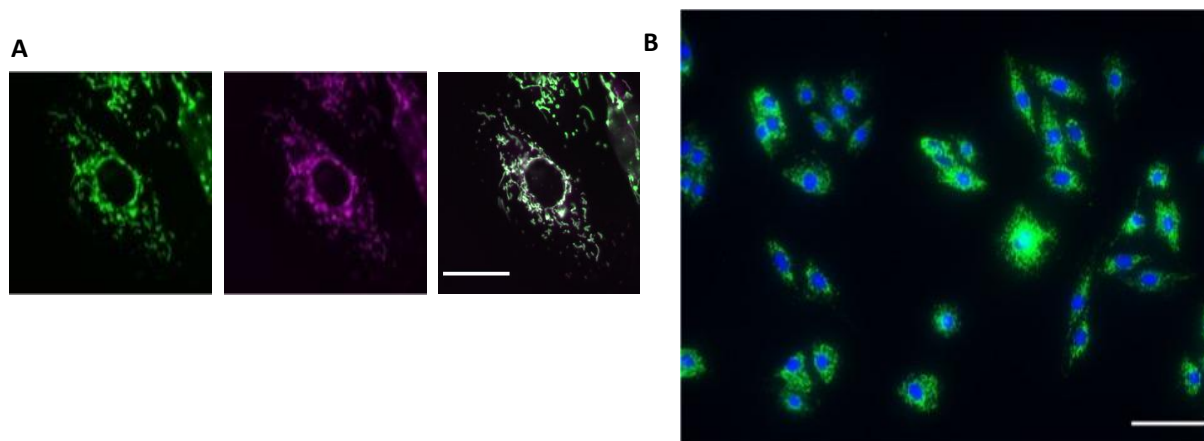
9. Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). All the statistical analysis was performed by using an unpaired *t*-test with two tails. Difference with $p < 0.05$ was considered to be significant.

Results

1. Characterization of Su-EGFP-expressing H9c2 cells

Figure 1 shows the fluorescent images of Su9-EGFP-expressing H9c2 cells. The red fluorescence of MitoBright LT DeepRed shows the mitochondria location. The green fluorescence of GFP was observed, which is corresponding with that of mitochondria. In addition, the



fluorescence of GFP was detected around nuclei (stained with Hoechst 33258).

Figure 1. A. fluorescent images of Su9-EGFP-expressing H9c2 cells stained with MitoBright LT Deep Red: GFP (left), MitoBright LT Deep Red (middle), and co-localization (right) images. Scale bar is 30 μ m. B. fluorescent images of Su9-EGFP-expressing H9c2 cells stained with Hoechst 33258 Blue: nuclei. Green: Su9-EGFP. Scale bar is 60 μ m.

2. Characterization of mitochondria isolated

Figure 2 shows the western blotting result of different cell fractions obtained during the mitochondria isolation. The COXIV of a mitochondria-specific protein was not detected in the cytosol fraction, while small amount of a cytosol-specific protein, α -tubulin was detected in the mitochondria fraction. The purity of mitochondria isolated was confirmed. On the other hand, the ATP amount of isolated mitochondria increased proportionately with an increase in the mitochondria amount (**Figure 3**).

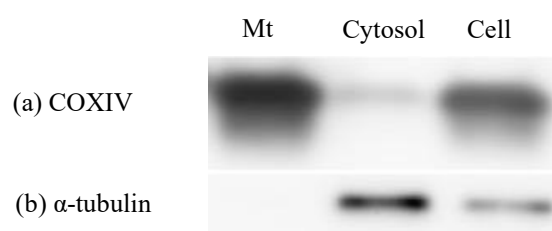


Figure 2. Western blotting images of different fractions obtained by the conventional differential centrifugation: Mt fraction (Mt), cytosolic fraction (Cytosol), and total cell extracts (Cell). Anti-COXIV (a) and anti- α -tubulin antibodies (b) are used as the markers of Mt and cytosol, respectively.

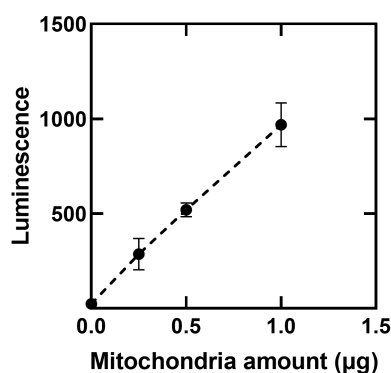


Figure 3. Relationship between mitochondria amount and the luminescence.

3. Characterization of mitochondria associated with or without cGNS

Table 1 summarizes the size and potential of cGNS used. The apparent size of cGNS increased with an increase of cG concentration. The value of zeta potential was positive for any size of cGNS. **Table 2** shows the zeta potentials of Mt-cGNS. After association with cGNS, the zeta potentials of Mt increased. The mitochondria labeled with MitoBright LT DeepRed was co-localized with FITC labeled cGNS, irrespective of the cGNS size (**Figure 4**). In addition, there was no difference between Mt and Mt-cGNS in terms of ATP amount (**Figure 5**).

Table 1. Physicochemical properties of cGNS prepared.

Code	The concentration of cG added (wt%)	Apparent size (nm)	Zeta potential (mV)
cGNS(50)	2.50	51.8 ± 0.12^a	6.21 ± 0.71^a
cGNS(100)	3.75	94.4 ± 0.39	8.75 ± 0.64
cGNS(200)	6.25	197.0 ± 2.26	7.51 ± 0.52
cGNS(400)	10.0	403.0 ± 4.97	6.99 ± 0.21

a) mean $\pm$ standard deviation

Table 2. Zeta potentials of Mt and Mt associated with cG and cGNS.

Sample	Zeta potential (mV)
Mt	-26.3 ± 1.79^a
Mt-cG	-9.09 ± 0.714
Mt-cGNS(50)	-14.4 ± 1.59
Mt-cGNS(100)	-20.4 ± 0.41
Mt-cGNS(200)	-20.4 ± 0.49
Mt-cGNS(400)	-20.7 ± 0.62

a) mean $\pm$ standard deviation

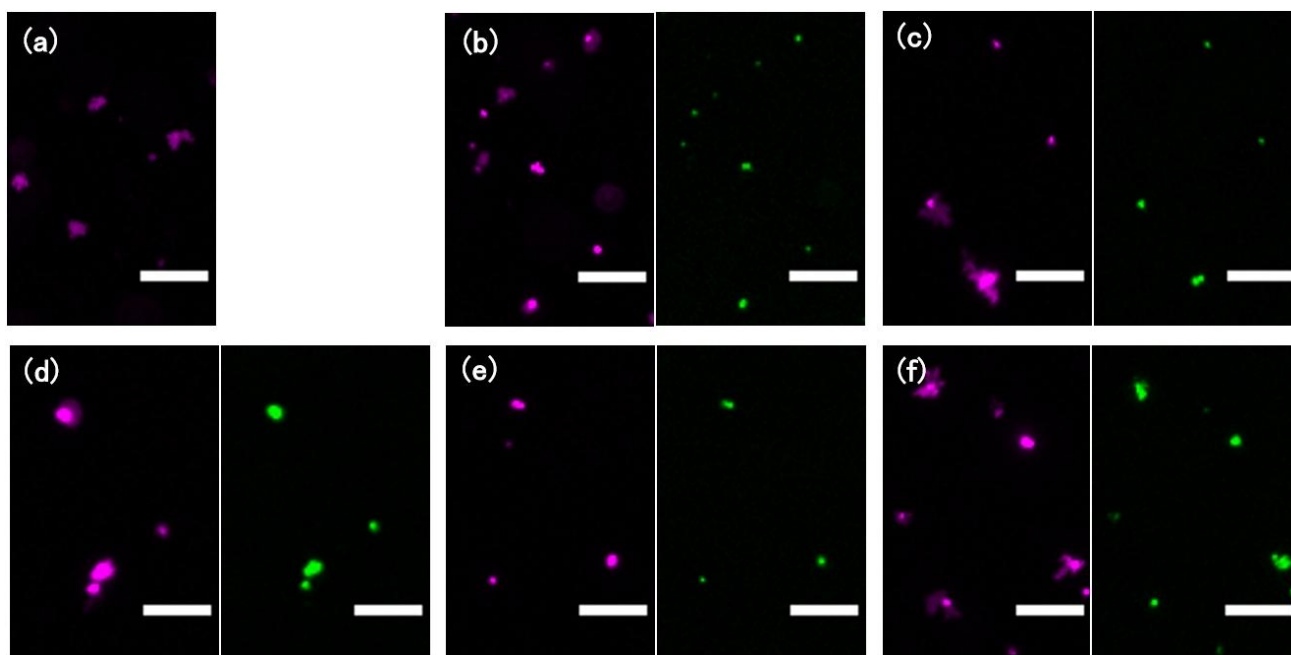


Figure 4. Fluorescent microscopic images of Mt (a), Mt-cG (b), Mt-cGNS(50) (c), Mt-cGNS(100) (d), Mt-cGNS(200) (e), and Mt-cGNS(400) (f). Magenta: Mt. Green: cG or cGNS. Scale bar is 5 μ m.

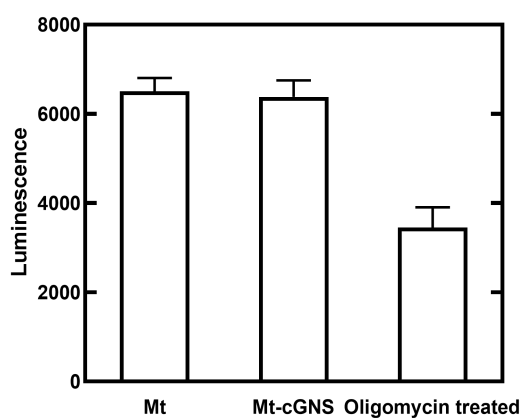


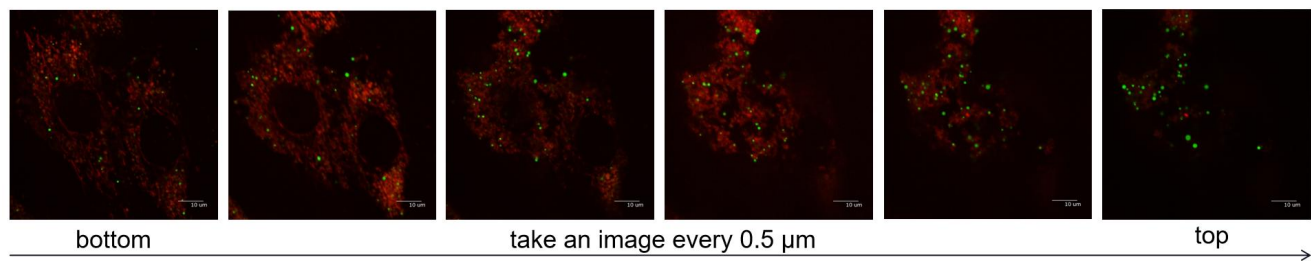
Figure 5. Luminescence of Mt isolated with and without cGNS coating. Mt treated with oligomycin are used as a positive control.

4. Cellular internalization efficiency of Mt associated with or without cG and cGNS

Mt-GFP were detected in every stack of recipient H9c2 cells after 6 hr co-incubation (**Figure 6**). **Figure 7** shows the efficiency of cellular internalization of Mt and Mt associated with cG and cGNS. The Mt-GFP internalization was observed in all the Mt-treated groups, indicating the successful cellular internalization of exogenous Mt. Significantly higher amount of GFP was observed for the

Mt-cGNS groups compared with that of only Mt group and Mt-cG groups. However, there was no significant difference in the internalization efficiency among any sizes of cGNS.

A



B

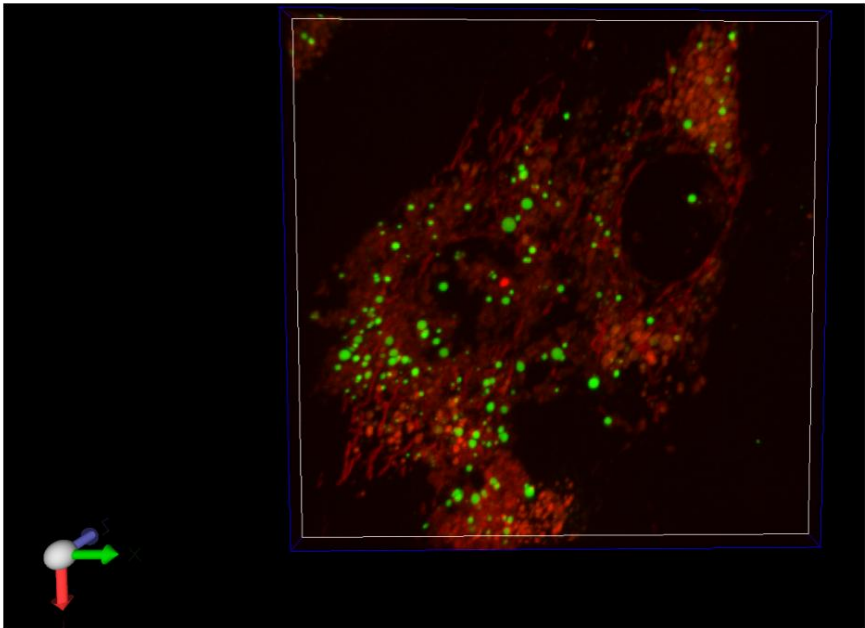
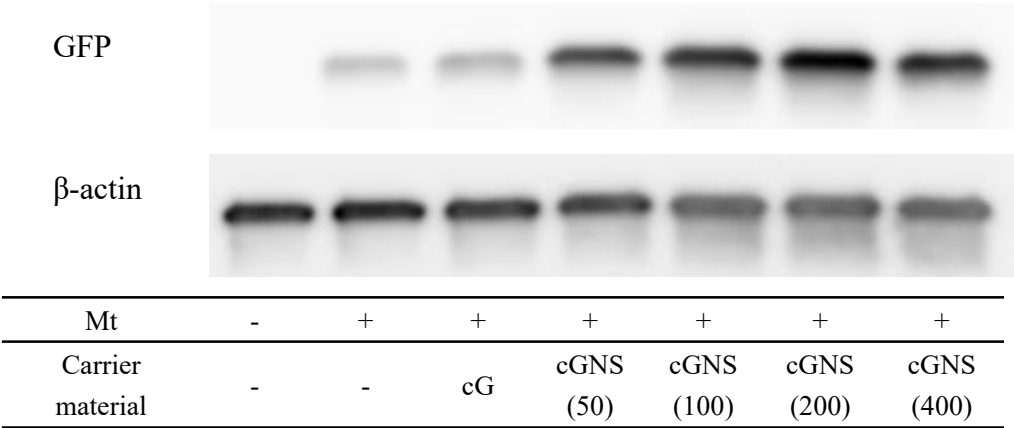


Figure 6. A. Fluorescence images of H9c2 cells 6 hr after incubation with Mt-GFP-cGNS at different z-positions. Red: endogenous mitochondria. Green: exogenous mitochondria. Scale bar: 1 μ m. B. A 3D image of H9c2 cells incubated with Mt-GFP-cGNS.

A



B

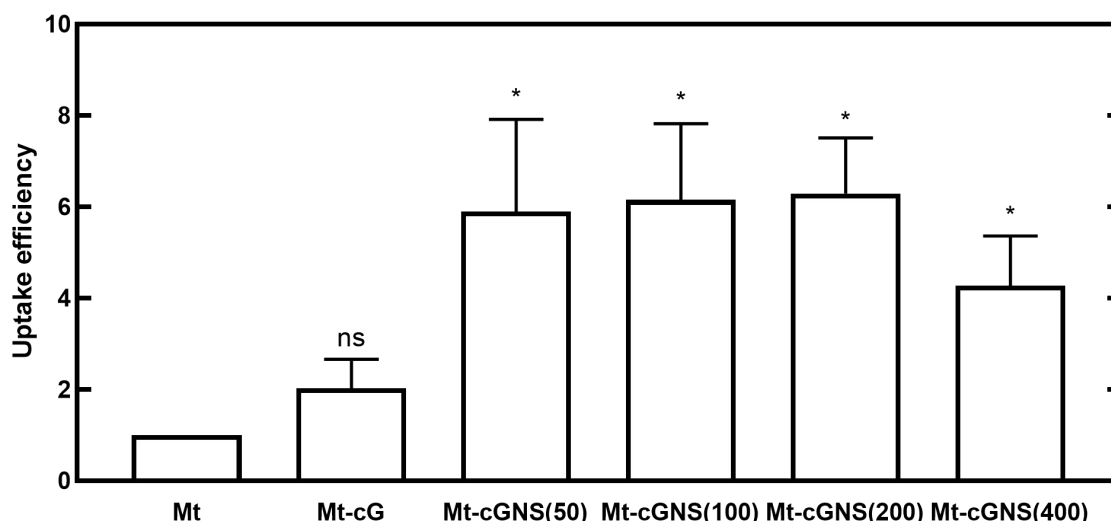


Figure 7. A. Western blotting image of cell lysates after cellular internalization of Mt and Mt associated with cG and cGNS. B. Efficiency of cellular internalization of Mt and Mt associated with cG and cGNS. The value for the Mt group is 1. *, $p < 0.05$; significant difference from the Mt group, ns; not significant.

5. Functions of cells internalized with Mt with or without cG and cGNS

Figure 8 shows the amount of superoxide anions. In comparison with the untreated cells, cells treated with menadione generated a higher amount of superoxide. The amount of superoxide generated was reduced for the cells incubated with the Mt to a significant great extent compared with those without treatment. In addition, the amount in cells incubated with the Mt-cGNS was significantly less than that of cells incubated with Mt and Mt-cG. No significantly higher reducing effect than Mt was observed for cells incubated with Mt-cG, which is different from the Mt-cGNS. The ATP amount in the cells incubated with Mt-cGNS was higher than that of no treated cells (**Figure 9**). Significantly higher ATP amount was observed after applying 7.5 and 15 μg Mt-cGNS compared with that of no treated cells. On the contrary, there was no significant difference between non-treated and 30 μg Mt-cGNS-cultured cells.

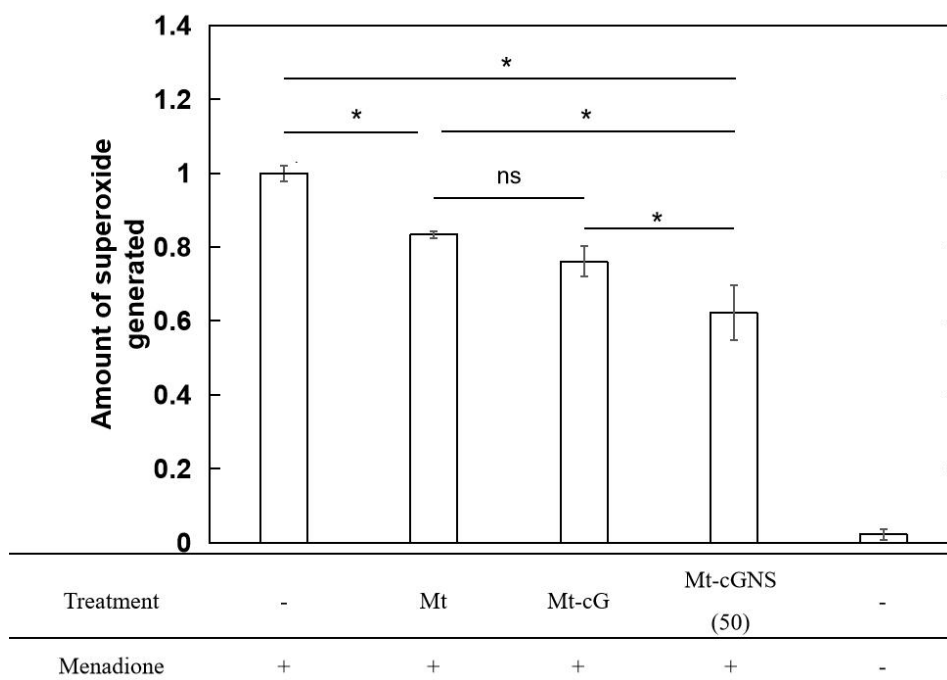


Figure 8. The amount of superoxide generated from cells by the menadione treatment. The value of cells treated with menadione is 1. *, $p < 0.05$; significant difference between the two groups, ns; not significant.

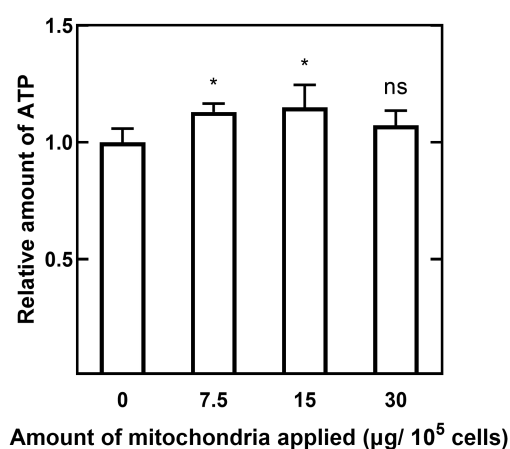


Figure 9. ATP amount of cells 6 hr incubated with 7.5, 15, and 30 μg Mt associated with cGNS. *, $p < 0.05$; significant difference from the value of mitochondria amount 0 μg , ns; not significant.

Discussion

In present study, we demonstrates the methodological feasibility of a cationized material, cGNS, to enhance the cellular internalization efficiency of Mt isolated into cells to increase cell functions. cG and cGNS with different sizes were prepared according to the method previously reported¹⁸. The Mt were isolated from H9c2 cells while the purity and activity of Mt isolated were experimentally

confirmed. The increased zeta potential of Mt by mixing cG and cGNS revealed the successful association of cG and cGNS with Mt. Significant higher internalization efficiency was observed after the internalization of Mt-cGNS compared with that of Mt-cG. In addition, the internalization of Mt-cGNS allowed cells to improve the metabolic functions in terms of a decreased superoxide amount and increased ATP production. Mitochondrial dysfunction is a hallmark of many types of diseases. An artificial mitochondria transfer gives a new strategy to rescue the mitochondrial dysfunction. However, the low transfer efficiency is still challenging for developing this research field. So far, the technology to enhance the mitochondria internalization efficiency can be divided into two categories. The first category is using physical force to allow more mitochondria to internalize into recipient cells, for instance, Mito-Ception⁹, Mito-Punch¹⁰, and Magnetomitotransfer²². However, they are challenging for Mt internalization in vivo. Another category is Mt surface modification. Cell-penetrating peptides¹⁵, cationic ligand conjugated-polymer²³, and cationic lipid modification¹⁶ has been reported can enhance the Mt internalization efficiency.

Comparing with other studies, just by a mixing procedure, both cG and cGNS can be easily modified on Mt surface by the electrostatic interaction. In addition, it is reported that spermine with secondary amino groups shows a buffering effect to enhance the endosomal escape²⁴. It is conceivable that cG and cGNS with spermine play a role in the pH-buffering effect to allow Mt to escape from the endosome. The biodegradability of cGNS was confirmed in a previous study, but should be optimized for the Mt internalization in the future.

To evaluate the cellular internalization efficiency of mitochondria isolated, GFP-expressing H9c2 cells were prepared as the exogenous mitochondria source. Although a fluorescence dye is often used to label mitochondria, mitochondria are dynamically fused and fissured, leading to loss of fluorescence signal²⁵⁻²⁷. In addition, a long time incubation might lead to the fluorescence detaching from mitochondria. As one trial to tackle this issue, mitochondria genetically labeled with GFP were prepared by transfecting plasmid to H9c2 cells. The fluorescence of Su9-EGFP was observed around each nucleus ubiquitously, indicating the successful selection of stable Su9-EGFP-expressing cells (**Figure 1**).

It is well recognized that the surface potential of Mt was negative, while that of cGNS was positive (**Tables 1 and 2**). After Mt were associated with cG or cGNS, their surface potential increased (**Table 2**). In addition, it is reported that the size of mitochondria is around 1 μm . However,

the sizes of cGNS used in this study ranged from 50 to 400 nm. It is highly conceivable that cG and cGNS were attached to the Mt surface through the electrostatic interaction force, leading to a reduced Mt zeta potential. It should be noted that the association of cGNS did not affect the mitochondrial function (**Figure 5**).

After culture for 6 hr, Mt-cGNS were internalized into cells (**Figure 6**). When cells were incubated with Mt-cGNS, the amount of Mt internalized was about 6 times higher than that incubated with Mt alone. It is reported that the cellular internalization efficiency of Mt modified with cell penetrated peptide (CPP) was 2 or 3 times higher than that of non-modified Mt^{15, 28}. Indeed, it is difficult to compare our result with that result because different cells and evaluation methods are used. However, it may be concluded that Mt-cGNS were a promising system for the enhanced cellular internalization of Mt.

A higher internalization efficiency of Mt-cGNS was observed than that of Mt-cG. This can be explained in terms of cationic charges flexibility. It is possible that the cG molecule of water-soluble polymer is flexible and tightly interacts with the negative surface of Mt. On the contrary, the positive charge of cGNS is rather fixed and less flexible because the charge is localized on the surface of nanospheres. It is likely that in addition to the positive charges on one side of nanospheres interacting with Mt, the positive charges on the other side remain to interact with the recipient cells, leading to an enhanced internalization of Mt into the cells.

No significant difference in the internalization efficiency was observed among different sizes of cGNS as the Mt carrier. However the internalization efficiency of Mt-cGNS(400) was relatively lower than that of other sizes of cGNS. The reason is still unclear. It might be possible that the larger size of cGNS is easier to contact with many Mt, to form an increased Mt aggregation, leading to a reduced Mt internalization. It is reported that isolated Mt are intracellularly internalized by macropinocytosis²⁹⁻³⁰. It is well known that macropinosomes range from 0.2 μm to 5 μm . Therefore, the Mt aggregation would affect the cellular internalization of Mt, resulting in the relatively lower internalization efficiency of Mt-cGNS(400). Further investigation is needed to make clear the internalization mechanism.

There have been reported on two different fates of exogenous mitochondria in recipient cells. The mitochondria without any modification might fail in endosomal escape and finally degrade. In the case of spontaneous mitochondria transfer, mitochondria are degraded after internalized into other cells³¹⁻³². This mitochondrial transcellular degradation is considered as a more advanced method to

degrade mitochondria and keep the mitochondrial homeostasis in recipient cells. On the other hand, in the case of artificial transfer of mitochondria, it is demonstrated that most of exogenous mitochondria can effectively fuse with the endogenous mitochondrial network, while some of mitochondria are destined to degrade³³. In our study, the amino groups are chemically introduced in cG and cGNS by reacting with spermine. It is recognized that the secondary amino groups show the pH buffering effect, which may lead to the endosomal escape of Mt-cG and Mt-cGNS. The cG and cGNS will gradually be degraded while the exogenous mitochondria will fuse into original mitochondria network. The fusion of mitochondria enlarges and rearranges the component inside the original mitochondria network, promoting the weak mitochondria to be excluded from the new mitochondrial network which result in an increase of mitochondrial function^{5, 34-36}. After the mitochondria transfer, the total volume of mitochondria and metabolic fitness of chimeric antigen receptor (CAR) T-cell are improved³⁷. Coincidentally, the internalization of exogenous mitochondria into adipose-derived mesenchymal stem cells switches the metabolism method from OXPHOS to glycolysis³⁸. In this study, the recipient cells used are healthy H9c2 cells. Therefore, an enhancement of metabolic abilities after internalizing mitochondria will be expected.

In this study, as two functions of mitochondria, the ability to maintain the homeostasis of ROS, and the ability to produce ATP. Mitochondria are known as the main source of reactive oxygen species (ROS)³⁹. Nearly 90 % of ROS are generated in the mitochondrial electron transportation chain. During the process of oxidative phosphorylation, an electron transfer and the interaction with molecular oxygen to form superoxide mainly in mitochondria complex I and III. On the other hand, mitochondria have an antioxidant enzyme system to eliminate ROS. Superoxide dismutase (SOD) and catalase convert superoxide to hydrogen peroxide in mitochondrial intermembrane space and mitochondrial matrix, respectively. Non-enzymatic antioxidants, such as ubiquinol, vitamin C, and uric acid, also assist to eliminate ROS⁴⁰. By keeping the balance between the production and elimination of ROS, the oxidative stress does not occur in healthy cells. Menadione is often used to prepare the oxidative stress and post-ischaemic damage models by generating superoxide anion⁴¹⁻⁴². After treated with menadione, cells generated superoxide, and the amount of superoxide was significantly higher than that of cells incubated with Mt, Mt-cG, and Mt-cGNS (**Figure 8**). This result indicates that the internalization of Mt assisted to scavenge ROS, which is well corresponding with previous reports⁴³. Zhang et al. demonstrate that via increasing the ATP supplement and reducing the subsequent activation of apoptosis signaling pathway, the mitochondrial internalization

can accelerate ROS scavenging⁴⁴. After the Mt internalization, the level of catalase antioxidant enzyme and the SOD2 protein expression were found upregulated, which contributes to the inhibition of ROS production.⁴⁵⁻⁴⁶. Combined with the previous result (**Figure 4**), we can say with certainty that a higher amount of Mt internalized into cells leads to a higher ability to scavenge superoxide.

ATP generation is one of the most essential functions of mitochondria. Around 95 % of ATP is produced by mitochondria. It is likely that the internalization of healthy mitochondria gives more ATP producer to cells to assist ATP production. After mitochondria internalization, the ATP production significantly increased compared with that of non-treated cells (**Figure 9**). It is possible to consider that exogenous Mt function to enlarge the original mitochondrial network and consequently promote the mitochondrial ability of ATP production. However, this enhancement of ATP production was not in a dose-dependent manner. Assuming 100 % applied Mt were internalized, these results indicated that there is a preferable range of exogenous Mt doses for internalization. It has been reported that as few as 100 cells were sufficient to rescue 1 cell clone via intracellular mitochondrial transfer⁵. According to this report, Chang and his group report that the applied Mt amount which lower than 21 $\mu\text{g}/10^5$ cells or higher than 105 $\mu\text{g}/10^5$ cells was ineffective for restoring the mitochondria membrane potential in MERRF cybrid cells under peptide-mediated mitochondrial delivery⁴⁷. Another research demonstrate that the neuroprotection of oxygen-glucose deprivation cells peaked under the treatment with an Mt dose of 50 $\mu\text{g}/10^5$ cells in vitro¹⁶. In this study, the Mt doses applied were 7.5, 15, and 30 $\mu\text{g}/10^5$ cells. However, the rescue effect of Mt internalization with the doses of 7.5 and 15 $\mu\text{g}/10^5$ cells was still observed in this study (**Figure 9**). It might be due to the recipient cells being healthy which do not require much more Mt supplement. Meanwhile, the enhancement of Mt internalization using cGNS as a carrier might also contribute to this effect. For further research to understand the Mt internalization mechanism and optimize the dose of Mt to apply, Mt-deficient cells should be used as the recipient cells.

Mitophagy plays an important role in controlling the quality of mitochondria. Inducing mitophagy is one of the methods to cure the dysfunction mitochondria. It is reported that the presence of healthy mitochondria is required to proceed this process⁴⁸. cGNS used as a potential tool to deliver healthy mitochondria also utilize the carrier of drug release. The controlled release mitophagy-inducing drugs may be attraction for this research area. Further investigation will be proceeded to make clear underlying mechanism.

Conclusion

Mitochondria internalization is an innovative strategy for the mitochondria dysfunction treatment. This study demonstrate that cGNS enabled to enhance the intracellular uptake of exogenous Mt, leading to the recovery of Mt functions. By the cGNS association with Mt, the internalization efficiency of Mt significantly increased. With an increased amount of Mt internalizing into cells, the cells functions of scavenging ROS and generating ATP were enhanced. In conclusion, cGNS are potential carriers to improve the therapeutic efficacy of mitochondria internalization.

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Chapter 2

Association with Cationized Gelatin Nanospheres Enhances Cell Internalization of Mitochondria and the Mitochondrial Membrane Potential

Introduction

Mitochondria (Mt) are one of the most essential organelles in cells. Mt not only produce energy to support various cell activities and functions, but also response to several signals. Mt have their own genome which encodes proteins important for oxidative phosphorylation. In addition, Mt always undergo fusion and fission to form a dynamic Mt network to meet energy demand in different situations. Therefore, damaged Mt cause many diseases, including diabetes, neurodegenerative diseases, cardiovascular diseases, ischemia, spinal cord injury, cancer, and senescence¹⁻⁴. Therefore, it is a urgent demand to develop an effective therapy for the functional rescue of mitochondrial dysfunction.

Recently, several evidences reveal that Mt can transfer between several types of cells under both physiological and pathological conditions⁵⁻⁷. There are 3 main routes of the Mt transfer, such as the intercellular transfer through the transient cellular connections⁸⁻¹⁰, via extracellular vesicles¹¹⁻¹³, and by directly captured from the recipient cells¹⁴⁻¹⁶. The effect of intercellular Mt transfer varies depending on the situation⁷. In general, the Mt intercellular transfer assists to support the metabolism of recipient cells^{15, 17-18}, control the Mt quality of donor cells^{12, 19}, and regulate the immune system^{14, 20-22}. In this connection, several researches have been reported to introduce exogenous Mt to recipient cells, named the Mt internalization, to rescue the Mt function.

One of the main problems for the Mt internalization is that the electrostatic repulsion between the cell and Mt membranes limits the efficiency of Mt internalization. There are some reports to utilize a physical force to promote the Mt internalization, such as Mito-Ception^{18, 23-24} and Mito-Punch²⁵⁻²⁶. The merit of the methods is the high efficiency and quick cellular internalization of Mt, irrespective of cell types. However, the physical force sometimes induces the cytotoxicity due to the pores formation of cell membranes. In addition, it is technically challenging for the Mt internalization in vivo. Another trial is to modify the Mt surface. Cell penetrating peptides (CPP)²⁷⁻²⁹,

polymers³⁰⁻³³, cationic lipids³⁴, and cardiomyocytes membrane³⁵ are utilized to modify the Mt surface and have been reported that can promote the Mt internalization.

Gelatin is a well-known biocompatible material and has been widely used in the field of food, pharmaceuticals, and medicine. It has many functional groups (amino, carboxyl, and hydroxyl groups) which allow to chemically modify gelatin. Cationized gelatin (cG) was prepared by chemically introducing spermine to the amino groups of gelatin³⁶. It is reported that cG enables to deliver nucleic acid (negative charge), such as pDNA³⁶, small interfering RNA³⁷⁻³⁸, and imaging probes³⁹⁻⁴⁰ by forming positive surface charge complexes. In addition, cG has biodegradability, which allows nucleic acid to controlled release with the degradation of cG-based hydrogels. Lipofactamine® is a commercial reagent commonly used for nucleic acid delivery. However, lipofactamine® cannot always release nucleic acid in a controlled manner.

In our previous study, the influence of water-soluble and nanosphere-type cG on the enhancement of Mt internalization efficiency was investigated⁴¹. It is demonstrated that the association with cationized gelatin nanospheres (cGNS) has a higher potential to enhance the efficiency of Mt internalization. However, since the cells used are normal cells in our previous study, it is difficult to assess the effect of Mt internalization on the Mt function. In this study, to evaluate the effect of Mt function, rho 0 cells of mitochondrial DNA (mtDNA)-depletion are used as the recipient cells⁴². The rho 0 cells are generated by the culture with medium containing ethidium bromide (EtBr)⁴³. A low concentration of EtBr can intercalate preferentially with mtDNA and interfere with replication enzymes, leading to a declined mtDNA during the cell division. Lack of mtDNA makes the rho 0 cells line a valuable tool to investigate the mechanism of Mt disorder.

The objective of this study is to evaluate the effect of exogenous Mt transplantation by the cGNS association on the Mt activity of rho 0 cells. The mixing ratio of Mt to cGNS was investigated to optimize, while the Mt membrane potential of rho 0 cells was assessed with or without the Mt transplantation. We examine the fate of exogenous Mt transplanted assessing the Mt internalization.

Materials and Methods

1. Preparation of cationized gelatin and cationized gelatin nanospheres

According to the preparation procedure previously reported⁴⁴ the carboxyl groups of gelatin were chemically converted by introducing amino groups to allow gelatin to cationize. Spermine was

added at a molar ratio of 50 to the carboxyl groups of gelatin into 50 ml of double-distilled water (DDW) containing 2.0 g of gelatin (isoelectric point (pI) = 9.0, weight-averaged molecular weight = 99000, derived from pig skin, Nitta Gelatin Inc., Osaka, Japan.). Immediately after that, the solution pH was adjusted to 5.0 by adding 11 M HCl aqueous solution. Next, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was added at a molar ratio of 3 to the carboxyl groups of gelatin. The reaction mixture was agitated at 37 °C for 18 hr, and then dialyzed against DDW for 3 days at room temperature. The dialyzed solution was freeze-dried to obtain cationized gelatin (cG). To determine the percentage of amino groups introduced into gelatin, the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) method was performed. The percentage was 44.5 mole% per the carboxyl groups of gelatin.

cGNS were prepared by the conventional coacervation method³⁷. In brief, 1.25 ml of cationized gelatin aqueous solution (2.5 wt%) was warmed up to 40 °C. Next, 5 ml of acetone was added to the solution for coacervate formation. Then, glutaraldehyde (25 wt%, 20 µl) was added to chemically crosslink cGNS for 6 hr. For the blocking of aldehyde groups unreacted, 2 ml of glycine aqueous solution (0.5 M) was added. The resulting solution was agitated overnight at 40 °C and the residual acetone was evaporated. cGNS were collected by the ultra-centrifugation (Optima MAX-XP ultracentrifuge, Beckman Coulter Inc., Indianapolis, IN, USA) of (250,000 g, 30 min 25 °C) and resuspended in DDW. cGNS were resuspended in 10 mM phosphate buffered-saline solution (PBS, pH7.4), and the apparent size of cGNS was measured by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). On the other hand, cGNS were resuspended in 10 mM phosphate buffer solution (PB, pH7.4), and the zeta potential was measured by electrophoresis light scattering (ELS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK).

2. Cell culture and rho 0 cells preparation

H9c2 cardiomyoblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The H9c2 cells were maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Inc., Massachusetts) supplemented with 10 vol% fetal bovine serum and 1 vol% penicillin/streptomycin mixture. The cells were incubated at 37°C in a humidified atmosphere at 5% CO₂. Stably green fluorescent protein (GFP) expressing H9c2 cells (GFP-H9c2) were generated to visualize the mitochondria isolated according to the reported method. A plasmid Su9- EGFP was

kindly provided by Dr. Ishihara⁴⁵ (Department of Biological Sciences, Graduate School of Science, Osaka University). According to the manufacturer's protocol, the pDNA was transfected by using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc., Massachusetts). After the transfection, cells were cultured in the medium containing a selective agent, G418 (Thermo Fisher Scientific, Inc., Massachusetts), for 3 weeks. Finally, GFP-expressing cells were selected and collected by Cell Sorter MA900 (Sony Inc., Tokyo, Japan).

Establishment of rho 0 cells was performed as previously described^{43, 46}. H9c2 cells (1×10^5 /dish) were seeded into 100 mm TC-treated culture dish (Corning Inc., Corning, NY, USA) over one night before the treatment. The culture medium were changed to Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Inc., Massachusetts) supplemented with 10 vol% fetal bovine serum, 1 vol% penicillin/streptomycin, 50 µg/ml uridine, and 200, 500, and 1000 ng/ml ethidium bromide (EtBr). The uridine and EtBr contained medium was changed twice per week. Then, the cells continued to culture in the same condition for at least 1 month to obtain rho 0 cells. The characterization of rho 0 cells were performed by TMRM (Tetramethylrhodamine, Thermo Fisher Scientific Inc., Waltham, MA, USA) staining and DNA staining, according to the method reported previously.

3. Isolation of mitochondria

Mitochondria (Mt) were isolated from H9c2, and the Mt-GFP were isolated from H9c2-GFP cells. After disrupting cells through a 27- gauge needle, the isolation was conducted based on the research procedure of Maeda et al³³. Briefly, cells were ruptured by 20 strokes using a syringe with 27-gauge needle in homogenization buffer [HB; 20 mM HEPES-KOH (pH 7.4), 220 mM mannitol 70 mM sucrose 100 µM PMSF (Phenylmethylsulfonyl fluoride)]. The homogenate was centrifuged at 400 g at 4 °C for 5 minutes to remove unbroken cells, and then the supernatant was further centrifuged at 6,000 g for 5 min to pellet the isolated Mt-enriched fraction (Mt fraction). The mitochondria isolated from H9c2 cells are expressed as Mt, while the mitochondria isolated from GFP-H9c2 are expressed as Mt-GFP. The supernatant was collected as a cytosolic fraction. The amount of protein in each fraction was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Massachusetts).

The activity of isolated Mt was confirmed by the conventional adenosine triphosphate (ATP) assay. The isolated Mt were suspended in respiration buffer [RB; 250 mM sucrose, 15 mM KCl, 1

mM EGTA, 30 mM K₂HPO₄, 5 mM MgCl₂, 25 mM succinate] and divided into three groups. One group was added with ADP (adenosine diphosphate, final concentration is 1.65 mM), another group was added with oligomycin (final concentration is 1 μM). The isolated Mt were incubated at 37 °C for 1 hr. And then, the same volume of CellTiter-Glo® 2.0 reagent (Promega Corporation, Madison, USA) was added to each group and mixed on the shaker for 5 min covered with a aluminum foil. The solution (100 μl) of each group was added to each well of 96 multi-well white wall plate (SUMITOMO BAKELITE Co. Ltd. Japan). After stabilization of the luminescent signal at room temperature for 10 min, the luminescence was recorded by Multi-mode Microplate Reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan).

4. Preparation of Mt associated with cGNS

The cGNS were added to the suspension of Mt and gently mixed, followed by incubation for 10 min on ice to form Mt-cGNS. The apparent size of cGNS or Mt-cGNS resuspended in 10 mM phosphate buffered-saline solution (PBS, pH 7.4) was measured by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). The zeta potential of cGNS or Mt-cGNS resuspended in 10 mM phosphate buffered solution (PB, pH 7.4) was measured by electrophoresis light scattering (ELS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK).

5. Incubation of rho 0 cells with Mt or Mt-cGNS for internalization

The rho 0 cells were seeded to cell culture plates or 35 mm glass bottom dishes over one night before Mt internalization. Mt or Mt-cGNS freshly isolated were added to the rho 0 cells and incubated for 6 hr. And then, the cells were washed with PBS twice and further cultured with the fresh medium for 2 weeks.

6. Western blotting to evaluate cellular internalization of Mt

The amount of GFP in the cell lysates was quantified by the western blotting. Rho 0 cells incubated with Mt or Mt-cGNS were collected by trypsinization and centrifugation (400 g, 3 min, 25 °C). The pellets were mixed with a radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Nacalai Tesque Inc., Kyoto, Japan) containing 1 mM Na₃VO₄ and 1 mM

phenylmethanesulfonyl fluoride (PMSF). The protein concentration of cell lysates was determined by BCATM Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacture's protocol. The protein concentration of all the samples were adjusted to be consistent. Next, the Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) supplemented with 2.5% 2-mercaptoethanol was mixed to cell lysates and heated to 95 °C for 5 min. The samples were loaded on Any kDTM Mini-PROTEAN TGXTM Precast Gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and electrophoretically run at 0.02 A for 15 min and 0.04 A for 40 min. Then, the samples were transferred onto a polyvinylidene difluoride (PVDF) membrane, blocked with the blocking solution (Immuno BlockTM, KAC Co. Ltd., Kyoto, Japan) for 1hr, and washed 3 times with Tris-buffered saline solution containing 1 vol% Tween-20 (TBST). For the detection, the primary antibodies were added and the membranes were incubated overnight at 4 °C. The antibodies used were mouse monoclonal anti-COXIV (ab14744, Abcam (Cambridge, UK), 1:1000), rabbit polyclonal anti- α -Tubulin (2144S, Cell Signaling Technology Japan, K.K. (Tokyo, Japan), 1:1000), rabbit polyclonal anti-GFP (ab290, Abcam, 1:3500), rabbit monoclonal anti- β -Actin (4970S, Cell Signaling Technology, 1:1400). The blots were washed 3 times with TBST before incubation with the HRP-conjugated secondary antibody (HRP-linked goat anti-rabbit IgG, 7074S, Cell Signaling Technology, 1:2000; HRP-linked horse anti-mouse IgG, 7076S, Cell Signaling Technology, 1:2000) for 1 hr at RT. Finally, the membranes were washed 3x with TBST and detected using a PierceTM ECL Plus Western Blotting Substrate (Thermo Fisher Inc., Waltham, MA, USA) with a chemiluminescence imaging system Molecular Imager[®] ChemiDocTM XRS+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The efficiency was calculated as the ratio of the amount of GFP contained in each sample to that of GFP in the sample cultured with Mt-GFP.

7. Evaluation of Mt internalization pathway

Rho 0 cells or H9c2 cells were pre-treated with chlorpromazine (CPZ, 15 μ M), cytochalasin D (CytoD, 1 μ M), nocodazole (Noc, 200 nM), methyl- β -cyclodextrin (M β CD, 1 mM), and wortmannin (Wort, 1 μ M) for 30 min. And then, the Mt-cGNS was added to the cell, followed by 6 hr incubation. The cells incubated with Mt-cGNS were collected by trypsinization and centrifugation, and analyzed by a FACSCanto II flow cytometer and FlowJoTM (v 10.8, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) software.

8. Visualization of exogenous Mt internalized in rho 0 cells

Before the Mt transplantation, rho 0 cells were stained with 100 nM of MitoTrackerTM Deep Red FM (Thermo Fisher Scientific, Inc., Massachusetts) for 20 min. And then, Mt-GFP-cGNS were incubated with MitotrackerTM Deep Red FM-stained rho 0 cells for 6 hr. After incubation with Mt, rho 0 cells were fixed with 4 vol % of paraformaldehyde (PFA) for 15 min, and washed with PBS for twice. The fluorescence images were taken by a confocal laser scanning microscope.

9. Evaluation of mitochondrial membrane potential

The rho 0 cells incubated with Mt or Mt-cGNS were further cultivated in the medium containing 50 nM of TMRM (Tetramethylrhodamine, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 30 min. And then, the stained cells were collected by trypsinization and centrifugation, and analyzed by a FACSCanto II flow cytometer and FlowJoTM software. The fluorescence intensity was normalized by that of H9c2 cells.

10. Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). All the statistical analysis was performed by using an unpaired *t*-test with two tails. Difference with $p < 0.05$ was considered to be significant.

Results

1. Characterization of rho 0 cells

The Mt membrane potential of rho 0 cells treated with different concentrations of EtBr was evaluated by the TMRM signaling (**Figure 1A**). The EtBr treatment significantly decreased the Mt membrane potential of cells, irrespective of the EtBr concentration. There was no significant difference in the Mt membrane potential of rho 0 cells between the treatment of 500 and 1000 ng/ml of EtBr. The co-localization of red and green fluorescences was observed, indicating the exist of mtDNA in H9c2 cell. For rho 0 cells, no green fluorescence was detected, in remarked contrast with H9c2 (**Figure 1B**).

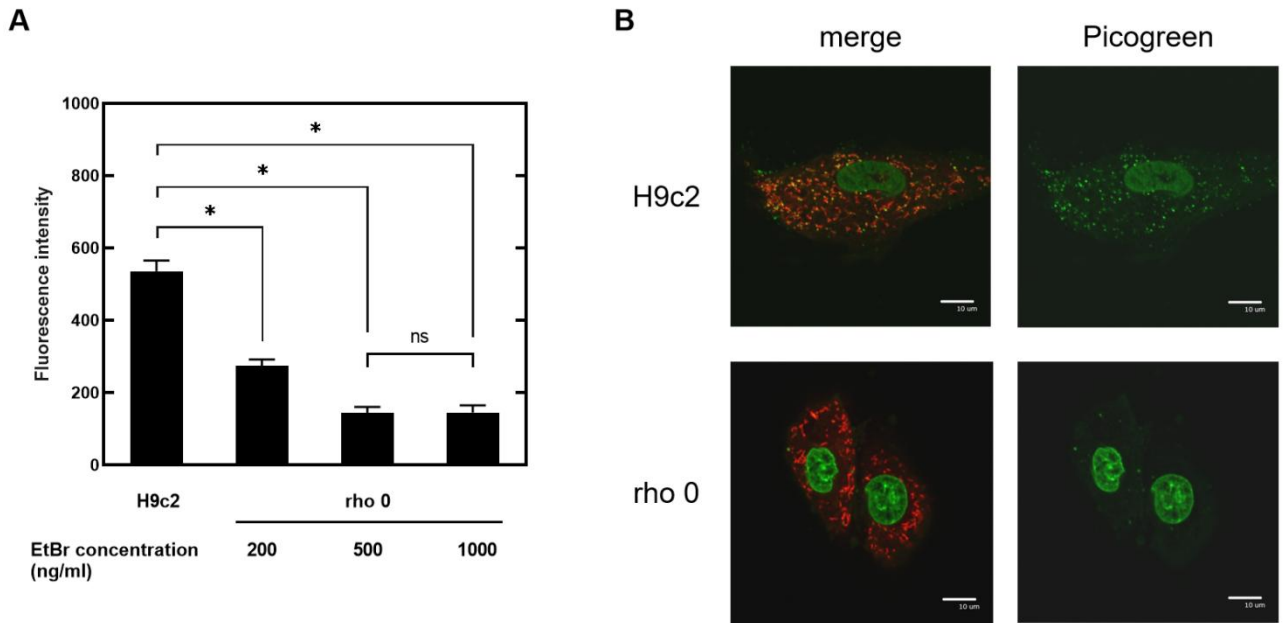


Figure 1. Characterization of rho 0 cells prepared. A. Mt membrane potential of H9c2 and rho 0 cells treated with 200, 500, and 1000 ng/ml of EtBr. $n = 3$ *, $p < 0.05$; significant difference between the two groups, ns; not significant, using one-way ANOVA followed by Tukey's multiple comparisons. B. Fluorescence microscopic images of rho 0 cells treated at 500 ng/ml of EtBr. Red: Mt. Green: DNA. Scale bar is 10 μ m.

2. Characterization of mitochondria isolated

Figure 2A shows the activity of Mt isolated. By the ADP addition, the ATP production of Mt isolated increased, while the oligomycin treatment suppressed the ATP production. In addition, after mixing with cGNS at the ratio of 3/1 and 2/1, Mt-cGNS showed the similar ADP-stimuli ATP production to that of Mt isolated. The significant decrease of ADP-stimuli ATP production was detected at the Mt-cGNS mixing ratio of 1/1, 1/2, and 1/3 (**Figure 2C**). **Figure 2B** shows the purity of Mt isolated. The COXIV of a mitochondria-specific protein was detected in the Mt fraction with no detection of cytosol-specific protein, α -tubulin. The size of Mt isolated was around 860 nm and the surface charge was negative. With the increase in the amount of cGNS association, the surface charge of Mt-cGNS increased whereas the size decreased (**Table 1**).

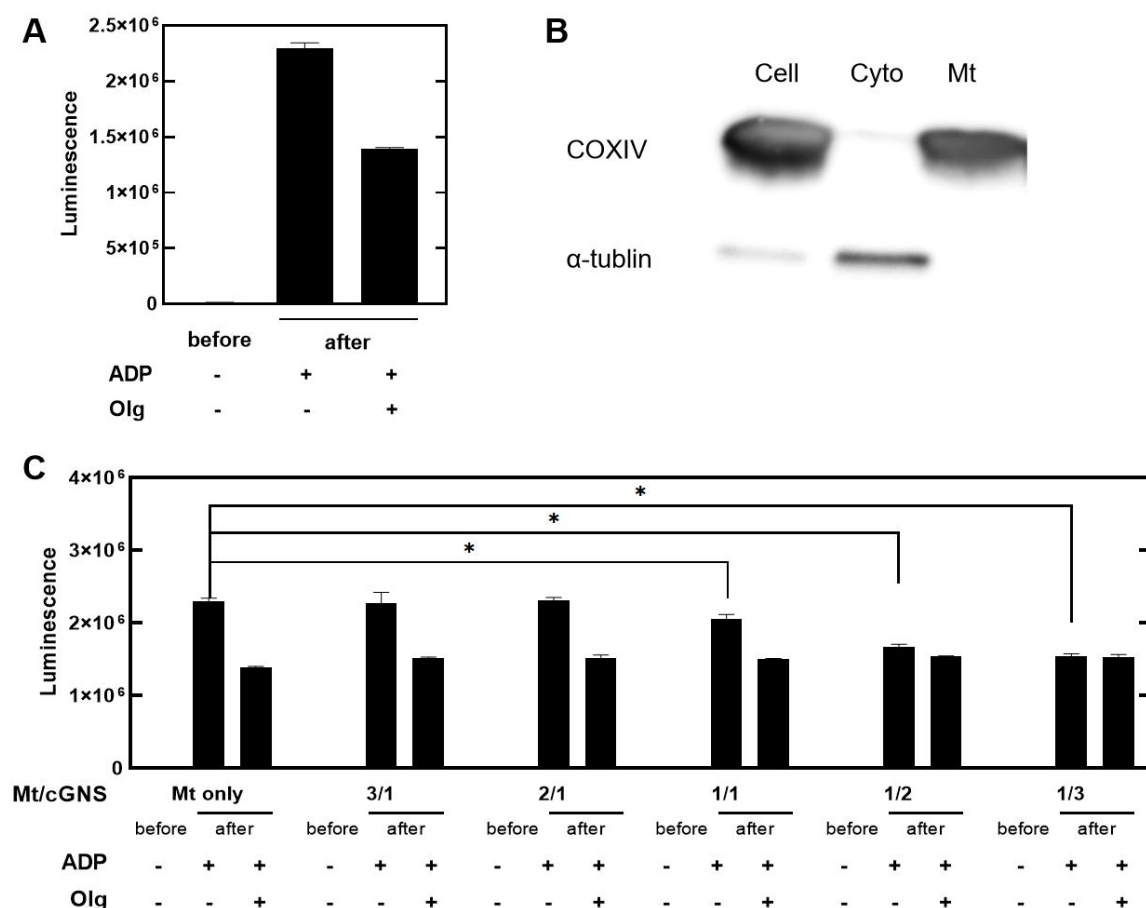


Figure 2. A. ATP production of Mt isolation before and after ADP addition with or without 1 μ M olygimycin (Olg). B. COXIV and α -tubulin western blotting images of different cell fractions obtained in Mt isolation; Mt fraction (Mt), cytosolic fraction (Cyto), and total cell extracts (Cell). C. ATP production of Mt isolation mixed with cGNS at different ratios before and after ADP addition with or without 1 μ M olygimycin (Olg). n= 6. *, p<0.05; significant difference between the two groups, using two-way ANOVA followed by Tukey's multiple comparisons.

Table 1. Physicochemical properties of cGNS and Mt-cGNS prepared at different mixing ratios of Mt to cGNS.

Mt (μ g/ml)	cGNS (μ g/ml)	Size (nm)	Zeta potential (mV)
0	100	60.4 $\pm$ 0.59 ^a	5.19 $\pm$ 0.93 ^a
200	0	862.0 $\pm$ 107.1	-11.73 $\pm$ 1.27
200	66.7	2147.7 $\pm$ 1337.3	-5.97 $\pm$ 0.13
200	100	1560.7 $\pm$ 442.6	-2.55 $\pm$ 0.07
200	200	720.8 $\pm$ 51.5	1.6 $\pm$ 0.77
200	400	371.0 $\pm$ 25.6	2.21 $\pm$ 0.74
200	600	268.0 $\pm$ 8.1	2.25 $\pm$ 0.41

a) mean $\pm$ standard deviation

3. Internalization of Mt-cGNS into rho 0 cells

The highest efficiency of Mt internalization was observed at the Mt/cGNS mixing ratio of 3/1. No significant difference in the internalization efficiency was observed among other groups (Figures 3A and 3B). Figure 3C shows the efficiency of Mt internalization under different pre-treatments to rho 0 cells and H9c2 cells. Significantly decreased of Mt internalization efficiency was observed after treatment with cytochalasin-D. However, there were no significant difference in the efficiency between the control and other groups.

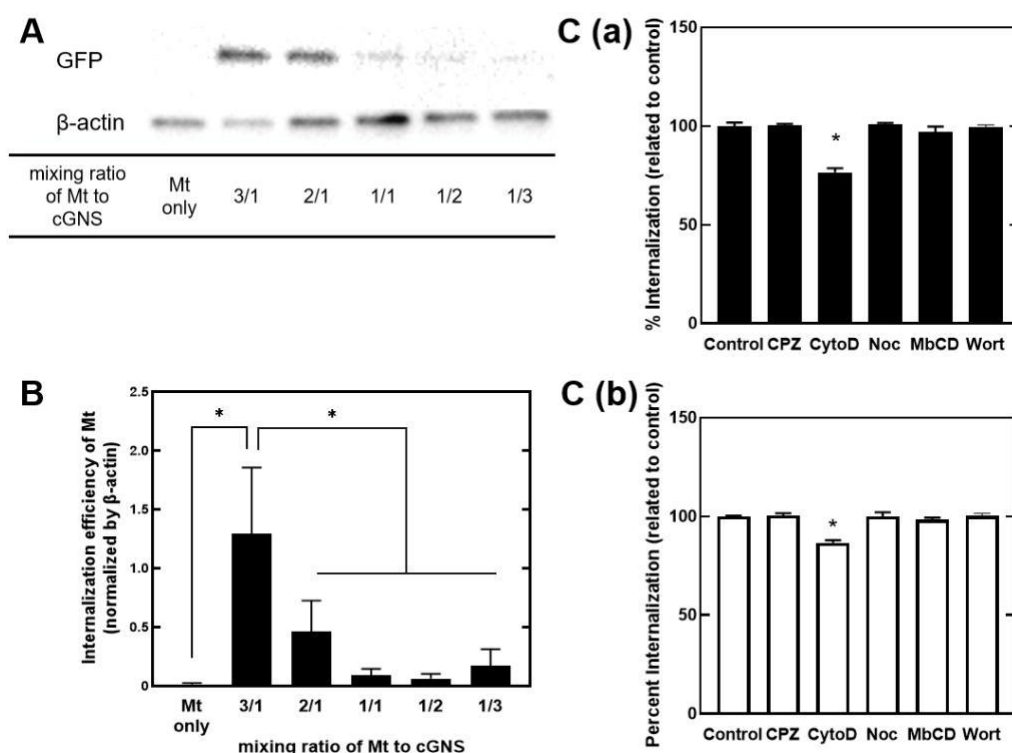
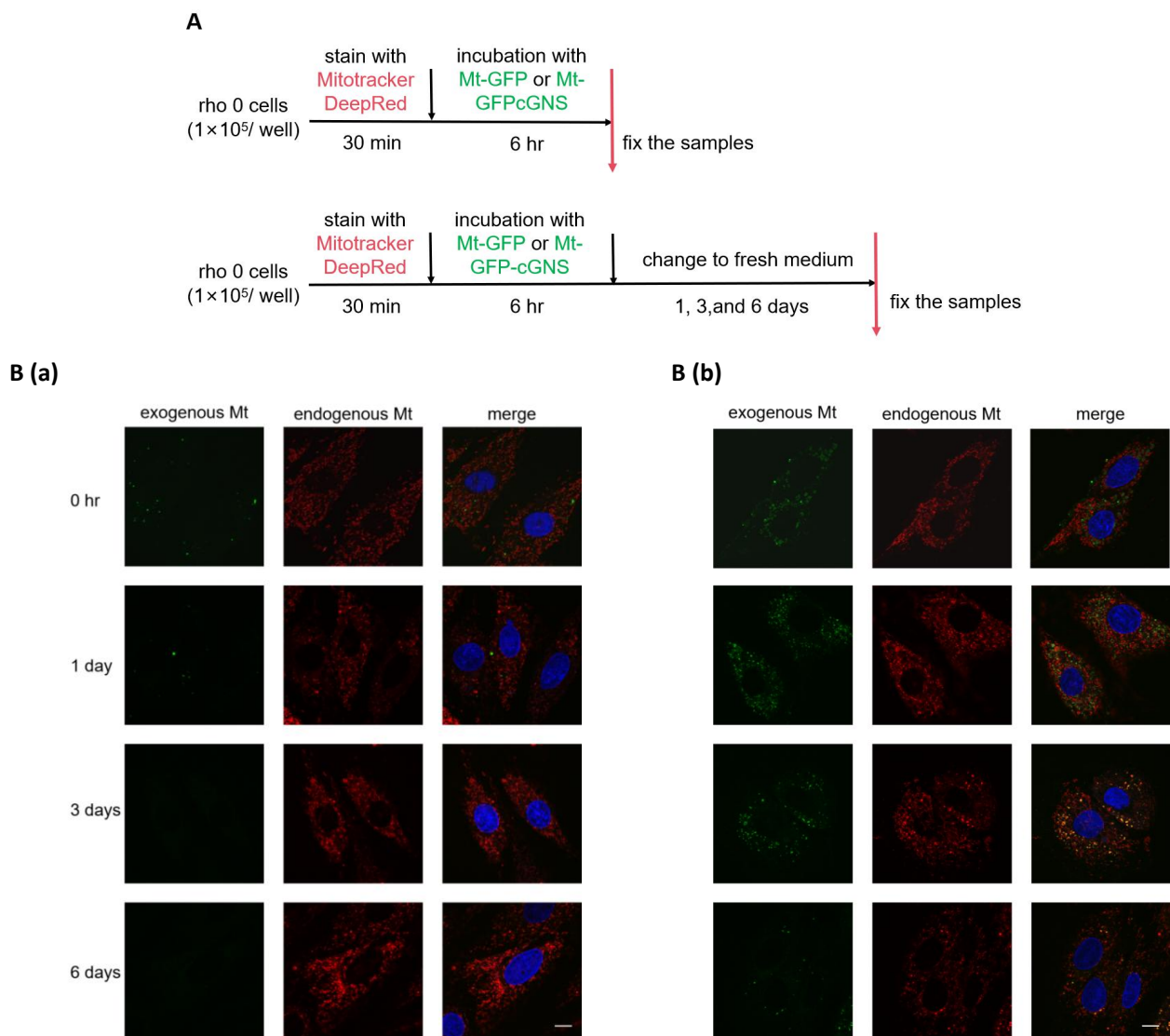


Figure 3. A. GFP and β-actin western blotting images of cell lysates after incubation with Mt and Mt-cGNS prepared at different mixing ratios of Mt to cGNS. B. Efficiency of cellular internalization of Mt and Mt-cGNS prepared at different mixing ratios of Mt to cGNS. n= 3. *, p<0.05; significant difference from the 3/1 group, ns; not significant, using one-way ANOVA followed by Tukey's multiple comparisons. C. Mt internalization into rho 0 cells (a) and H9c2 cells (b) pre-treated with chlorpromazine (CPZ), cytochalasin D (CytoD), nocodazole (Noc), methyl-β-cyclodextrin (MβCD), and wortmannin (Wort). The value for the control group is 100%. n= 3. *, p<0.05; significant difference from the control group without any pretreatment, using one-way ANOVA followed by Tukey's multiple comparisons.

4. Fusion of exogenous Mt in the recipient cells

After 6 hr incubation with Mt, the green fluorescence of exogenous Mt was observed in the recipient cells, although the amount was low (**Figure 4B (a)**). The medium was changed into the fresh medium and continued to culture the recipient cells. The green fluorescence decreased with time to disappear.

On the other hand, the green fluorescence in the recipient cells incubated with Mt-cGNS was much higher than that of incubated with Mt only. The green fluorescence (exogenous Mt) and red fluorescence (endogenous Mt) were merged 1 days after culturing in the fresh medium, indicating that the fusion of exogenous Mt and endogenous Mt occurred. (**Figure 4B (b)**). **Figure 4C** shows the quantification of fluorescence merge area in the rho 0 cells incubated with Mt and Mt-cGNS at different time point. In the rho 0 cells incubated with Mt-cGNS, the merge area of green and red fluorescence significantly higher than that of rho 0 cells incubated with Mt after culturing from 0 to 3 days. In addition, sharply decreased of merge fluorescence area after culturing from 3 to 6 days was observed in the rho 0 cells incubated with Mt-cGNS.



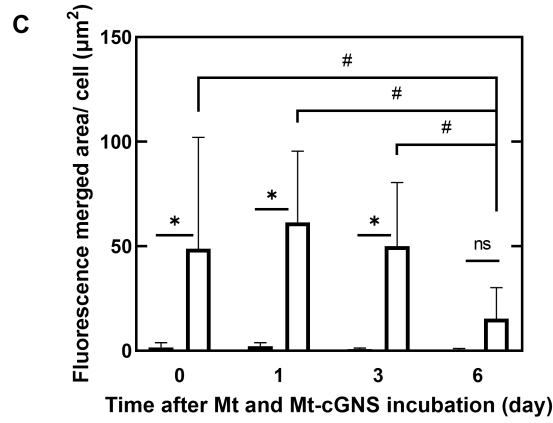
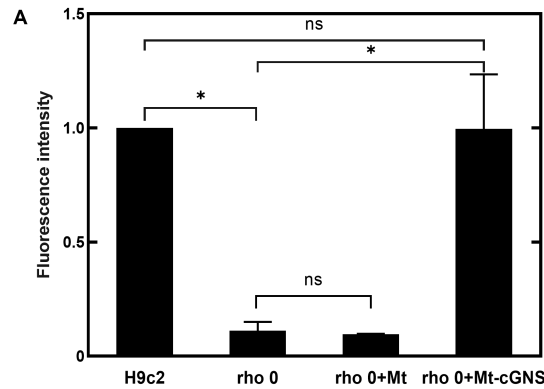


Figure 4. A. Scheme of sample collection. B. Fluorescence images of exogenous Mt and endogenous Mt for rho 0 cells after incubated with Mt (a) Mt-cGNS (b) prepared at the mixing ratio of 3/1 at 0, 1, 3 and 6 days. Red: endogenous mitochondria. Green: exogenous mitochondria. Scale bar is 10 μm. C. The area of merged fluorescence in each rho 0 cell incubated with Mt (■) Mt-cGNS (□) prepared at the mixing ratio of 3/1 at 0, 1, 3 and 6 days. n= 10. *, #, p < 0.05; significant difference between the two groups, ns; not significant, using two-way ANOVA followed by Tukey's multiple comparisons.

5. Mt membrane potential of rho 0 cells internalized with Mt and Mt-cGNS

Figure 5 shows the TMRM fluorescence intensity of H9c2 cells and rho 0 cells after 6 hr incubation with Mt and Mt-cGNS. The Mt membrane potential of rho 0 cells was significantly lower than that of normal H9c2 cells. The Mt membrane potential of rho 0 cells did not change after incubation with the original Mt. On the other hand, incubation with Mt-cGNS significantly increased the Mt membrane potential of rho 0 cells, and the level was similar to that of H9c2 cells. The enhance potential maintained or at least 6 days after incubation with Mt-cGNS (**Figure 5B**). The Mt membrane potential of rho 0 cells incubated with Mt-cGNS gradually decreased with time, but still maintained to be at significantly higher level than that of rho 0 cells over 6 days culture.



B

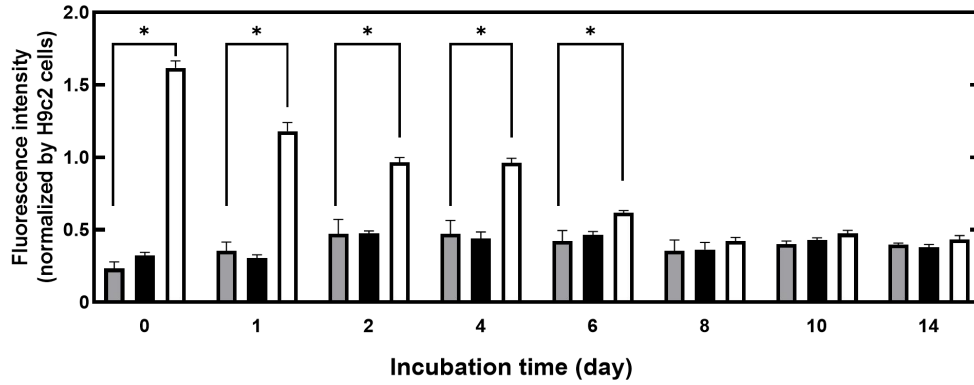


Figure 5. A. TMRM fluorescence intensity of H9c2 and rho 0 cells after 6 hr incubation with Mt and Mt-cGNS prepared at the mixing ratio of 3/1. $n = 3$. *, $p < 0.05$; significant difference between the two groups, ns; not significant, using one-way ANOVA followed by Tukey's multiple comparisons. B. Time course of TMRM fluorescence intensity of rho 0 cells 0, 1, 2, 4, 6, 8, 10, and 14 days after incubation with Mt (■), Mt-cGNS (□), and original rho 0 cells (▨). The value of H9c2 cells is 1. $n = 3$. *, $p < 0.05$; significant difference between the two groups, using two-way ANOVA followed by Tukey's multiple comparisons.

Discussion

The present study demonstrates that the cGNS association enables to enhance the internalization of exogenous Mt into rho 0 cells and maintain the Mt membrane potential for 6 days. It is possible that this phenomenon results from the efficient fusion of exogenous Mt exogenously internalized into original Mt network in the recipient cells.

The recipient cells used in this study is rho 0 cells. The rho 0 cells were generated by a traditional treatment of EtBr which can intercalate preferentially with mtDNA and interplay with the replication enzymes^{43, 46}. Due to the lack of mtDNA, the mitochondrial functions are deficient⁴⁷. The Mt membrane potential of cells treated with 200, 500, and 1000 ng/ml of EtBr decreased, and significantly lower than that of normal cells, irrespective of EtBr concentration treated. There was no significant difference in the Mt membrane potential of rho 0 cells between the treatments of 500 and 1000 ng/ml of EtBr (**Figure 1A**). However, the higher concentration tended to suppress the proliferation of rho 0 cells. Therefore, the rho 0 cells generated at 500 ng/ml of EtBr were used in the following experiments. The staining observation of cells was performed to confirm the elimination of mtDNA⁴⁸⁻⁴⁹. The DNA was stained by picogreen, and the Mt was stained by

MitoTracker™ Deep Red FM. The co-localization of green fluorescence of DNA and red fluorescence of Mt indicates the exist of mtDNA. The mtDNA was only observed in the normal cells. This finding experimentally indicates that mtDNA was eliminated in rho 0 cells generated.

The previous study investigated the influence of water-soluble and nanosphere-type cG on the enhancement of Mt internalization efficiency⁴¹. To demonstrate a higher potential of cGNS to improve the Mt internalization. It is likely that the cGNS associated to the Mt surface decreased the surface charge, resulting in an enhanced Mt internalization. Since the amount of cGNS associated with Mt affects the enhancement efficiency of Mt internalization, in this study, the effect of mixing ratios of Mt to cGNS on Mt internalization was evaluated. The increasing amount of cGNS led to an increase of zeta potential of Mt-cGNS. This result indicates that the increased amount of cGNS allows more cGNS to associate with Mt by the electrostatic interaction (**Table 1**). On the other hand, the lower ratio of Mt to cGNS resulted in a decrease of Mt-cGNS size. It is conceivable that strong interaction of many cGNS with Mt surface may destroy of Mt. The ATP assay of Mt mixed with different ratios of cGNS may support this conclusion. The increased ATP amount after the ADP addition means the healthy Mt ability to produce ATP. The decreased ATP amount after the oligomycin treatment indicates the intact Mt ability to response to ATP synthase inhibitor⁵⁰. Mt-cGNS at mixing ratios of 1/1, 1/2, and 1/3 had significantly lower ADP-stimulated ATP production, indicating the damaged Mt function (**Figure 2C**).

In this study, Su9-EGFP expressed-H9c2 cells are selected as the source of Mt for easier Mt tracing. The EGFP is tagged with the subunit 9 of the F0-ATPase on the Mt inner membrane⁵¹. The Mt destruction would result in the damage of Mt inner membrane to lose EGFP. To evaluate the internalization efficiency of exogenous Mt, the EGFP amount in the recipient cells was assessed by the western blotting. The highest amount of EGFP in the recipient cells was obtained when the ratio of Mt to cGNS was 3/1. This experimentally confirms that Mt/cGNS mixing ratio of 3/1 is suitable to deliver intact Mt to the recipient cells at a high efficiency even though the surface potential of Mt showed a slightly negative charge (**Figures 3A and 3B**). It is highly conceivable that the cGNS is associated on Mt surface at a discontinuous point manner. The positive charge of cGNS is locally concentrated at a micro-order level. Taken together, it is likely that even though Mt-cGNS at the mixing ratio of 3/1 showed totally a slightly negative charge, the some sites of Mt surface associated with cGNS is of a strongly positive charge. This discontinuous and locally strong positive points would enable to interact of Mt-cGNS with the cell membrane of negative charge, leading to an

enhanced internalization of Mt-cGNS. The increasing association of cGNS results in the large positive points to enhance the internalization efficiency. Therefore, the Mt-cGNS at Mt/cGNS mixing ratios of 6/1, 3/1 showed higher internalization efficiency (data not shown). On the other hand, higher amount of cGNS at the mixing ratios of 1/1, 1/2, and 1/3 strongly interacts with Mt which causes the damage in Mt structure and Mt dysfunction (**Figure 2C**), which is not suitable for Mt internalization. In addition, it is reported that the internalization of Mt is by a heparan sulfate-dependent process^{16, 52}. It is possible that higher amount of cGNS associated on Mt surface affects the interaction of Mt and heparan sulfate on the cell membrane, leading to a decreased internalization efficiency compare with Mt/cGNS at the mixing ratios of 3/1 and 2/1.

Some researches suggest that the internalization of exogenous Mt is carried out by the macropinocytosis pathway⁵²⁻⁵⁴. However, another report reveals that the Mt internalization involves in an actin-dependent endocytosis⁵⁵. It should be noticed that, different cell types and conditions might lead to different results. In this study, the rho 0 cells were pre-treated with different endocytosis inhibitors. Chlorpromazine (CPZ) can block the clathrin-mediated endocytosis by blocking the endosome recycling. Cytochalasin D (CytoD) can block actin polymerization⁵⁶⁻⁵⁷. Nocodazole (Noc) can block the microtubule polymerization^{10, 58}. Methyl- β -cyclodextrin (M β CD) can block caveolae-mediated endocytosis by cholesterol depletion⁵⁹. Wortmannin (Wort) can block macropinocytosis by inhibiting macropinosome closure⁶⁰. The internalization of Mt-cGNS was not suppressed by the treatment with CPZ, Noc, M β CD, and Wort. On the contrary, a significant decrease of Mt internalization was observed for cells pre-treated with CytoD, but not with Wort (**Figure 3C(a)**). This result suggests that the internalization of Mt-cGNS involves in an actin-dependent pathway, but in the macropinocytosis. The similar phenomenon was observed for the recipient H9c2 cells (**Figure 3C(b)**). McCully's groups compare the internalization efficiency of Mt in CytoD, M β CD, Noc, and 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) treated cardiomyocytes⁵⁵. It is demonstrated that Mt internalization in cardiomyocytes are not involved in tunneling nanotubes, caveola-dependent-clathrin dependent endocytosis, and macropinocytosis, but involved in an actin-dependent endocytosis. Other groups investigating the internalization mechanism of Mt experimentally confirm the Mt macropinocytosis in human osteosarcoma⁵⁴, hepatocellular carcinoma⁵², and human uterine endometrial gland-derived mesenchymal cells⁵³. The different types of recipient cells may cause different results. Based on the finding, we can say with certainty that the macropinocytosis is not always the pathway of Mt internalization.

There are two fates of exogenous Mt after the internalization into the recipient cells. It is suggested that some of exogenous Mt achieve endosomal escape, and then fuse into the original Mt network⁶¹. However, the clear mechanism remains unknown. On the other hand, another exogenous Mt might fail to escape and be degraded in the lysosome to maintain the mitochondrial homeostasis⁶²⁻⁶³. In this study, it is apparent in **Figure 4B(b)** that the exogenous Mt can fuse into the endogenous Mt network 1 day after incubation. Just after the 6 hr co-incubation of Mt-cGNS, the green fluorescence of exogenous Mt was observed in the recipient cells. On the contrary, very few green fluorescence was observed when co-incubated the rho 0 cells with Mt only (**Figure 4B(a)**). This result consistent with those that the cGNS association assists the internalization of Mt (**Figures 3A and 3B**). It is also noticed that the most of Mt-cGNS were internalized into the recipient cells, whereas the Mt fusion was not occurred yet. After culture for 1 day, the fluorescence of exogenous Mt (green) was merged with endogenous Mt (red). Because the green fluorescence is expressed from the EGFP tagged with a Mt inner membrane protein, and the red fluorescence of MitoTracker™ Deep Red FM is accumulated in the Mt matrix. This result indicated possibility that Mt-cGNS internalized escaped from the endosome, and then fused into the endogenous Mt network both outer and inner membranes.

However, the number of fused Mt was low and decreased with time (**Figure 4C**). There are three possible reasons. Firstly, few amount of Mt-cGNS escaped from the endosome. As demonstrated by another paper⁶⁴, the secondary amino groups provided by cGNS showed a pH buffering effect to allow the endosomal escape. It provides possible chance for Mt-cGNS to escape from edosome. Therefore, the lost of exogenous Mt in endosome might be few. Secondly, the mitochondrial dynamic of rho 0 cells is abnormal. In response to the stress from mtDNA mutation, the Mt network is remodeled in rho 0 cells. Mt in rho 0 cell are more fragmented which is not observed in the normal cells, and the shape of Mt are short tubules and spheres⁶⁵⁻⁶⁶. In addition, fusion is inhibited whereas fission is promoted in rho 0 cells, and the Mt dynamics becomes slower⁶⁵. It is highly possible that the declined fusion in rho 0 cells led to a decreased fusion of exogenous Mt and endogenous Mt in rho 0 cells. Thirdly, the H9c2 derived rho 0 cells still have an ability to proliferate. It is highly possible that the fused Mt were divided into cells with cell proliferation.

Mt is an organelle which has two membranes, outer membrane and inner membrane. Due to the double-membrane structure, the Mt inside space is divided into two independent spaces. The space between outer membrane and inner membrane is inter-membrane space, and the space inside the

inner membrane is Mt matrix. Energy is produced by the oxidative phosphorylation (OXPHOS) complexes and ATP synthase, which are located on the Mt inner membrane. The complexes transfer the electrons and pump hydrogen ions from the Mt matrix to the inter-membrane space. This movement of hydrogen ions generates a pH gradient and a voltage gradient, so-called Mt membrane potential. This electrochemical gradient drives ATP synthase to synthesis ATP and replace the protons from inter-membrane space to Mt matrix. In addition, transportation of metabolites and inorganic ions into Mt matrix is also driven by the electrochemical proton gradient⁶⁷⁻⁷⁰. Therefore, the Mt membrane potential is one of the most important factors to evaluate the mitochondrial activity. There are several researches to show the evidence that a positive correlation among mtDNA damage, mitochondrial fragmentation, and decline of Mt membrane potential⁴⁷. The internalization of exogenous Mt enhanced the declined of Mt membrane potential in the rho 0 cells. Just 6 hr after co-incubation of Mt-cGNS, the Mt membrane potential recovered in the rho 0 cells to the level which is similar with that of the normal cells. On the other hand, the low internalization of Mt naked did not enhance the Mt membrane potential in rho 0 cells (**Figure 5A**). In this study, the Mt membrane potential was evaluated by the TMRM staining. The TMRM is accumulated in active Mt in a Mt membrane potential-dependent manner due to the cationic and lipophilic nature. The staining was proceeded after the Mt transplantation. Therefore, the high Mt membrane potential might originate from the exogenous Mt present in the recipient cells although of they fuse into the original Mt network or not. To confirm whether the enhancement of Mt membrane potential maintains, the Mt membrane potential was continually investigated after Mt internalization for 2 weeks. The Mt membrane potential decreased with time in the rho 0 cells incubated with Mt-cGNS. The significant higher Mt membrane potential was maintained over 6 days after cultured with Mt-cGNS. At the initial stage of culture, exogenous Mt remained in the recipient cells and contributed to an high Mt membrane potential. However, with the incubation time, some of exogenous Mt fail to fuse with the original Mt network and degraded, while others were divided into cells by the cell proliferation. In addition, it is suggested that activity of damaged Mt is rescued by intact respiratory chain proteins and crucial DNA sequences which provided by fresh exogenous Mt⁷¹. Therefore, the limited Mt fusion would lead to an insufficient material (proteins and mtDNA) exchange, which might be responsible to the decrease of Mt membrane potential. Although the maintenance of enhanced Mt activity is essential in the Mt transplantation research, very few studies are focused. Pour's group reported that the Mt co-incubation promoted the Mt respiration and

cellular bioenergy in healthy cardiomyocytes internalized with non-autologous Mt in 2 days. However, the significant enhancement disappeared for 7, 14, and 28 days⁷². It is reported that the energy demand inside the cells regulates the amount of Mt. Insufficient and excessive Mt will cause a pathological condition. Therefore, normal cells might eliminate excessive Mt to maintain the Mt homeostasis. Jui-Chih Chang's group used the cell penetrating peptide, Pep-1, to promote the internalization of exogenous Mt into an A3243G MELAS cell line²⁷. The improvement of Mt function to produce ATP was limited to 4 days and the normal mtDNA level sharply decreased 11 days after post treatment with Pep-1 conjugated Mt. This might be related to the rapidly proliferation of cells and the increase in mtDNA replication⁷³. In Emma R Dawson's research, MitoPunch is used to introduce exogenous Mt into rho 0 cells²⁶. It is confirmed that the transferred Mt disappeared between one and two weeks after the Mt internalization. However, exogenous mtDNA stably maintained and replaced the endogenous mtDNA in the recipient cells with the mismatched nuclear DNA and mtDNA genomes. The findings strongly suggest the possibility that the exogenous Mt retention is related to the interplay between nuclear and mitochondrial genomes. In this study, both the cell sources of Mt and the recipient cells are same, H9c2 cells line. The matched nuclear DNA and mtDNA genomes might be one of the reasons that the retention of exogenous Mt was limited. The exogenous Mt internalization, endosomal escape, and the fusion of Mt are essential elements for successful Mt internalization. To obtain better therapeutic effect of enhanced Mt internalization, further conditioned investigation should be carried out in future.

The cGNS used as a potential tool to intracellularly deliver healthy mitochondria can also utilize the carrier of drug release. A combination with the controlled release technology may be promising to enhance the Mt therapeutic efficacy.

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Chapter 3.

A Potential Method of Autophagy Imaging with Cationized Gelatin Nanospheres Incorporating Molecular Beacon

Introduction

Recently, regenerative medicine has been noted as a new medical treatment for various diseases of heart, nerve, and senescence or tissue deficit which cannot be completely cured by the conventional therapies, such as the surgical or drug treatment.¹⁻⁴ The basic idea of regenerative medicine is to activate and regulate the biological functions of cells which promote the self-healing potential of body itself for tissue regenerating and repairing. In this connection, it is technically important to evaluate the cell functions which physiologically relate to the cell-based healing potential. In addition, the time course of functions should be evaluated for the same cell in the alive condition. Living cell imaging is one of the promising technologies to visualize cell functions.⁵⁻⁷ Temporally and spatially detailed data can be obtained by using the imaging technology comparing with the conventional evaluation methods, such as immunofluorescence (IF), western blotting (WB), polymerase chain reaction (PCR), and single molecule fluorescence in situ hybridization (smFISH). However, to prepare the sample for the above methods, cells are needed to destroy. A new technology is expected to develop for the visualization of cell functions in the living state which enables us to maintain the integrity of cells and evaluate their functions during experiments.

Molecular beacon (MB) is known as one of the imaging probes which can convert a gene expression to a fluorescent signal.⁸⁻⁹ MB is a nucleotide-based material with a loop-stem structure. A fluorophore is conjugated on the end of the sequence while a quencher is conjugated on the other side. Normally, MB cannot emit fluorescence because the fluorophore and quencher are adjacent to each other due to the stem structure. After hybridization with the target nucleotide which has the complementary sequence toward that of loop structure, the conformation of MB changes and emit fluorescence because the fluorophore and quencher are separated. In other words, by varying the loop sequence, the target gene expression is available to be visualized. This technique is applied to PCR and has achieved the high precision detection of gene expression.¹⁰ Recently, MB is applied as an imaging probe to visualize the target gene expression in living cells.¹¹⁻¹⁵ However there is a

problem to be resolved to allow MB to internalize into cells because of the electrorepulsion between MB and cell membrane which both of them are negative charged. Thus, it is essential to develop an effective method of MB into living cells for their imaging.

Gelatin is one of the natured and biocompatible polymers. Cationized gelatin of a positive charge can be prepared by introducing spermine of a polyamine present in the body to gelatin.¹⁶ The cationized gelatin enables to electrostatically interact with negative charged materials. Based on this feature, the cationized gelatin has been used for a carrier to internalize gene-based materials into cells.¹⁷⁻¹⁹ It is reported that the cationized gelatin was effective in the internalization of MB into cells.²⁰ In the previous researches, cationized gelatin nanospheres were prepared by the coacervation method of cationized gelatin for MB incorporation. When applied to cells to evaluate their cell functions, the cationized gelatin nanospheres incorporating MB can visualize the apoptosis, the proliferation, and the differentiation in the time dependent manner.²¹⁻²²

Autophagy is one of the important cell functions as the intracellular degradation system.²³ For example, autophagy plays an important role in starved cells. Nutrition lacking triggers cells to degrade their components to generate amino acids by the autophagy to overcome the starvation. In addition, another important role of autophagy is to maintain the quality of cell components. Autophagy assists to degrade the cell components damaged, such as damaged mitochondria or lysosomes and protein aggregates. Autophagy selectively eliminates the damaged components. Recent researches demonstrate that autophagy relates to several serious diseases of cancer, nervous diseases, and chronic diseases.²⁴⁻²⁶ Therefore, autophagy is considered as a promising therapeutic target.²⁷⁻²⁹ To develop autophagy researches, it is of prime importance to develop the technology for the temporal and spatial visualization of autophagy.

The objective of this study is to develop an imaging method to visualize autophagy activity in living cells. Autophagy was induced by incubating cells in the serum and amino acids-free medium. Autophagy activity was confirmed by immunofluorescence (IF) and polymerase chain reaction (PCR). On the other hand, the availability of cationized gelatin nanospheres incorporating MB (cGNS_{MB}) were designed as the imaging probe to visualize and evaluate the autophagy activity. In addition, after incubating autophagy induced cells with the cGNS_{MB} for the internalization, the autophagy activity was visualized to compare with the conventional evaluation methods of PCR. Upon applying the cGNS_{MB} to cell culture systems with a gradient of autophagy state, the feasibility of cGNS_{MB} to visualize the autophagy activity is evaluated.

Materials and Methods

1. Materials

Gelatin with an isoelectric point of 9.0 and the weight-averaged molecular weight of 99,000, prepared by an acidic treatment of pig skin, was kindly supplied from Nitta Gelatin Inc., Osaka, Japan. MB for mouse mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sequestosome 1 (SQSTM1), and cathepsin F (CTSF) were designed by Beacon Designer™ (PREMIER Biosoft, Palo Alto, CA, USA), and synthesized by Integrated DNA Technologies, Inc. Coralville, IA, USA. The sequences of MB were listed in **Table 2**. Glutalaldehyde (GA 25 wt% in water), glycine, concentrated hydrochlonic acid (HCl), acetone, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) were purchased from Nacalai Tesque. Inc., Kyoto, Japan. Spermine was purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. The reagents were used without further purification.

Table 2. Sequence of MB used.

MB	Sequences (5' to 3')
SQSTM1 MB	[TYE®665]-CGC GAT CAC CTT TCA CTC ACT ATG GAC AGG ATC GCG-[IBRQ]
CTSF MB	[TYE®665]-CGC GAT CAG ACA GTC AAT CGC CAC TGG ATC GCG-[IBRQ]
GAPDH MB	[TYE®665]-CTG GTA ATC CGT TCA CAC CGA CCT TCA CCA G-[IBRQ]
SQSTM1 MB not conjugated quencher	[TYE®665]- CGC GAT CAC CTT TCA CTC ACT ATG GAC AGG ATC GCG
CTSF MB not conjugated quencher	[TYE®665]-CGC GAT CAG ACA GTC AAT CGC CAC TGG ATC GCG
GAPDH MB not conjugated quencher	[TYE®665]-CTG GTA ATC CGT TCA CAC CGA CCT TCA CCA G

MB: molecular beacon

SQSTM1: Sequestosome 1

CTSF: Cathepsin F

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IBRQ: Iowa Black® RQ

underline: stem structure

2. Preparation of cationized gelatin

Cationized gelatin was prepared according to the method previously reported.¹⁷ In brief, spermine was added into 50 ml of double-distilled water (DDW) containing 2.0 g of gelatin at the molar ratio of 50 to the carboxyl groups of gelatin. The pH of mixture solution was adjusted at 5.0 with 11 M HCl. To perform the condensation reaction, EDC was added at the molar ratio of 3 to the carboxyl groups of gelatin. Then, the solution was stirred at 40°C for 18 hr, and then dialyzed against DDW for 3 days with a dialysis membrane (molecular weight cut off = 12,000 to 14,000, Viskase Companies, Inc., Willowbrook, IL, USA) at room temperature. After dialysis, the solution was freeze-dried to obtain cationized gelatin. To determine the percentage of spermine introduced into the carboxyl groups of gelatin, the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS, FUJIFILM Wako Pure Chemical Inc., Osaka, Japan) method was performed.⁷¹ The percent introduced of spermine was 45.3 ± 4.6 %.

3. Preparation of cationized gelatin nanospheres incorporating MB (cGNS_{MB})

Cationized gelatin nanospheres (cGNS) were prepared by the coacervation method previously reported.³⁸ Briefly, to form coacervate, 5 ml acetone was added to 1.25 ml of 50 mg/ml cationized gelatin aqueous solution at 40 °C. Immediately after that, 20 µl of 25 wt% GA was added to crosslink cationized gelatin. After 6 hr incubation, an excessive amount of 0.5 M glycine was added for the blocking of aldehyde groups unreacted. The resulting solution was agitated overnight at 40 °C and the residual acetone was evaporated. Then, cGNS was collected by the centrifugation of 16,000 g for 30 min at 25 °C and resuspended in DDW. The centrifugation and resuspension procedure were repeated three times to wash cGNS. cGNS containing MB (cGNS_{MB}) were prepared by simply mixing 1 mg/ml cGNS and 100 µM MB at the ratio of 20 pmole of MB/ 1 µg of cGNS. The sequences of MB used in this study were listed in **Table 2**. Following the incubation for 15 min at room temperature, the mixture was centrifuged at 16,000 g for 15 min at room temperature. The supernatant was removed and the precipitated cGNS_{MB} was resuspended in DDW.

4. Characterization of cGNS with or without MB incorporation

The apparent size of cGNS or cGNS_{MB} was measured by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). The zeta potential of cGNS or cGNS_{MB} was measured by electrophoresis light scattering (ELS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). The amount of MB incorporated into cGNS was determined

by using MB without a quencher. Briefly, after mixing cGNS and the quencher-free MB, the mixture was centrifuged and the supernatant was collected similarly. The fluorescence intensity of supernatant was measured with Multimode Microplate Reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan). The amount of MB incorporated into cGNS was calculated in comparison with the fluorescence intensity of aqueous MB solution.

5. Cell culture experiments

NIH/3T3 cells (JCRB0615) of a mouse embryo-derived fibroblast line were purchased from JCRB Cell Bank (National Institute of Biomedical Innovation, Health and Nutrition, Osaka, Japan). The cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10 vol% bovine fetal calf serum (FCS, GE healthcare Life Science Hyclone laboratories inc., Logan, UT, USA) and 1 vol% of penicillin and streptomycin (Nacalai Tesque. Inc., Kyoto, Japan) at 37 °C in a 5 % CO₂ 95 % air atmospheric condition. The cells were detached with 0.25 wt% trypsin-containing 1 mM EDTA solution (Nacalai Tesque Inc., Kyoto, Japan) and continued to culture in 100 mm cell culture dish (Corning Inc., Corning, NY, USA) to allow them to grow until to 80 % confluency.

6. Evaluation of cGNS_{MB} internalization into cells

Cells were seeded at the density of 1×10^5 cells/well in 6 well multi-dish culture plate (Corning Inc., Corning, NY, USA) with 2 ml/well of DMEM. After the incubation for 24 hr, the medium was changed to 1 ml/well of Opti MEM (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 1 µg of cGNS_{MB}. After 3 hr internalization incubation with cGNS_{MB}, cells were washed with PBS, detached with the trypsin solution, and resuspended in cold 10 mM phosphate buffered saline solution (PBS). In addition, naked MB targeting GAPDH (GAPDH naked MB) and Lipofectamine 2000 containing MB targeting GAPDH (GAPDH Lipo) were used as the negative and positive control, respectively. The naked MB (200 pmole) was co-cultured with cells for 3 hr in Opti-MEM to allow to internalize into cells. Lipofectamine 2000 was used as the manufactured protocol. The cell suspension was analyzed by fluorescence activated cell sorting FACSCanto II (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

7. Evaluation of cell viability after incubation with cGNS_{MB}

Cells (1×10^4) were seeded on each well of 96 well multi-dish culture plate (Corning Inc., Corning, NY, USA) with 100 μ l/well of DMEM. After the incubation for 24 hr, the medium was changed to 100 μ l/well of Opti MEM (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 1 μ g/ml of cGNS_{MB}. Following 3 hr internalization with cGNS_{MB}, 10 μ l of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Nacalai Tesque. Inc., Kyoto, Japan) was added and incubated for 4 hr according to the manufacture's protocol. After that, the absorbance at 450 nm was measured with plate reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan). In addition, Lipofectamine 2000 containing MB targeting GAPDH (GAPDH Lipo) was used as the control. Lipofectamine 2000 was used as the manufactured protocol. The percent survival of cells was calculated as 100 % for that cultured without cGNS_{MB}.

8. Autophagy induction

Cells were seeded at the density of 5×10^4 cells/well in 6 well multi-well culture plate with 2 ml/well of DMEM. After the incubation for 24 hr, the medium was changed to 1 ml/well of Opti MEM containing 1 μ g/ml of cGNS_{MB}. After 3 hr incubation with cGNS_{MB}, the medium was changed to DMEM, followed by an additional for 24 hr incubation. The cells without the cGNS_{MB} incubation were incubated in DMEM for 48 hr. Next, the medium was changed to 2 ml of Earle's Balanced Salts (EBSS, SIGMA ALDRICH, Co., St. Louis, MO, USA) which is serum and amino acids-free medium (starvation medium) for different time periods up to 450 min. The cells incubated in the starvation medium were used in the following experiments.

9. Immunofluorescent staining

Cells were seeded at the density of 5×10^4 cells/dish in a glass bottom dish of 35 mm in diameter (Matsunami Glass Industries Ltd., Tokyo, Japan) and incubated in DMEM (2 ml/dish) for 24 hr. The cells internalization culture with or without cGNS_{MB} were incubated with of EBSS (2 ml/dish), starvation medium for 0, 90, 180, 270, 360, and 450 min as described above. Following the starvation medium incubation, the cells were fixed with 4 vol% of paraformaldehyde for 15 min, permeabilized with 50 μ g/ml of digitonin for 15 min, and blocked with ImmunoBlockTM (KAC Co. Ltd., Kyoto, Japan) for 60 min. Every treatment was performed at room temperature. Next, the cells were incubated with a primary antibody, rabbit polyclonal anti-LC3B (Light Chain 3B) antibody (PM036, MBL CO., LTD, Tokyo, Japan) overnight at 4 °C. Then, the cells were incubated with the

secondary goat anti-rabbit IgG (H + L) antibody conjugated with Alexa Fluor 488TM (A11008, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 hr at room temperature. The cell nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 min at room temperature. The fluorescent images were taken by a fluorescent microscope (BZ-X700, KEYENCE Co., Ltd., Osaka, Japan). To evaluate the autophagy activity, the number of LC3B located vesicles (LC3 dots) in a cell was counted with an ImageJ software (NIH, Bethesda, MD, USA).

10. Western Blotting analysis

Cells were seeded to 6 well multi-well culture plate with 2 ml/well of DMEM at the density of 1×10^5 of cells/well. After the incubation for 24 hr, cells were separated into two groups. One group was cultured in the serum containing medium supplied 250 nM of bafilomycin A1 (Cayman Chemical Company, MI, USA) as a vacuolar-type H⁺-ATPase inhibitor. The other group was cultured in the serum containing medium without bafilomycin A1. After 2 hr incubation with or without bafilomycin A1, each groups of cells were cultured in the serum-free medium with or without bafilomycin A1. Following the culture in the starvation medium as described above, the cells were lysed with a radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Nacalai Tesque Inc., Kyoto, Japan) containing 1 mM Na₃VO₄ and 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration of cell lysates was determined by BCATM Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) as the manufacture's protocol. After the determination of protein concentration, the cell lysates were mixed with Laemmli sample buffer (277.8 mM Tris-HCl pH6.8, 44.4% lithium dodecyl sulfate, 0.02% bromophenol blue, Bio-Rad Laboratories. Inc., Hercules, CA, USA) supplemented with 2.5% 2-mercaptoethanol. The cell lysates (10 µg/lane) were applied to a sodium lauryl sulfate-poly(acrylamide) gel to carry out the electrophoresis (SDS-PAGE). After the SDS-PAGE, the separated protein bands were blotted to a polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked with ImmunoBlockTM for 60 min at room temperature, incubated with primary antibodies, rabbit polyclonal anti-LC3B (PM036, MBL CO., LTD, Tokyo, Japan) antibody or rabbit monoclonal anti beta-Actin antibody (4970L, Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4 °C, and incubated with secondary horseradish peroxidase-conjugated anti-rabbit antibody (#7074, Cell Signaling Technology, Inc., Danvers, MA,

USA) for 1 hr at room temperature. Following washing with Tris buffered-saline with Tween 20 (TBST), the membrane was incubated with a chemiluminescence reagent, Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 5 min at room temperature in a dark condition. The bands were detected by a chemiluminescence imaging system LAS-4000 (FUJIFILM Co., Tokyo, Japan). The autophagy flux index was determined by the difference of protein expression levels between LC3 conjugating phosphatidylethanolamine (LC3-II) in the presence of bafilomycin A1 and that in the absence of bafilomycin A1.

11. mRNA expression analysis

The cells were induced autophagy by the incubation in the starvation medium as described above. The total RNA of cells was extracted by RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. The extracted RNA was reverse-transcribed to the complementary DNA (cDNA) by SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, quantitative real-time polymerase chain reaction (qPCR) was performed by Prism 7500 real-time PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction was performed in the presence of cDNA, forward and reverse primers, and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used for qPCR were listed in **Table 3**. The gene expressions were normalized with a GAPDH expression. The gene expression levels were analyzed by the $\Delta\Delta C_t$ method compared with that of cells incubated in the serum-containing medium which are expressed as the autophagy induction for 0 min.

Table 3. PCR primers used.

PCR primers	Sequences (5' to 3')
GAPDH forward	TGA CCT CAA CTA CAT GGT CTA CA
GAPDH reverse	CTT CCC ATT CTC GGC CTT G
SQSTM1 forward	GAA CTC GCT ATA AGT GCA GTG T
SQSTM1 reverse	AGA GAA GCT ATC AGA GAG GTG G
CTSF forward	TGA CCA CCT ATA ACC GGA CTT
CTSF reverse	TGG TGA TCC CAT ACT GAG CTG

12. Fluorescent imaging of autophagy activity of cells incubated in the starvation condition

Cells were seeded to the glass bottom dish, incubated with cGNS_{MB} for internalization, and then induced autophagy as described above. The fluorescent images were taken by a fluorescent microscope (BZ-X700, KEYENCE Co., Ltd., Osaka, Japan). The fluorescence intensity of MB was quantified by an ImageJ software (NIH, Bethesda, MD, USA) and normalized with that of cells without autophagy induction.

13. Fluorescent imaging for a solute gradient in μ -Slide I

A solute gradient sample was prepared with fluorescein isothiocyanate isomer I (FITC, Sigma-Aldrich Inc., St Louis, MO, USA) and the channel slide, μ -Slide I Leur Glass Bottom (ibidi GmbH, Grafeling, Germany). To prepare the concentration gradient of FITC, PBS containing FITC and PBS were used. First, the channel was completely filled with PBS. Then, the half channel volume of PBS containing FITC was added from one side, while the same a volume of PBS was aspirated from the other side. Following incubation for up to 72 hr at 37°C in a dark condition, the fluorescent images were taken at the predetermined points by a fluorescent microscope (BZ-X700, KEYENCE Co., Ltd., Osaka, Japan).

14. Fluorescent imaging of autophagy activity of cells cultured in the concentration gradient of amino acids

The sample of an amino acids concentration gradient was prepared with a channel slide, μ -Slide I Leur Glass Bottom (ibidi GmbH, Grafeling, Germany) according to manufacturer's instructions. In brief, the cell suspension in DMEM at the density of 5×10^5 cells/ ml was injected to the slide chamber and incubated for 24 hr. To internalize cGNS_{MB} into cells, the medium was changed to Opti MEM containing cGNS_{MB} and incubated for 3 hr. After the internalization incubation, the Opti MEM was removed and then, cells were further incubated in DMEM for 24 hr. To prepare the concentration gradient of amino acids, DMEM (serum-containing medium) and EBSS (starvation medium without serum and amino acids) were used. First, the channel was completely filled with EBSS. Then, the half channel volume of DMEM was added from one side, while the same a volume of PBS was aspirated from the other side. The cells were incubated in the gradient condition for 0, 180, and 360 min. The fluorescent images were taken at the predetermined points by a fluorescent

microscope (BZ-X700, KEYENCE Co., Ltd., Osaka, Japan). The fluorescence intensity of MB was determined by an ImageJ software (NIH, Bethesda, MD, USA).

15. Statistical analysis

The data were expressed as the average $\pm$ standard deviation (SD). All the statistical analysis were performed using one-way analysis of variance (ANOVA) with a post-hoc Tukey-Kramer multiple comparison test. *P* values less than 0.05 were considered to be statistically significant.

Results

1. Characterization of cGNS_{MB} prepare

Table 1 shows the physicochemical properties of cGNS or three types of cGNS_{MB} targeting to GAPDH, SQSTM1 or CTSF gene. Irrespective of the type of incorporated MB, the amount of MB incorporated into cGNS was around 20 pmole per 1 μ g of cGNS. The mixing ratio to prepare the cGNS_{MB} was 20 pmole of MB per 1 μ g of cGNS, indicating nearly 100 % of MB incorporation into cGNS. The apparent size was around 200 nm, while all cGNS_{MB} were charged positively and the zeta potentials was around 10 mV.

Table 1. Physiochemical properties of cGNS_{MB}

	Amounts of MB incorporated (pmole/ μ g)	Apparent size (nm)	Zeta potential (mV)
cGNS		175.3 $\pm$ 0.23	8.44 $\pm$ 0.95
cGNS _{GAP MB}	19.9 $\pm$ 0.0 ^{a)}	227.3 $\pm$ 7.40	10.3 $\pm$ 0.51
cGNS _{SQS MB}	20.0 $\pm$ 0.0	217.7 $\pm$ 2.47	10.7 $\pm$ 0.75
cGNS _{CTSF MB}	19.9 $\pm$ 0.0	219.2 $\pm$ 3.70	10.8 $\pm$ 0.49

a) Average $\pm$ SD.

2. Cell internalization of cGNS_{MB}

Figure 1 shows the percentage of cGNS_{MB} incorporating different MBs without the quencher. Since the quencher-free MB always emits fluorescence, irrespective of the target gene expression, the fluorescence intensity is directly correlated with the amount of MB internalized into cells. All types of cGNS_{MB} were internalized into cells at the similar level of about 100 %. The internalization efficiency of Lipofectamine 2000 containing GAPDH MB was also around 100 %. There was no

significant difference in the internalization efficiency between Lipofectamine 2000 and cGNS_{MB}. On the other hand, the internalization efficiency of naked MB as the negative control was 44.7 %. When the cytotoxicity of cGNS_{MB} was evaluated (**Figure 2**), no cytotoxicity was observed, irrespective of the type of MB incorporated.

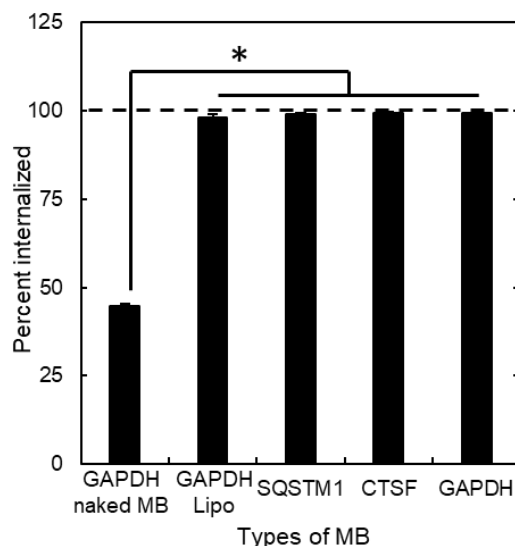


Figure 1. The percentage of naked MB, Lipofectamine 2000 containing MB (Lipo), and cGNS_{MB} internalization into cells. The cells were incubated with 200 pmole of MB, Lipofectamine containing 200 pmole of MB, or 10 μ g/ml of cGNS_{MB} for 3 hr. The number of cells detected by the flow cytometer is expressed as 100%. *, $p < 0.05$; significant against the naked MB targeting GAPDH.

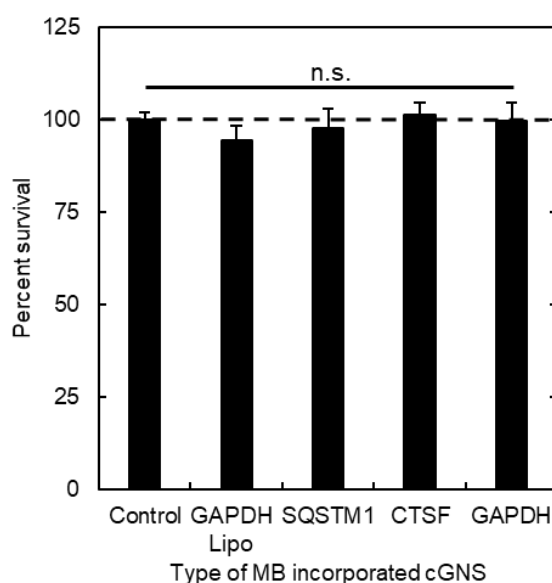


Figure 2. The percent survival of cells incubated with Lipofectamine 2000 containing 200 pmole of MB targeting GAPDH (GAPDH Lipo) or 10 μ g/ml of cGNS_{MB} incorporating different MBs for 3 hr. The viability of cells incubated without cGNS_{MB} is expressed as 100%. n.s.; not significant.

3. Effect of starvation induction time on the autophagy activity and autophagy-related gene expression

Figure 3A shows the fluorescent microscopic images of cells incubated in the starvation medium for 0, 90, 180, 270, 360, and 450 min. For the incubation time of 90 to 450 min, the cytoplasmic fluorescence of LC3 dots was observed. On the other hand, at 0 min incubation where cells were not starvation incubated, LC3 dots were not observed. From the quantitative results (**Figure 3B**), the number of LC3 dots increased with time after the starvation incubation. The number was the highest at 90 min, and then gradually decreased. **Figures 3C and D** show the results of autophagy flux assay. In the absence of bafilomycin A1, the protein expression of LC3-II was the highest at 90 min, and then gradually decreased. In contrast, in the presence of bafilomycin A1, the protein expression increased with time after starvation incubation. The autophagy flux index increased with the time of autophagy induction. **Figure 4** shows the effect of autophagy activity on the expression of autophagy-related SQSTM1 and CTSF genes. Both of SQSTM1 and CTSF gene expressions were significantly increased with the increase of autophagy induction time.

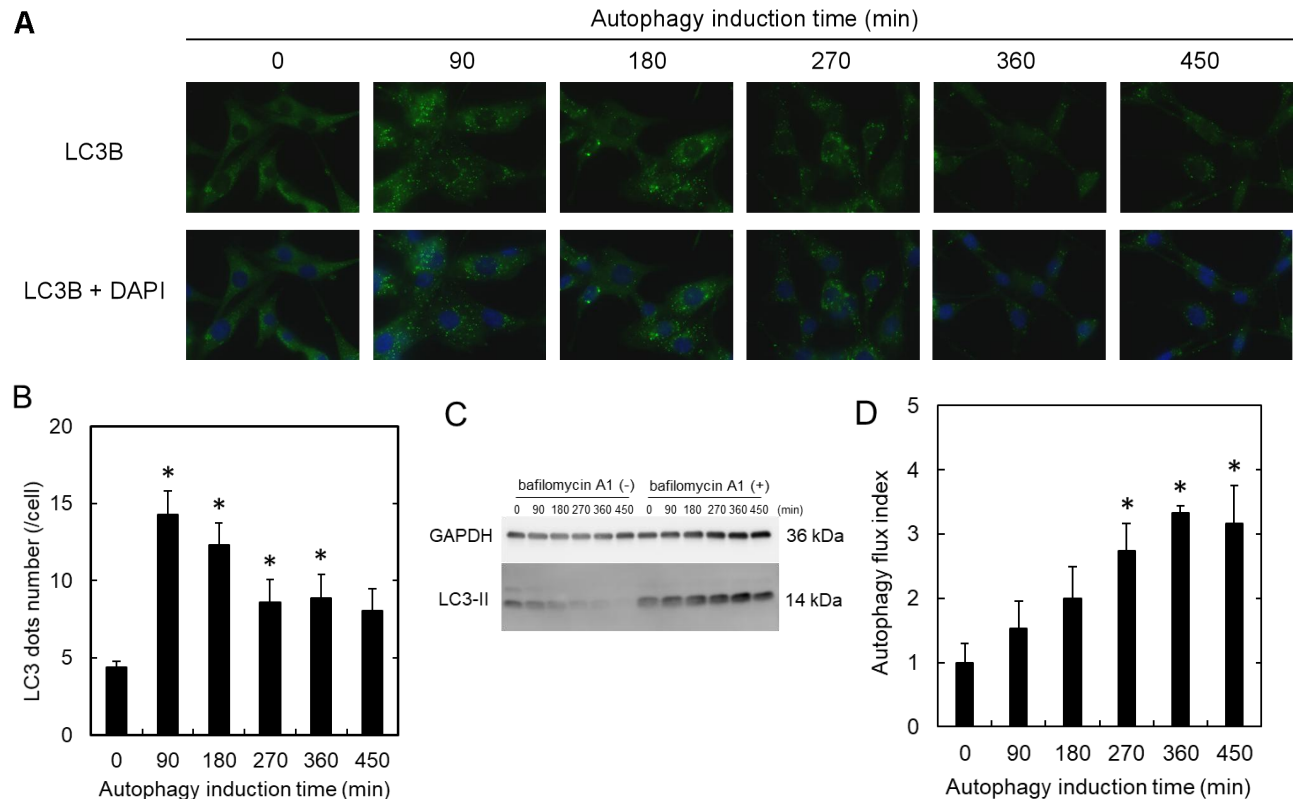


Figure 3. Immunofluorescent staining of LC3B for cells before and after autophagy induction. (A) Immunofluorescent images of LC3B in cells incubated with the serum-free medium for 0, 90, 180, 270, 360, and 450 min. Green: LC3B. Blue: nuclei by DAPI. Scale bar is 20 μ m. (B) Effect of

autophagy induction time on the number of LC3 dots. *, $p < 0.05$; significant against the number of LC3 dots of the cells cultured in serum-containing medium. (C) Western blotting analysis of LC3-II for autophagy induced cells. GAPDH was used as the loading control. (D) Autophagy flux analysis. *, $p < 0.05$; significant against the index of cells cultured with the serum-containing medium.

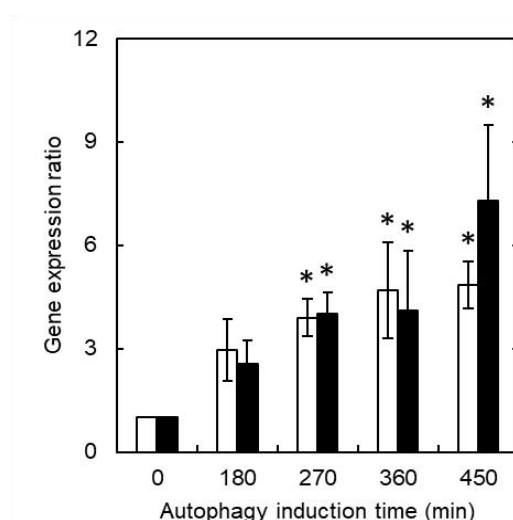


Figure 4. Autophagy-related gene expression levels of cells as a function of autophagy induction time: SQSTM1 (□) and CTSF (■) genes. The gene expression level is normalized to that of cells cultured in the normal medium. *, $p < 0.05$; significant against the expression level of cells cultured in the serum-containing medium at the corresponding gene.

4. Fluorescent imaging of autophagy activity

To reveal the availability of MB as an imaging probe of autophagy activity, the fluorescence intensity change of cGNS_{MB} in autophagy-induced cells was observed (**Figure 5A**) and the quantified data are shown in **Figure 5B**. The MB targeting to autophagy-related SQSTM1 (a) and CTSF genes (b), and GAPDH gene (c) which is constantly expressed in cells, were used as the autophagy imaging and internal control probes respectively. For the cGNS_{SQS MB} and cGNS_{CTSF MB}, the fluorescence intensity increased with the time of autophagy induction. On the other hand, the fluorescence of cGNS_{GAP MB} hardly changed at any induction time. The fluorescence intensity of cGNS_{SQS MB} and cGNS_{CTSF MB} were significantly increased as the cell autophagy proceeded. **Figure 6** indicates the effect of cGNS_{MB} internalization on the autophagy activity. The number of LC3 dots in the cells increased with the autophagy induction time. There was no significant difference in the LC3 dots number between the cells with and without cGNS_{MB} internalization.

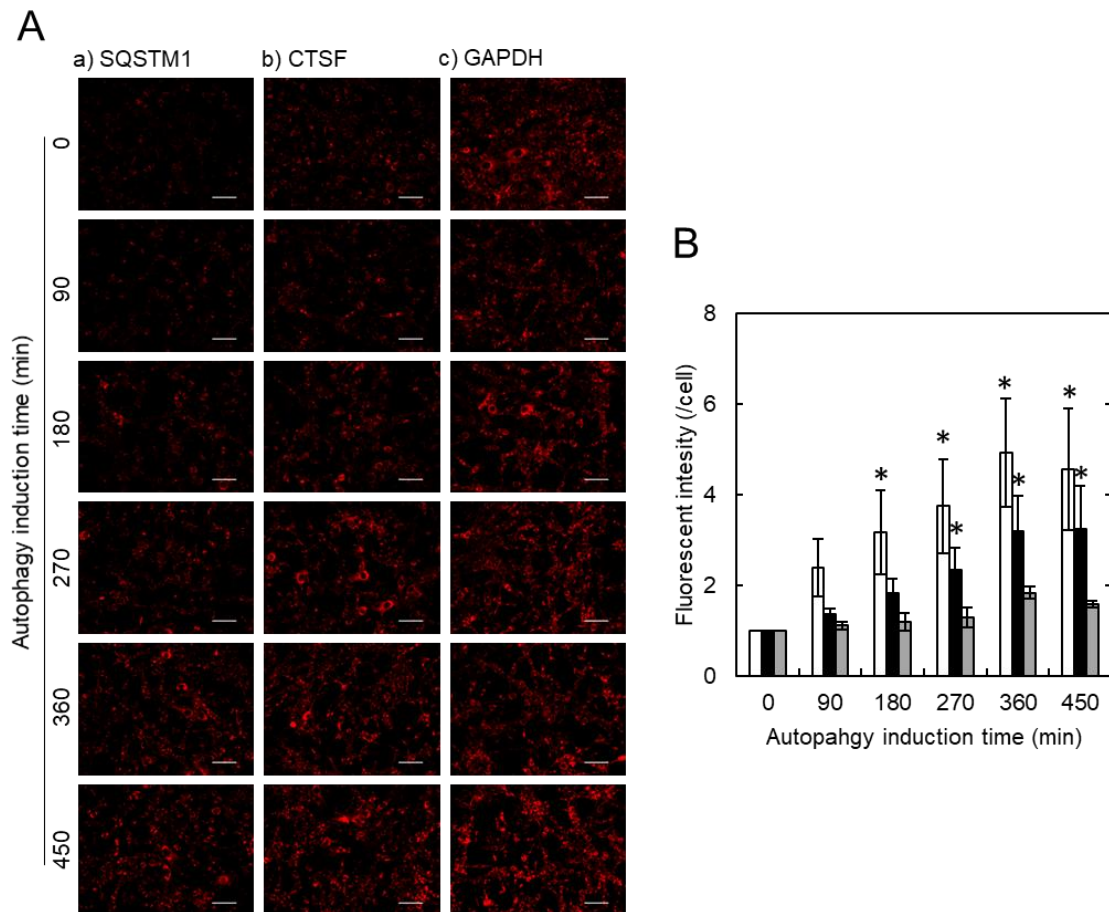


Figure 5. Fluorescent imaging of cGNS_{MB} internalized cells as a function of autophagy induction time. (A) Fluorescent images of cells incubated for the internalization of cGNS_{MB} targeting to SQSTM1 (a), CTSF (b), and GAPDH (c) after culturing in the serum-free medium for 0, 90, 180, 270, 360, and 450 min. Scale bar is 100 μ m. (B) Effect of autophagy induction time on the fluorescence intensity of cells incubated for the internalization of cGNS_{MB} incorporating SQSTM1 (□), CTSF (■), and GAPDH (■). The fluorescence intensity is normalized by that of cells incubated in the serum-containing medium (time = 0 min). *, $p < 0.05$; significant against fluorescence intensity of cells cultured with the serum-containing medium at the corresponding gene.

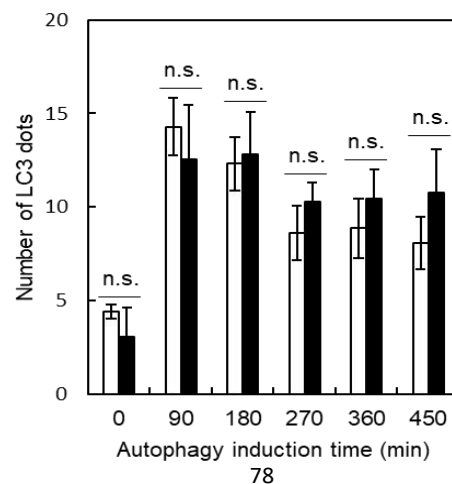


Figure 6. Time profile of LC3B staining images of cells with (□) or without cGNS_{MB} internalization culture (■). The cells were incubated with 10 µg/ml of cGNS_{MB} for the internalization, and then incubated with the serum-free medium, followed by the anti-LC3B antibody immunostaining and fluorescent microscopic observation. The number of LC3 dots in cells were counted by an ImageJ software and compared to that of the autophagy induced cells cultured without cGNS_{MB}. n.s.; not significant.

5. Fluorescent imaging of autophagy activity in condition of an amino acids gradient concentration

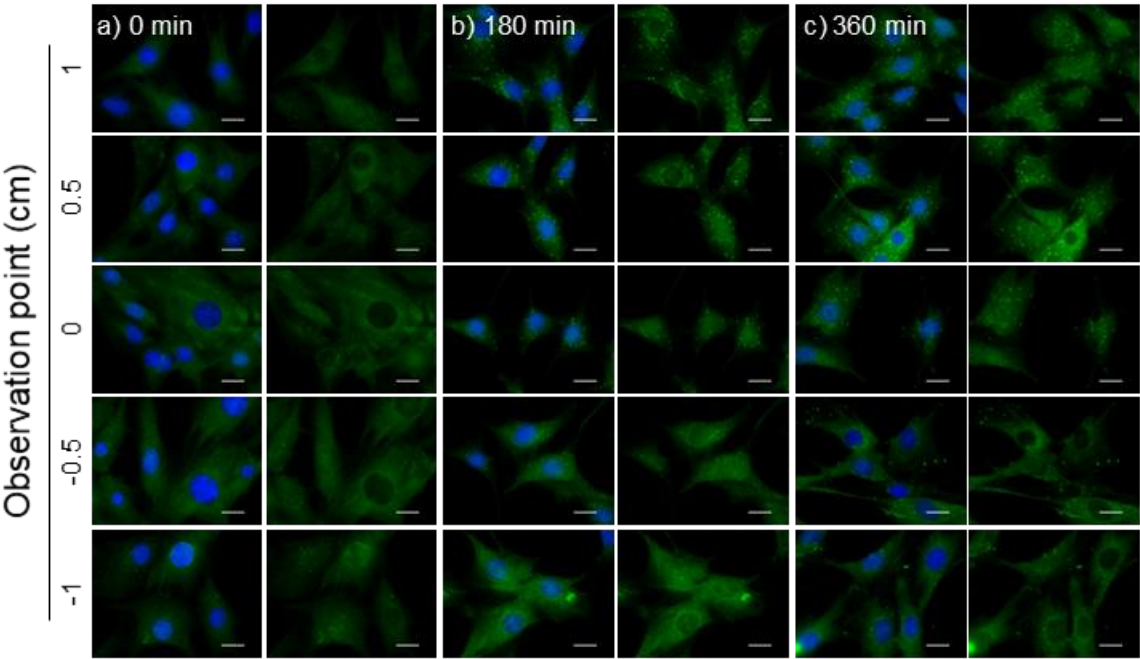
To indicate that imaging methodology using cGNS_{MB} enables to provide more detailed, temporal, and spatial information, an amino acids-gradient condition was prepared to induce autophagy and visualized by cGNS_{MB}. µ-Slide I is known as a channel slide to prepare the gradient condition and reported in several researches. Using this channel slide and two types of solution, each of them are contained solute at different concentrations, gradient of solute are formed in the channel. **Figure 7** shows the fluorescence image of channel slide with a concentration gradient of solute were formed. FITC as the visible solute. The fluorescent signal gradually increased toward the FITC-rich side. This gradient condition maintained for at least 72 hr. Based on this finding, a concentration gradient of amino acids was prepared by using amino acids-rich DMEM and amino acids-free EBSS. Following the gradient preparation, cells were immunostained for LC3B to evaluate the autophagy activity. In **Figure 8**, 0 cm of observation point means the center of slide channel. Toward the side of an increased positive number, the concentration of amino acids is higher in the medium, where the autophagy is not induced. On the other hand, toward that of an increased negative number, the amino acids concentration is lower where the autophagy is induced. **Figure 8A** shows the fluorescent images of cells stained with anti LC3B antibody. The number of LC3 dots increased as the increase of autophagy induction time. This tendency became strong toward the amino acids-poor side. **Figure 8B** shows the fluorescent images of cells internalization incubated with cGNS_{MB} cultured. The fluorescence intensity of cGNS_{MB} in cells increased with the autophagy induction. Interestingly, the fluorescence intensity tended to increase gradually as the amino acids concentration became low, which means stronger autophagy induction. The quantitative result of cGNS_{MB} fluorescence intensity in cells (**Figure 8C**) confirmed the fluorescence intensity to a

significantly strong extent in the side of autophagy induction. In addition, at the site close to the amino acids-free side, a higher cell proliferation was observed.

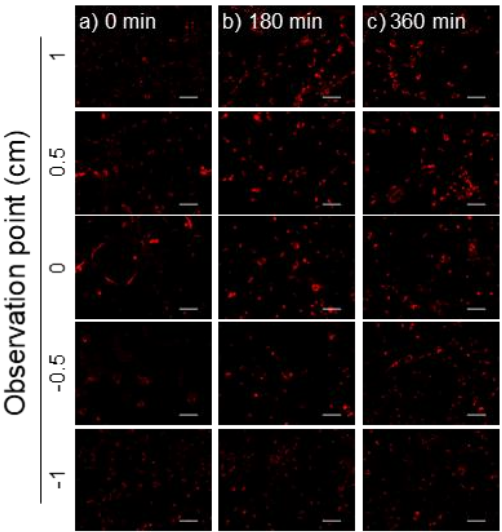


Figure 7. A merged fluorescence and bright field image of channel slide in a solute-gradient condition. FITC-PBS solution was used to visualize the gradient situation. Scale bar is 5 mm.

A



B



C

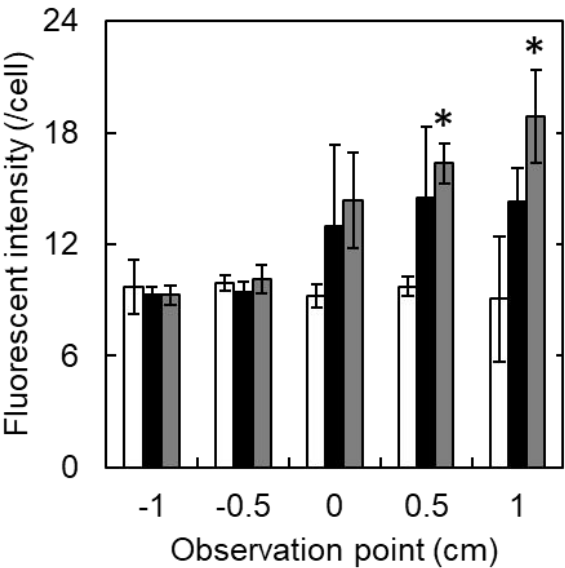


Figure 8. Fluorescent image patterns of MB in cells incubated in the amino acids-gradient condition. (A) Immunofluorescent images of LC3B in cells cultured in the channel slide filled with serum-free medium for 0 (a), 180 (b), and 360 min (c). The images were taken at different observation points. The center of channel slide was defined as 0 cm. Green: LC3B. Blue: nuclei by DAPI. Scale bar is 20 μ m. (B) Fluorescent image patterns of cells internalized cGNS_{MB} targeting to CTSF and induced autophagy for 0 (a), 180 (b), and 360 min (c). Scale bar is 100 μ m. (C) The quantitative analysis of fluorescence intensity of MB in the cells induced autophagy for 0 ($\square$), 180 ($\blacksquare$), and 360 min ($\blacksquare$). *, $p < 0.05$; significant against fluorescence intensity of cells at -1 cm at the corresponding observation time.

Discussion

The present study demonstrates that cGNS_{MB} can be applied as an imaging probe to visualize autophagy which is one of the important cell functions. In addition, temporally and spatially detailed information without cells disruption is obtained by using the cGNS_{MB} system. In this study, the autophagy of cells was induced by culturing with the serum and amino acids-free starvation medium. The expression of autophagy-related SQSTM1 and CTSF genes increased in autophagy-induced cells. cGNS_{MB} were effectively internalized into cells just by the simple incubation, and the fluorescence intensity of cGNS_{MB} in cells increased associated with an increase of autophagy activity. In addition, in the culture system of amino acids concentration gradient, the autophagy-dependent change of fluorescence intensity of cGNS_{MB} was observed.

The cGNS_{MB} used had around 200 nm of apparent size and 10 mV of zeta potential, irrespective of the type of MB incorporated (**Table 2**). In the DLS analysis, the particle size of cGNS hydrated was measured. Because cGNS were internalized in the hydrated state by cells. The values were decided based on the result previously reported to investigate an optimal condition for effective cGNS_{MB} internalization.³⁰ Therefore, it is likely that cGNS_{MB} prepared in this study also showed a high efficiency of cellular internalization. To prepare cGNS_{MB}, 20 pmole of MB was incorporated with 1 μ g of cGNS. In this concentration, almost 100 % of MB were incorporated into cGNS without any loss. It is apparent from **Figure 1** that cGNS_{MB} was internalized into almost 100% of cells and while the type of MB did not affect the percent cGNS_{MB} internalized. The internalization efficiency was similar to that of Lipofectamine 2000 which is a popular transfection reagent. The

internalization efficiency of cGNS_{MB} was high compared with that of naked MB. These findings indicate that cGNS is well-suited carrier of MB which allows MB to incorporate and deliver into cells effectively. For the carrier application, the cytotoxicity of carrier itself is one of the important factors to be considered. It is generally recognized that the nanospheres of a positive charge showed a cytotoxicity.³¹⁻³² However, cGNS_{MB} did not have significant cytotoxicity (**Figure 2**). It can be explained by the following two characteristic of cGNS. First, the main component of cGNS is a biocompatible gelatin. The bio-safety of gelatin has been widely recognized, which allows gelatin to apply in various fields of food, pharmaceutical, and medical researches.³³ Secondly, the molar (N/P) ratio of amino groups to the phosphate groups of cGNS_{MB} used is lower than that of other cationic nanospheres. In other studies of cationic nanospheres, a high N/P ratio around 10 was used to transfect genetic materials into cells.³⁴⁻³⁶ Usage of nanospheres at a high N/P ratio is needed for the effective transfection although they result in cytotoxicity. The N/P ratio of cGNS_{MB} in this study is 1.2 which is much lower than that of other nanospheres. Therefore, it is highly conceivable that cGNS_{MB} does not affect the cell survival. In this study, even though the N/P ratio of cGNS_{MB} was such low, the high percentage of cGNS_{MB} was internalized into cells. In addition to the electrostatic interaction, hydrogen and hydrophobic bonds by various functional groups, such as amino, carboxy, hydroxy, and hydrophobic groups of gelatin would contribute to the interactions between cGNS_{MB} and the cell membrane. Regarding on the intracellular dynamics of cGNS_{MB}, the following findings were experimentally confirmed in the previous study.³⁰ cGNS_{MB} were internalized into cells through the endocytosis, and then, cGNS_{MB} internalized are escaped from the endosomal degradation, and then released to cytoplasm by the proton sponge effect of spermine. cGNS_{MB} in cytoplasm are degraded by enzymes like collagenase, and then, the complex of fragmented of cationized gelatin and MB was generated. In general, spermine molecules have amino groups with acid dissociation constants (pKa) of 7.9-10.9. The spermine conjugated to gelatin facilitates an endosomal escape of complexes by the pH buffering effect of secondary amino groups in spermine.³⁷ However, at present, it is unclear how MB hybridize with target mRNA in a cell. However, it is well known that cationized gelatin and various kinds of nucleic acid materials form complexes by the electrostatic interaction.^{30, 38-39} Therefore, it is highly conceivable that the fragmented cationized gelatin complexed plays an important role in the suppression of MB enzymatic degradation in cells and consequently the mRNA specific hybridization.

The cGNS are useful for the controlled release of MB. The controlled release technology enables MB to achieve a long-term imaging of gene expression. This cGNS_{MB} system is available to visualize cells in the aggregates which require a long time period for formation.⁴⁰⁻⁴¹ The time period of MB controlled released depends on that of cGNS degradation which can be modified by the crosslinking degree. The crosslinking degree of cGNS is changed by varying the amount of glutaraldehyde (GA).³⁰ The higher concentration of GA, the higher crosslinking degree of cGNS. With the high crosslinking degree of cGNS, a long-term controlled release of MB can be achieved. On the other hand, the lower crosslinking degree, the more amount of MB can be incorporated into cGNS while the zeta potential of cGNS_{MB} becomes higher. Based on the findings, cGNS crosslinked with a low amount of GA is adaptable for effective MB internalization. It is reported that PLGA nanospheres were effective in the controlled release of MB.⁴²⁻⁴³ Compared with the PLGA nanospheres, cGNS have been widely used for controlled release applications. It is because the degradation and released profiles can be easily regulated only by changing the crosslinked degree. In addition, more interestingly, it is observed that the cGNS_{MB} internalized were evenly divided into each daughter cells during cell division through real time living cell imaging.²² cGNS_{MB} have an interest property to be internalized into every cells at the similar level of 100 %, and evenly divided into each daughter cells during cell division. Taken together, we can say with certainty that cGNS_{MB} is a suitable imaging probe for the long-term visualization of cell function. Therefore, by using cGNS_{MB}, a long-term imaging of cell function can be achieved even in the cells dividing process during the imaging experiments. In this study, autophagy was induced with serum and amino acids-free medium. By using the starvation medium, autophagy can be induced for a few hours. In this connection, it is not necessary to design the long-term release system. In this study, 20 μ l of GA was selected to prepare cGNS which allows more amounts of MB to incorporate into cGNS. In case the autophagy is induced during the cell culture process, the intracellular release of MB and consequent autophagy visualization will be a useful research tool of cell biology.

In this study, serum and amino acids-free media were used to create a starvation condition which can induce the autophagy of cells.⁴⁴ This method is widely used for autophagy induction experiments.⁴⁵⁻⁴⁷ To certainly and reproducibly induce the autophagy, this standard method was selected. According to The number of LC3 dots were increased for cells cultured with the starvation medium (**Figure 3**), indicating an induction of cell autophagy. The number of LC3 dots was the highest at 90 min, and then decreased with the autophagy induction time. The similar time profile

was reported in the previous research.⁴⁸ It is well known that the starvation stimulates cells to start the autophagy process and isolate the cell components by the autophagosomal membrane where the LC3B is localized.⁴⁹ Therefore, by staining with anti-LC3B antibody, autophagosome can be detected as LC3 dots (**Figure 3A**). Following the isolation, the autophagosome fuses with lysosomes while the isolated cargo is degraded. Because LC3B is also degraded along with the cargo, the number of LC3 dots is gradually decreased with time. Therefore, the decrease in the LC3 dots number (**Figure 3B**) does not always indicate the decreased autophagy activity. The LC3 dots increase implies the promotion of autophagy. To eliminate the effect of component degradation steps, the autophagy flux assay was performed (**Figures 3C and D**). By inhibiting the autophagosome-lysosome fusion with bafilomycin A1, the LC3-II expression can be regarded as the index of autophagy activity.⁵⁰⁻⁵¹ The autophagy index increased with time after the starvation incubation (**Figure 3D**). In other words, the autophagy activity increased as the increase of autophagy induction time.

Autophagy-related gene expression was evaluated as a measure of autophagy activity. By the starvation culture, the autophagy is induced while the transcription factor EB (TFEB) is transferred into nuclei. The TFEB promotes the essential gene expression in autophagy. The genes promoted by TFEB are called autophagy-related genes.⁵² Based on the previous reports,⁵³⁻⁵⁴ compared with other autophagy-related genes, the gene expression of SQSTM1 and CTSF significantly increased associated with autophagy induction. The gene expression of SQSTM1 and CTSF increased as the autophagy activity increased (**Figure 4**). Consequently, in this study, SQSTM1 and CTSF autophagy-related genes were chosen as the target genes for the following visualization of autophagy.

The fluorescence intensity of cGNS_{MB} increased as the autophagy induction time increased, irrespective of the MB type (**Figure 5**). On the other hand, the fluorescence intensity of cGNS_{MB} incorporating GAPDH of an internal control hardly changed. The change in the fluorescence intensity of cGNS_{MB} well corresponded to that of gene expression of SQSTM1 and CTSF determined by the PCR (**Figure 4**). To confirm the reactivity of MB to the target oligonucleotide, a hybridization assay was performed. It is experimentally confirmed that the fluorescence intensity of three types of MB increased as the increase of target oligonucleotide concentration, and each MB can specifically react with the target sequence. Considering the above results, it is concluded that the autophagy-related gene expression can be detected in the living cells by cGNS_{MB}. In this study, two

types of MB targeting to respective autophagy-related genes were designed and applied to the autophagy imaging. The present results suggested that cGNS_{MB} were well-suited to obtain more detailed information of autophagy. It is promising to evaluate the change of autophagy-related gene expression level as the measure of autophagy activity by applying the corresponding MB into living cells. A dual color imaging method with two types of MB to simultaneously evaluate two types of gene expression has been reported. The application of cGNS_{MB} to the dual imaging will provide a new insight into autophagy visualization in the further research. Counting the number of LC3 dots and the autophagy flux assay are utilized as the standard methods to evaluate the autophagy. LC3 dots and the autophagy flux can be regarded as the ideal index for the autophagy activity because these indexes sensitively increase with the autophagy induction. In addition, by applying the fluorescent protein technique, the expression time and place of autophagy related protein including LC3B can be observed in living cells.⁵⁵⁻⁵⁷ However, in addition to the proteins expression, it is also important to evaluate the gene expression which reveals the autophagy mechanism and utilize autophagy as the drug target. Recently, some researches are reported to discover the drugs targeting the respective specific autophagic events, for example mitophagy.⁵⁸⁻⁵⁹ In the studies, the evaluation methods are required to distinguish each type of autophagy. It is known that in the conventional method of LC3 dots and autophagy flux, the indexes increase in autophagy. cGNS_{MB} used in this study are an imaging probe to visualize the gene expression in living cells. If the gene related to the specific autophagy is selected and combined with cGNS_{MB} system, temporal and spatial information about the specific autophagy can be obtained. This method will become a strong tool for the drug screening and the pharmacokinetics. In the general autophagy research, a naked MB was used to reveal the mechanism of autophagy.⁶⁰ However, as described above, increase the naked MB is used, the observation time is limited because the internalized efficiency of MB is low and MB is rapidly degraded in cells. On the contrary, in the cGNS_{MB} system, MB can be internalized into cells and prevents from the intracellular degradation and released for a long time period, leading in an enhanced and prolonged visualization of autophagy.

One of the essential points in autophagy imaging is to show no effect of cGNS_{MB} on autophagy. It is reported that the internalization of nanospheres, such as liposome, and gold nanoparticles, activates autophagy.⁶¹⁻⁶³ cGNS_{MB} had no cytotoxic effect on the autophagy (**Figure 5B**), which means the biocompatibility and suitability of cGNS as the carrier to internalize MB into cells. The recent research of the cGNS_{MB} effect on the cell differentiation revealed that cells can be

differentiated, irrespective of cGNS_{MB} internalization. This is due to the characteristics of gelatin itself. Since gelatin of a denatured collagen is one of the extracellular matrix components, gelatin is not recognized as a foreign substance by cells and consequently does not affect cell functions. In addition, the distribution of cGNS_{MB} in the cells is one of the major factors to visualize the cell functions. In the previous research, the homogenous distribution of cGNS_{MB} is shown.³⁰

To prepare a culture system with a gradient of amino acids concentration, the μ -Slide I of a channel slide product was used. This slide is originally developed to observe cell in a flow condition.⁶⁴⁻⁶⁵ It is apparent in **Figure 7** that a simple application of two solutions allows to prepare a concentration gradient in the slide. In this research an amino acids-gradient condition was prepared with two types of media: the amino acids-rich and amino acids-free media. The autophagy activity increased, at the amino acids-poor area while the fluorescence intensity of cGNS_{MB} also increased. However, no change in the autophagy activity and the fluorescence of cGNS_{MB} was detected at the amino acids-rich area. In this study, toward the amino acids-poor side, autophagy was strongly induced. This result corresponds with the previous finding.⁶⁶ The autophagy activity increased as the decrease of amino acids concentration. It is clearly indicated that cGNS_{MB} is available to visualize the autophagy state of cells even in heterogeneous condition of cell starvation. Recently, it is important to develop 3D cell culture for the design of an in vitro models that are closer to an in vivo environment.⁶⁷⁻⁶⁹ The local condition of cells is different in the localization of cells in 3D culture systems, such as cell aggregates and multi-layered cell sheets. In other words, in the 3D cell culture, the heterogeneity of cell states is normally generated and needs to be accurately evaluated.⁷⁰ The imaging technology with cGNS_{MB} will enable to obtain the accurate data on the heterogeneity or localizations of cell functions in a time-dependent manner without destroying the samples.

In this research, the feasibility of cGNS_{MB} to visualize the autophagy activity was experimentally confirmed. As described above, this imaging technology enabled to visualize the autophagy function in living cells over time. In addition, this technology was effective in the detection of autophagy activity in the heterogeneous culture system of different autophagy activities. This cGNS_{MB} system can apply to visualize the autophagy activity in 3D cell aggregates. Other researches indicate that cGNS_{MB} were applied to visualize various cell functions.²¹⁻²² Moreover, cGNS_{MB} are also available to visualize not only the autophagy but also other cell functions. With the combination of this autophagy imaging and other cell functions imaging, a multiple evaluation can

be achieved at the same time to obtain more detailed information of cell biological processes, which scientifically assists to clarify the unsolved puzzles inside cells.

Conclusion

The present study demonstrates that the cGNS_{MB} is available to visualize the autophagy activity in living cells. By incubating NIH/3T3 cells with cGNS_{MB} prepared at the mixing ration of 20 pmoale of MB / 1 µg of cGNS, cGNS_{MB} were internalized into almost 100 % cells without significant cytotoxicity and the effect on the autophagy activity. This is because the cationized gelatin of cGNS is the compatible materials. It is confirmed that the gene expression of SQSTM1 and CTSF increased with the autophagy induction by culturing in the serum-free medium. The fluorescence intensity of cGNS_{MB} internalized into cells increased with the autophagy induction. In addition, this visualization method can be applied to the amino acids-gradient culture system. By culturing NIH/3T3 in the slide channel with the concentration gradient of amino acids, autophagy activity gradient was shown. A correlation between the fluorescence intensity of cGNS_{MB} and the gradient of autophagy activity was observed. In conclusion, the cGNS_{MB} is a promising imaging methods in the autophagy research field.

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Chapter 4

A Reverse Transfection System with Cationized Gelatin Nanospheres Incorporating Molecular Beacon as A Tool to Visualize Cell Function

Introduction

In biological research, the viability, proliferation, differentiation, and vitality of cells are all important indicators to analyze and access the biological functions of cells. An environment change often results in the regulation of gene expression which is transcribed to the messenger RNA (mRNA), translating to the protein coded. As the important components of biochemical reactions, mRNA and protein are usually used to determine cell functions. There have been reported several methods to measure mRNA and proteins so far, such as polymerase chain reaction (PCR) and western blotting ¹. Because of their high sensitivity, these two methods are widely used in cell research. However, the time-consuming procedures and the destruction of cells necessary often prevent us from evaluating the time course of biological functions for the same cell.

As one trail to tackle this problem, molecular imaging (MI) is served as a potential approach. MI is a technique that enables us to visualize, characterize, and measure the biological processes and phenomena at the cellular or molecular level spatiotemporally. In this technique, imaging probes are introduced into the target cell of interest and detected by the diagnostic imaging equipment ².

Molecular beacon (MB) is one of the candidates as an activatable fluorescent imaging probe. MB is a nucleic acid derivative modified with a fluorophore at the 5' end and a quencher at the 3' end and can hybridize with the corresponding nucleic acid specifically. MB has a stem-loop structure due to the hybridization between the complementary base pairs. Individual MB is in the “fluorescence off” stage. The MB loop hybridization with the specific sequence causes the structure change of MB, resulting in the fluorescence emission which is the “fluorescence on” stage. Thus, the high specificity is one of the advantages of MB. Another advantage of MB is its versatility. By changing the target sequence, varied MB can be designed to detect varied targets related to different cell functions, such as proliferation, differentiation, apoptosis, and metabolism. MB is widely used in chemical, biological, biotechnological, and medical science fields. It is reported that MB is available to detect mRNA ³, microRNA ⁴, and virus ⁵, which show great potential as a useful activate

fluorescent probe.

However, due to the negative charge of MB, it is not always easy to allow MB to internalize into cells at a high efficiency. Thus, it is of prime importance to develop a transfection method to enhance the efficacy of MB imaging. Several MB transfection methods have been reported⁶⁻⁹. The physical transfection methods, such as electroporation and cell injection, require a special equipment and time-consuming procedures, which is not always practically suitable for cell function visualization. On the other hand, some transfection carriers including viral vectors, cationized polymers, liposomes, and gold nanoparticles, are widely used. However, these materials sometimes show cytotoxicity¹⁰ and may induce mutation in the host cells¹¹. These points should be considered carefully to design the carrier material.

Cationized gelatin nanospheres (cGNS) are one of the ideal candidates for MB delivery. Gelatin is a natural polymer derived from collagen which has been widely applied for food, medicine, and clinical uses. Compared with other materials, it has excellent biocompatibility and biodegradability which shows the potential to be a safety carrier material. The hydrogel of gelatin has permitted the controlled releases of proteins¹²⁻¹³, peptides¹⁴, low-molecular weight drugs¹⁵, imaging probes¹⁶⁻¹⁷, and siRNA¹⁸ with the biological activity remaining. In addition, due to the functional groups of gelatin, it is easy to chemically modify gelatin to meet the research goals. For example, cationized gelatin was successfully synthesized by adding spermine to introduce the amino groups¹⁹⁻²⁰. Positively charged hydrogels of cationized gelatin can incorporate nucleic acids of siRNA^{18, 20} and pDNA²⁰ to achieve the high transfection efficiency. In addition, gelatin nanospheres with sizes ranging from 1 to 1000 nm can be easily internalized into cells via an endocytotic pathway²¹. Since it is necessary to detect the cell functions for a long time, the detection period is also an important factor for fluorescent probe delivery. Another benefit of gelatin nanospheres as a carrier material enables the imaging probe of MB to achieve the controlled release and prolong the imaging time of MB. It is reported that incorporation into cGNS enables MB to visualize cell functions, such as cell proliferation²²⁻²³, autophagy²⁴, and energy metabolism for a long time²⁵.

For the conventional transfection method, first cells are seeded on the culture dish, following by incubating in the culture medium containing the transfection complex. In the culture medium, there exist proteins of the serum component which easily interact with the transfection complex, resulting in suppression of transfection efficiency. To break through this problem, a reverse transfection system has been explored. In the reverse transfection system, first, the transfection complex is

immobilized on the culture substrate, and then cells are seeded onto them. In the reverse transfection technique, the transfection complex is always exposed to cells, which reduces the interaction of complex with serum. This reverse transfection system has been reported that can increase the transfection efficiency of plasmid DNA ²⁶⁻²⁸, siRNA ²⁹⁻³⁰.

Based on the result obtained in the 2D cell culture, the reverse transfection system was expected to apply to a more complicated culture system. For biological investigations, 3D cell aggregates are widely utilized to mimic the in vivo environment ³¹. The conventional transfection is challenging to visualize the cell functions of cell aggregates. The reason is that the dense structure of cell aggregate creates an obstacle for transfection complex to penetrate into the center of cell aggregates. To this end, the reverse transfection system is promising to allow cells to transfect homogeneously in cells of aggregates.

The objective of this study is to design a reverse transfection system with cGNS incorporating MB (cGNS_{MB}) to visualize a cell function. In the 2D cell culture, the pre-coating condition for the cell culture substrate was optimized by evaluating the cGNS immobilization efficiency and internalization efficiency. And then the fluorescence intensity of cGNS_{MB} in the reverse transfection system was compared with those of conventional transfection system. In the 3D cell aggregate culture, the size of GMS and the ratio of cells to GMS were optimized by in terms of preparation of cell aggregates with sizes smaller than 200 μm and the homogeneous distribution of GMS in the cell aggregates. The ratio of cGNS_{MB} to GMS was optimized by the signal-to-noise ratio (S/N ratio). We compared the fluorescence intensity of cGNS_{MB} and its distribution in the reverse transfection system with those of conventional transfection system.

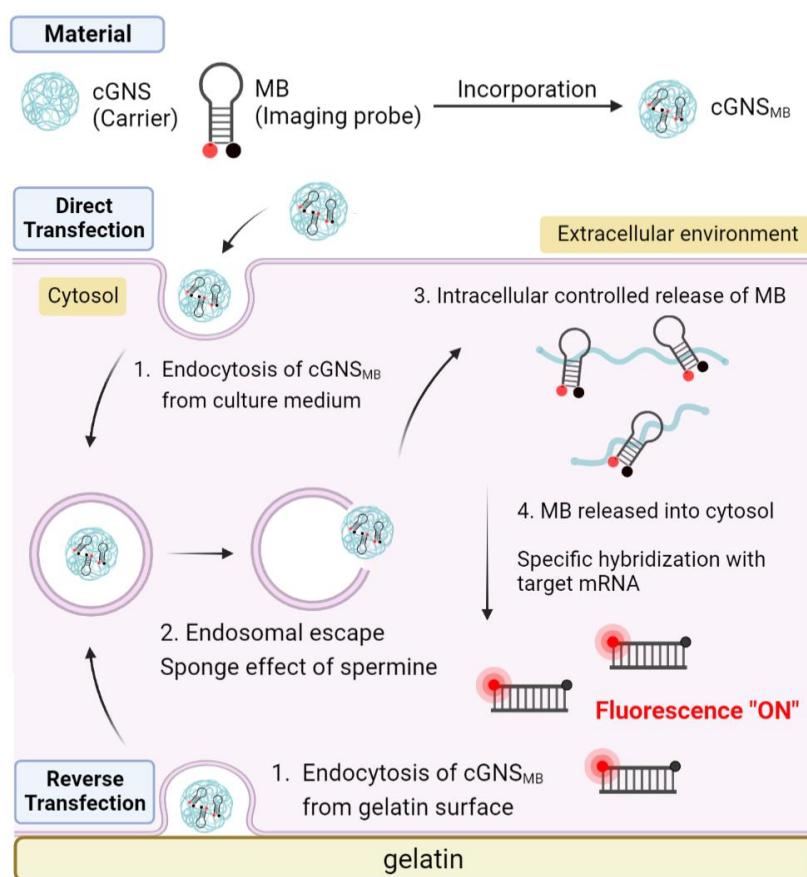


Figure 1. A schematic diagram describing the mechanism of direct transfection and reverse transfection.

Materials and Methods

1. Preparation of cationized gelatin and cationized gelatin nanospheres (cGNS)

Based on the procedures previously reported [5], 2 g of gelatin (isoelectric point (pI) = 9.0, weight-averaged molecular weight = 99000, derived from pig skin, Nitta Gelatin Inc., Osaka, Japan.) was dissolved in 50 ml double-distilled water (DDW) and mixed with spermine at the molar ratio of 50 to the carboxyl groups of gelatin. The solution pH was immediately adjusted to pH 5.0 by adding HCl aqueous solution. Then, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was added at the molar ratio of 3 to the carboxyl groups of gelatin. The total volume was adjusted to 100 ml by adding DDW. The solution was agitated at 40 °C for 18 hr and then was dialyzed against DDW for 3 days at room temperature. The dialyzed solution was freeze-dried to obtain the cationized gelatin. The percentage of spermine introduced into carboxyl groups of gelatin was determined by the conventional 2,4,6- trinitrobenzenesulfonic acid (TNBS, FUJIFILM Wako Pure Chemical Inc.,

Osaka, Japan) method. The percent was $42.6 \pm 2.14\%$.

To prepare cGNS, 5 ml of acetone was added into 1.25 ml cationized gelatin solution in DDW (50 mg/ml) drop by drop by a needle (24 gauge) at 40 °C to form the coacervate. Next, 20 µl of 20 wt% glutaraldehyde was added. After 6 hr chemical crosslinking reaction at 40 °C, 2 ml of 0.5 M glycine aqueous solution was added to block the aldehyde groups unreacted. After stirring for 1 hr at room temperature to evaporate acetone, cGNS were collected by centrifugation at 16,000g for 30 min 3 times and re-suspended in DDW.

2. Preparation of cGNS incorporating molecular beacon (cGNS_{MB})

Two types of MB targeting to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a green and red fluorophores (green and red) were prepared and named as green and red, respectively. in this study. Each type of MB (100 µM) was mixed with cGNS solution (1 mg/ml) at the ratio of 20 pmol/µg cGNS. The mixture solution was incubated at room temperature for 15 min to prepare cGNS_{MB}. They were collected by centrifugation of their mixture solution at 16,000 g for 15 min and adjusted to the final concentration of cGNS is 100 µg/ml, and the final concentration of MB is 2 µM. The sequences of MB used in this research are shown in Table 3.

Table 3. Sequences of MB and target oligonucleotides used.

MB and target oligonucleotides	Sequences (5' to 3')
mGAPDH MB	[TYE [®] 563]-CTG <u>GTA</u> ATC CGT TCA CAC CGA CCT T <u>CA CCA G</u> -[IAbRQSp]
mGAPDH MB	[Alexa Fluor [®] 488]-CTG <u>GTA</u> ATC CGT TCA CAC CG A CCT TCA <u>CCA G</u> -[IABkFQ]

MB: molecular beacon

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

IAbRQSp: Iowa Black RQ quencher

IABkFQ: Iowa Black FQ quencher

Underline: stem structure

3. Characterization of cGNS with or without MB incorporation

cGNS and cGNS_{MB} (cGNS incorporating MB) were resuspended in phosphate-buffered saline solution (PBS, pH 7.4) and to measure the size by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). The suspension of cGNS and cGNS_{MB} in 10 mM phosphate-buffered solution (PB, pH 7.4) was also prepared. Then, the zeta potential of

cGNS and cGNS_{MB} was measured by electrophoresis light scattering (ELS, Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK).

The amount of MB incorporated in cGNS was determined by using MB without a quencher. In brief, cGNS were mixed with the quencher free MB as described above. The mixture was centrifuged and washed two times by DDW. The fluorescence intensity of supernatant was determined on a multimode microplate reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan).

The stability of free MB and cGNS_{MB} against DNase I (Takara Bio Inc., Japan) was determined. Free MB or cGNS_{MB} (100 nM) were mixed with different concentrations of DNase I (0, 2.5, 5, 10, 20, 40 U/ml) in PBS containing 0.5 mM MgCl₂ and 0.9 mM CaCl₂. After incubation in 37 °C for 15 min shading from light, the fluorescence intensity was measured by a microplate reader described above.

4. Preparation of gelatin microspheres (GMS)

From gelatin (pI = 9.0, weight-averaged molecular weight = 99000, derived from pig skin, Nitta Gelatin Inc., Osaka, Japan.), 20 ml of 10 wt% gelatin aqueous solution was prepared and pre-warmed at 40 °C. Next, the solution was added into the pre-warmed 600 ml of olive oil at 40 °C stirring at 800 rpm for 10 min. Then, the resulting water-in-oil emulsion was cooled down in the ice bath for 2 hr to obtain non-crosslinked GMS. Acetone (200ml) was added to rinse the residual olive oil. Then, non-crosslinked GMS were washed 3 times and collected by centrifugation at 5,000 rpm for 5 min. Finally, GMS of different sizes were classified by the sieves with different apertures (5, 20, and 32 µm, Iida Seisakusyo, Osaka, Japan) and air-dried at 4 °C overnight. Dried GMS (100 mg) were dehydrothermal crosslinked for 72 hr at 140 °C by a vacuum oven. Crosslinked GMS were sterilized by ethylene oxide gas before using in the following cell culture experiments, and stored at 4 °C refrigerator.

5. Preparation of GMS binding cGNS_{MB} (GMS-cGNS_{MB})

GMS sterilized (2 mg, diameter=18 µm) were suspended by 200 µl of sterilized ethanol in a 1.5 ml plastic tube to avoid the aggregation of GMS. After removing ethanol by centrifugation at 3,000 rpm for 3 min, 400 µl of PBS was added into the tube and incubated at 37 °C for 1 hr to allow GMS to swell. The needle (29 gauge) and 40 µm cell strainer were used to remove the GMS aggregates.

The concentration of GMS was calculated by counting the number of GMS suspended in 1 μ l solution on a microscope (CKX41, Olympus, Tokyo, Japan). cGNS_{MB} were prepared as previously described. And then, 10 μ l of cGNS_{MB} solution was mixed with GMS in the number of GMS is 10,000, and the total volume was adjusted to 100 μ l by adding PBS. After shaking the mixture at 500 rpm for 1 hr, GMS-cGNS_{MB} were collected 2 times by centrifugation at 5,000 rpm for 5 min to remove the residual cGNS_{MB}. Then, the concentration of GMS-cGNS_{MB} was determined based on the same procedure as described above for GMS.

6. Optimization of gelatin and cGNS pre-coating

To optimize the gelatin coating procedure, two types gelatin of solution at different concentrations (pI=5.0 or 9.0, 0.01, 0.1, 1, 2,4, and 8 mg/ml) was added into each well of 96 multi-well culture plates (Corning Inc., Corning, NY, USA) and incubated for 3hr. The amount of gelatin pre-coated in the well was measured by Micro BCA™ Protein Assay Kit (Thermo Scientific, Inc., Massachusetts) according to the manufacture's protocol.

FITC-labeled cGNS (FITC-cGNS) were prepared by incubated 5 mg/ml cGNS with 200 μ g/ml fluorescein isothiocyanate isomer-I (FITC, Sigma-Aldrich Inc., St. Louis, MO, USA) in carbonate-bicarbonate buffer solution (0.1 M, pH 9.6) for 6 hr at room temperature. After incubation, FITC-cGNS were collected by centrifuged at 14,000 rpm for 30 min, and washed with DDW three times. 10 μ g/ml FITC-cGNS solution was added in each well gelatin pre-coated of culture plate. After incubation for 1 hr, the supernatant was collected to measure the fluorescence intensity of FITC-cGNS by Microplate Reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan). The percentage of cGNS remaining in the culture plate was calculated with the following formula: Percentage of cGNS remained= $100\% - \text{FI}_{\text{sup}} / \text{FI}_{\text{orig}}$ (FI: fluorescence intensity; sup: supernatant; orig: original)

7. Cell culture experiments

MC3T3-E1 cells of an osteoblast precursor cell line derived from mouse calvaria were cultured in α -minimum essential medium (α -MEM, Gibco Life technologies Co., Carlsbad, USA) supplemented with 10 vol% fetal calf serum (FCS, Hyclone Laboratories, Inc., Utah, UT, USA) and 1 vol% penicillin and streptomycin at 37 °C in the 95 % air/ 5 % carbon dioxide atmosphere. The

culture medium was changed every 3 days while the subculture was performed at 80 % confluency by 0.25 wt% trypsin - 1 mM EDTA solution (Nacalai Tesque, Inc., Kyoto, Japan.).

8. Reverse and conventional transfection systems in 2D cell culture

For the reverse transfection, gelatin pre-coated glass bottom dishes (diameter= 35mm, Matsunami Glass Industries Ltd., Tokyo, Japan) were prepared as mentioned before. Different concentrations of cGNS_{MB} (5, 10, and 20 µg/ml) were added into the gelatin pre-coated dishes and incubated for 1 hr after washing the dish with PBS. MC3T3-E1 cells were seeded at the density of 1×10^5 cells/dish.

For the conventional transfection, MC3T3-E1 cells were seeded in a glass-bottom dish with a density of 1×10^5 cells/dish. After 24 hr culture, the medium was changed into 1 ml/dish of α -MEM containing 5, 10, or 20 µg/ml of cGNS_{MB}. After 1 hr incubation with cGNS_{MB}, the medium was changed by fresh α -MEM. The cells were observed by fluorescence microscope (BZ-X700, KEYENCE Co., Ltd., Osaka, Japan) with a 10× objective lens. The mean fluorescent intensity was calculated by Image J software (national institutes of health, USA).

9. Evaluation of internalization efficiency in different pre-coating conditions

To evaluate the internalization efficiency of cGNS, first 1 ml of different gelatins pI=5.0 or 9.0 solution at concentration of 0.1, 1, and 2 mg/ml was added into a glass-bottom dish of 35 mm in diameter (Matsunami Glass Industries Ltd., Tokyo, Japan). After 3 hr incubation, the pre-coating solution was aspirated, followed by washing with PBS. The FITC-cGNS solution (1ml/dish) was added into pre-coated dishes at a concentration of 10 µg/ml. After 1 hr incubation, the FITC-cGNS solution was aspirated, followed by washing with PBS. The MC3T3-E1 cells were seeded into the FITC-cGNS immobilized dish at a density of 1×10^5 cells/dish.

After overnight incubation, the cells were collected by trypsinization. The cells suspension was washed with cold PBS and resuspended in 200 µl/tube of PBS. Trypan blue solution (0.2 µl/tube) was added to quench the fluorescence from the FITC-cGNS that only attached on the cells surface. The internalization efficiency was measured and analyzed by a FACSCanto II flow cytometer and a FlowJo™ software (v 10.8, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

10. Reverse and conventional transfection systems in cell aggregates

MC3T3-E1 cells subcultured were collected by trypsinization and mixed with the GMS or GMS-cGNS_{MB} swollen in PBS at the 4/1 ratio of cells to particles in the medium. The solution of poly (vinyl alcohol) (PVA) (1 wt%, the degree of polymerization = 1800 and the saponification = 88 mol %, Unichika, Tokyo, Japan) was added into each well of U-bottomed 96 multi-well multi-culture plate (100 μ l/well) and incubated at 37 °C for 15 min. After washed 2 times with PBS, the mixed cells and GMS or GMS-cGNS_{MB} solution was added into well pre-treated (100 μ l/well, 500 cells/well and 125 spheres/well).

For the reverse transfection, first cGNS_{MB} were bound on the GMS surface to prepare GMS-cGNS_{MB}, and then MC3T3-E1 cells were incubated in the U-bottomed well with the GMS-cGNS_{MB} to form the cell aggregates incorporating GMS-cGNS_{MB}. For the conventional transfection, after 3 days culture of cells and GMS to form cell aggregates containing GMS, the aggregates were incubated with α -MEM containing 10 μ g/ml of cGNS_{MB} for 1 hr.

The cells were stained with the SYTOTM 13 green fluorescent nucleic acid stain according to the producer's manual, and the no fluorescence area inside the cell aggregates refer GMS. The cell aggregate images were divided into four sections with the same area, and the area percentage of alive cells to the cell aggregate area ($A_c\%$) was measured by an image J software (national institutes of health, USA). The standard deviation of $A_c\%$ was calculated among four sections in the same image.

The fluorescence of MB from both of 3D cell aggregates was observed by confocal laser scanning microscope (FV1000D, Olympus, Japan). The fluorescent image was divided into 5 concentric ring regions, and the mean fluorescent intensity was calculated in every ring by Image J software (national institutes of health, USA).

11. Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). All the statistical analysis was performed by using unpaired *t*-test with two tails. Difference with $p < 0.05$ was considered to be significant.

Results

1. Material property

Table 1 shows the size and zeta potential of cGNS with or without MB incorporation. The MB-free cGNS had the size of 152.5 ± 0.85 nm and the zeta potential of 5.47 ± 0.42 mV. The MB incorporation increased the size of cGNS, but the zeta potential decreased. The incorporation of two different fluorophores did not affect both of the size and zeta potential. $88.7 \pm 1.43\%$ of MB were incorporated into cGNS. **Figure 2** shows the nuclease stability of free MB and cGNS_{MB}. Free MB gradually degraded with the increasing of DNase I concentration, leading to increasing fluorescence intensity. On the other hand, the fluorescence intensity of cGNS_{MB} was constant at any DNase I concentration.

Table 1. Apparent size and zeta potential of cGNS with or without MB (green or red)

	cGNS	cGNS _{green}	cGNS _{red}
Size (nm)	153 ± 0.85^a	160 ± 2.07	162 ± 1.35
Zeta potential (mV)	5.47 ± 0.42	4.17 ± 0.71	4.20 ± 0.70

a) mean $\pm$ standard deviation

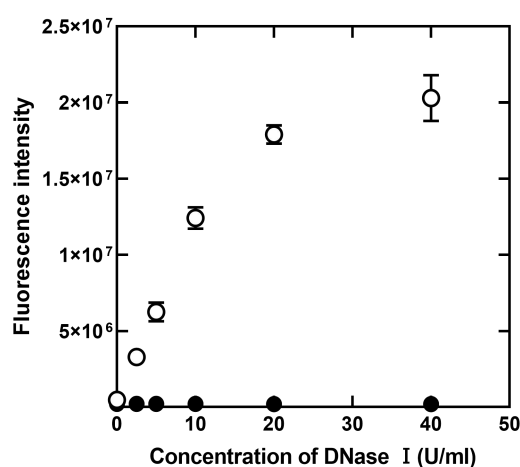


Figure 2. Stability of (○) free MB and (●) cGNS_{MB} against nuclease.

2. Optimization of gelatin and cGNS pre-coating

The percentage of gelatin pre-coated increased as the increasing of gelatin concentration, but tended to saturate at 50% when gelatin concentration was larger than 1 mg/ml, irrespective of gelatin types (**Figure 3**). By pre-coating, significant higher percentage of cGNS was immobilized (**Figure 4**). Around 50 % cGNS applied on the substrate pre-coated internalized into cells, whereas only 20 % of cGNS was done for the non-precoated substrate. In addition, there was no significant

difference in the percent immobilized among different conditions of gelatin pre-coating.

As shown in **Figure 5A**, there were two peaks of FITC-cGNS internalized for the pI 5 gelatin pre-coating whereas a single peak was observed under other conditions. This finding indicates that the internalization of cGNS in the cells cultured in the dish pre-coated with pI 5 gelatin was not homogeneous. The internalization efficiency of cGNS at 0.1 mg/ml pI 9 gelatin pre-coating showed the highest internalization efficiency among all the conditions, although there was no significant difference between the pI 9 gelatin coating and 2 mg/ml pI 5 gelatin coating (**Figure 5B**)

Green fluorescence of cGNS_{MB} was observed for all the groups (**Figure 6A**). Significantly low fluorescence intensity of cGNS_{MB} was observed in the no pre-coating group compared with that of gelatin pre-coating groups. A significantly high fluorescence intensity of cGNS_{MB} was observed for the pre-coating group at 0.1 mg/ml of pI 9 gelatin compared with all the gelatin coating groups (**Figure 6B**). The fluorescence intensity of cGNS_{MB} decreased and then increased with the increasing concentration of pre-coated gelatin solution, irrespective of the gelatin type.

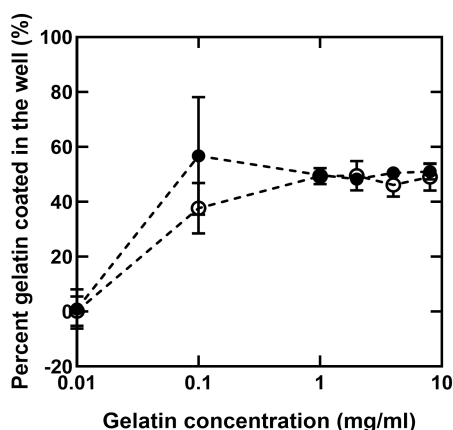


Figure 3. Percentage of gelatin pre-coated as a function of gelatin concentration: (○) pI 5 and (●) pI

9.

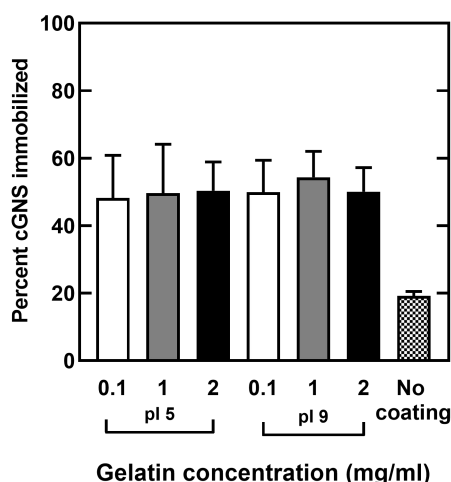


Figure 4. Percentage of FITC-cGNS immobilized on culture dishes pre-coated with or without pI 5 and pI 9 gelatin: (□) 0.1, (■) 1, and (■) 2 mg/ml of gelatin used in coating.

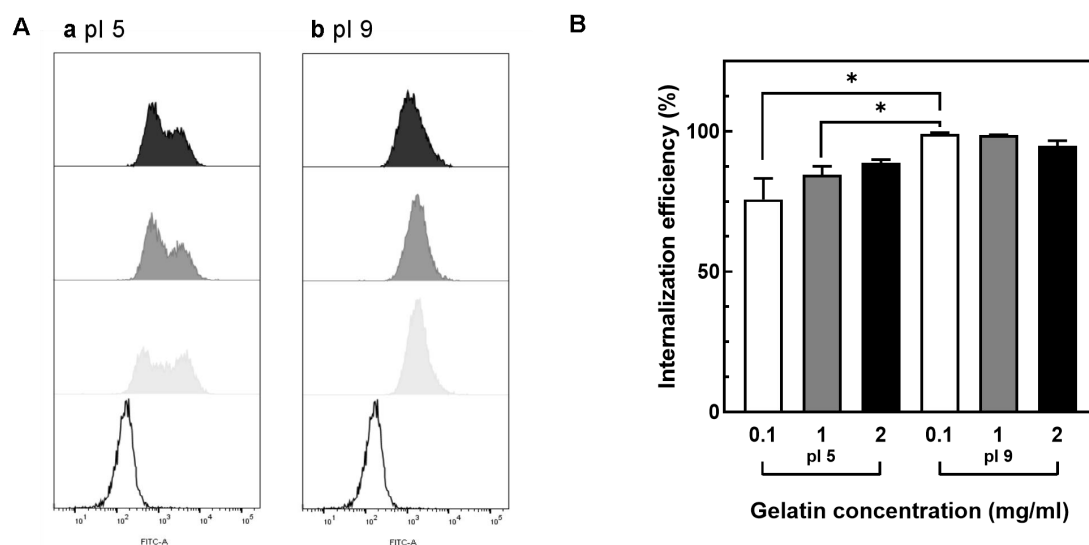


Figure 5. (A) FACS diagrams of cells cultured with FITC-cGNS for 1 hr in the reverse transfection system. The culture dishes were coated with (a) pI 5 gelatin and (b) pI 9 gelatin: (■) 0.1, (■) 1, and (■) 2 mg/ml. The cGNS_{MB} concentration is 10 μ g/ml. The cells cultured without FITC-cGNS (□) served as a negative control group. (B) Percentage of FITC fluorescent-positive cells cultured with FITC-cGNS in the reverse transfection system. The culture dishes were coated with (□) 0.1, (■) 1, and (■) 2 mg/ml. The cGNS_{MB} concentration is 10 μ g/ml. *, p < 0.05: Significant difference against the value of cells cultured on the dishes pre-coated with 0.1 mg/ml of pI 9 gelatin.

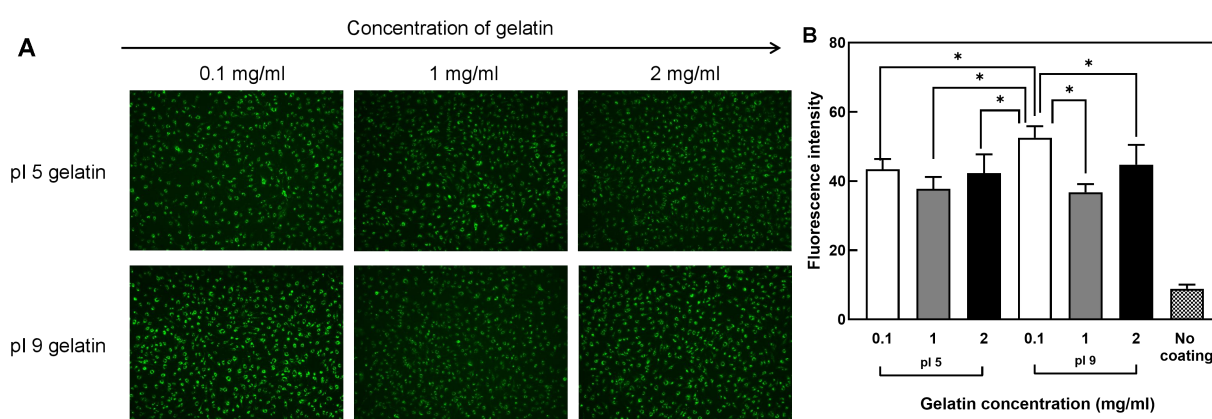


Figure 6. (A) Fluorescent microscopic images of cells cultured with cGNS_{green} in the reverse transfection system on culture dishes pre-coated with 0.1, 1, and 2 mg/ml of pI 5 and pI 9 gelatin. (B) Fluorescence intensity of cells cultured with cGNS_{green} in the reverse transfection system on culture dishes pre-coated with pI 5 and pI 9 gelatin: (□) 0.1, (■) 1, and (■) 2 mg/ml of gelatin or (■) No coating.

without gelatin. *, $p < 0.05$. Significant difference against the value of cell cultured on the dishes pre-coated with 0.1 mg/ml pI 9 gelatin.

3. Visualization of GAPDH mRNA in 2D culture

Figure 7A shows the fluorescence of cells incubated with cGNS_{MB} in different transfection methods. The fluorescence emission was observed for all groups. It is apparent in **Figure 7B** that the fluorescence intensity of reverse transfection system was significantly higher than that of conventional transfection system, irrespective of the cGNS_{MB} concentration. In addition, the fluorescence intensity tended to increase with an increase in the amount of cGNS_{MB} in the reverse transfection system, in remarked contrast to the concentration independence in the conventional transfection system.

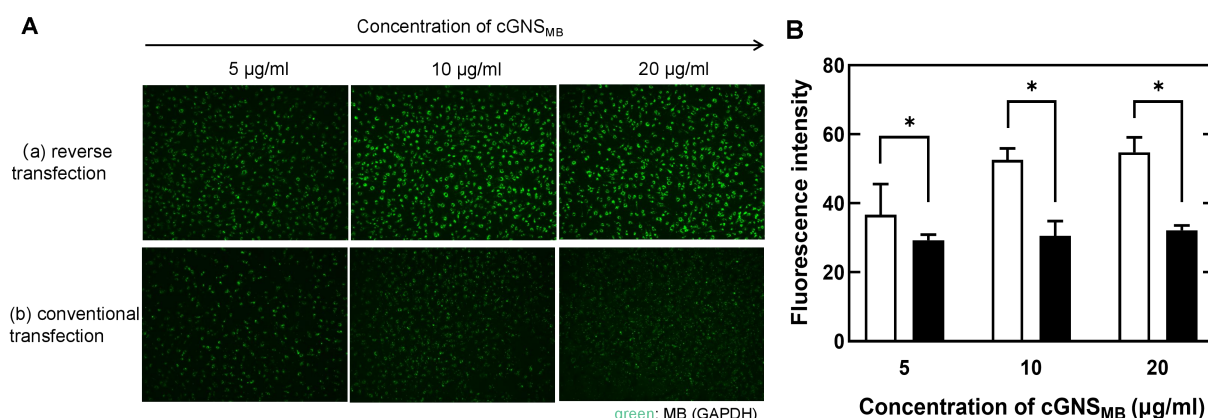


Figure 7. (A) Fluorescent microscopic images of cells cultured with 5, 10, and 20 µg/ml of cGNS_{green} in the (a) reverse transfection system and (b) direct transfection system on culture dishes pre-coated with 0.1 mg/ml of pI 9 gelatin. (B) Fluorescence intensity of cells cultured with different concentrations of cGNS_{green} on culture dishes pre-coated with 0.1 mg/ml of pI 9 gelatin: (□) reverse and (■) direct transfection systems. *, $p < 0.05$: significant difference between the two groups.

4. Optimization of reverse transfection system condition in 3D cell culture

Figure 8A shows the size of cell aggregates formed at different ratios of cells to GMS and different sizes of GMS. The size of cell aggregates decreased with an decrease in the ratio of cells to GMS, irrespective of the GMS size. **Figure 8B** shows the aspect ratio of cell aggregates at different ratios of cells to GMS and different sizes of GMS. Higher ratio of cells to GMS tended to become relatively larger aspect ratio. On the other hand, GMS of larger sizes tended to show larger error bars

which indicates the unstable size and shape of aggregates.

Figure 9A shows the representative images of cell aggregates as different ratios of cells to GMS, while **Table 2** shows the area percentage of alive cells to the total cell aggregate area in the cell aggregates prepared with the ratio of cells to GMS of 4/1 and 8/1. When the GMS were distributed homogeneously, the percentage should be ideally same in four sections. It is apparent in the **Figure 9A** that GMS tended to accumulate in one side of cell aggregates with the ratio of GMS to cell of 8/1. As shown in **Figure 9B**, the standard deviation at the ratio of 4/1 was smaller than those of 8/1. These results reveal that the distribution of GMS in cell aggregates seemed to be better at the ratio of 4/1 compared with those of 8/1 ratio. The fluorescence intensity of GMS-cGNS_{MB} significantly increased, irrespective of the cGNS_{MB} concentration (**Figure 10A**). On the other hand, the S/N ratio significantly decreased with an increase in the cGNS_{MB} concentration (**Figure 10B**).

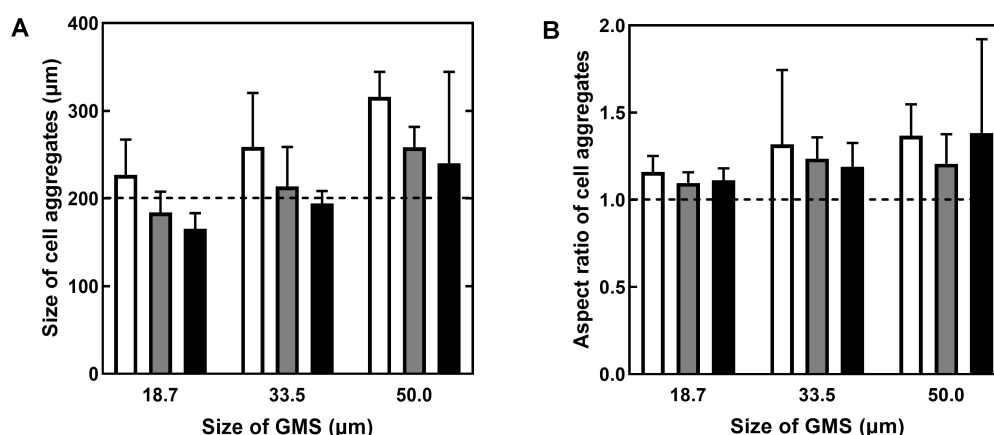


Figure 8. The size (A) and the aspect ratio (B) of cell aggregates cultured with different sizes of GMS and different ratios of cells to GMS: (□) 2/1, (■) 4/1, and (■) 8/1 of cells/ GMS ratios.

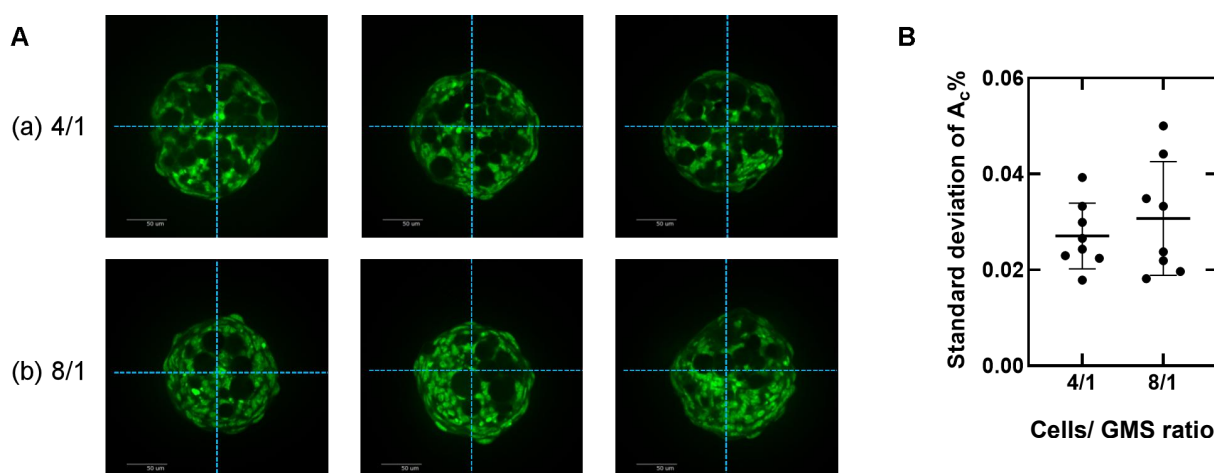


Figure 9. (A) Fluorescent microscopic images of cell aggregates cultured with 18.7 μm GMS and different ratios of cells to GMS of (a) 4/1, and (b) 8/1. (Green: nuclei. Scale bar: 50 μm .) (B) Percentage of cell area of total aggregate area ($A_c\%$) in each section. The standard deviation of $A_c\%$ among four sections are shown in B.

Table 2. Cell area percentage of alive cells to the total cell aggregates area.

Ratio of cells to GMS	Number of sample	Cell area as a percentage of the total area ($A_c\%$)				Standard deviation of $A_c\%$
		Section 1	Section 2	Section 3	Section 4	
4/1	1	9.08	13.3	3.75	9.35	3.93
	2	3.04	6.01	7.62	9.28	2.66
	3	9.32	6.17	10.1	7.51	1.79
	4	4.70	5.05	8.13	9.22	2.24
	5	4.21	10.5	6.05	9.76	2.99
	6	4.18	4.97	11.3	8.73	3.33
	7	9.24	6.53	12.3	8.27	2.43
	8	11.1	7.27	5.72	8.80	2.30
8/1	1	14.1	4.39	12.7	12.6	4.42
	2	12.2	15.5	13.7	11.4	1.82
	3	11.8	17.0	5.48	7.90	5.00
	4	12.8	9.31	15.1	12.4	2.38
	5	8.75	8.29	14.8	13.6	3.33
	6	10.1	10.0	12.7	7.89	1.97
	7	13.1	12.4	7.84	16.3	3.49
	8	5.02	7.01	8.28	10.2	2.19

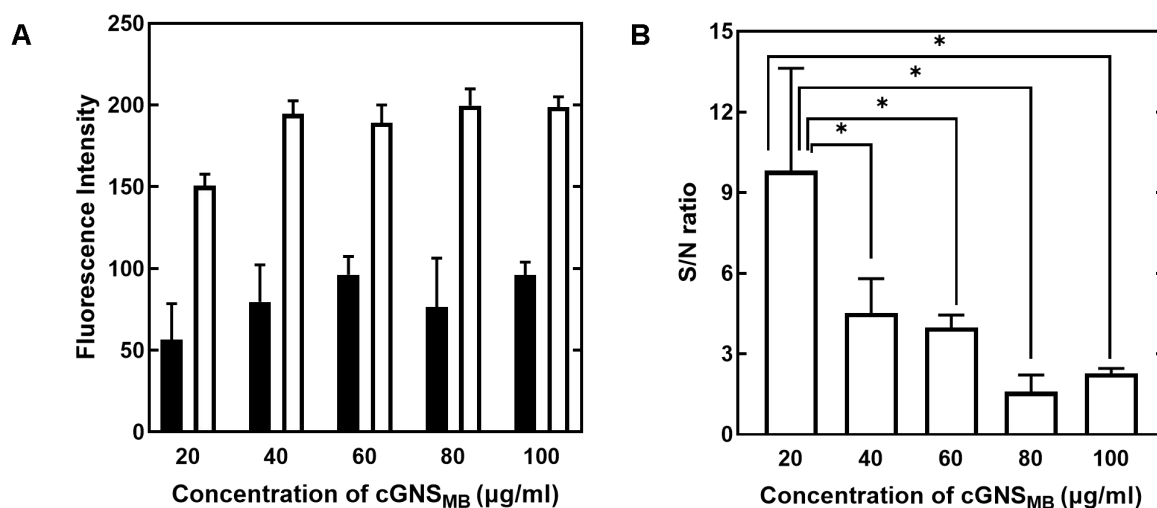


Figure 10. (A) The fluorescence intensity of cells cultured with cGNS_{MB} with (□) or without target sequence (■). (B) The signal-to-noise ratio of GMS-cGNS_{MB}. *, $p < 0.05$: Significant difference against the value of 20 $\mu\text{g/ml}$ cGNS_{MB} group.

5. Visualization of GAPDH mRNA in 3D culture

Figure 11A shows the fluorescence images of cell aggregates in the reverse and conventional transfection groups. The red fluorescence of MB was observed both in the reverse transfection and conventional transfection groups. The apparently different fluorescence distribution of cell aggregates was shown in **Figure 11B**. In the reverse transfection system, the fluorescence intensity was homogeneous over the ranges from the inside to outside of cell aggregates. On the contrary, for the conventional transfection group, the fluorescence was observed only around the surface of cell aggregates.

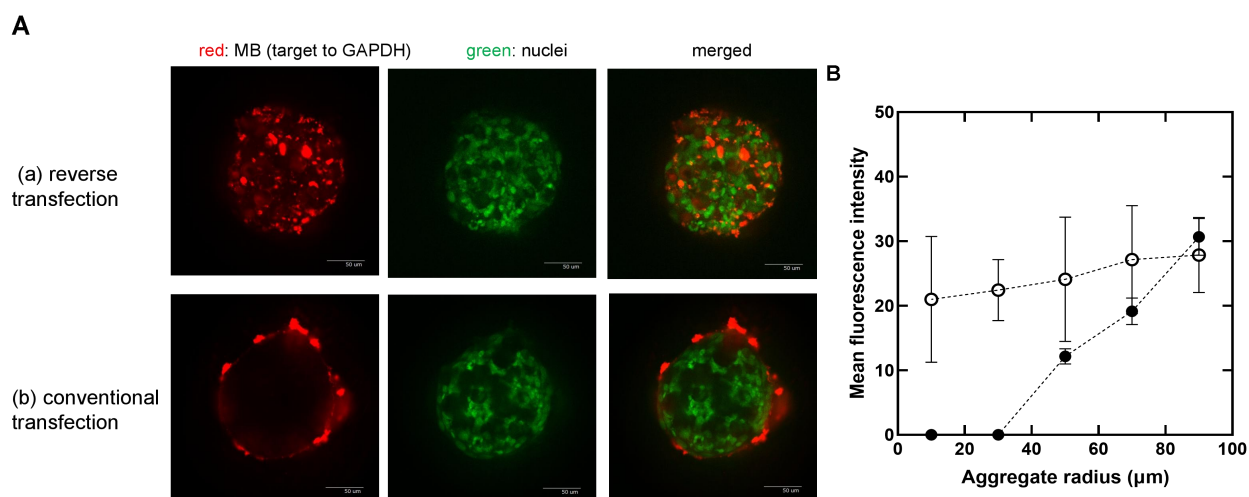


Figure 11. (A) Fluorescent microscopic images of 3D aggregates of cells transfected with cGNS_{red} plus GMS in (a) reverse, and (b) direct transfection methods. (Red: MB, Green: nuclei. Scale bar: 50 μm .) (B) The fluorescence intensity of 3D cell aggregates from cells cultured in the reverse transfection system of cGNS_{red} (○) and direct transfection (●) as a function of aggregates radius.

Discussion

MB is a powerful tool to visualize cell functions without destroying the samples. To internalize MB into cells, several methods have been reported, including microinjection ⁶, electroporation ⁷, and

streptolysin O usage ⁸. However, these methods require a specific equipment or a long time for MB internalization. As one trial, it is necessary to design carrier materials for MB to enhance the MB internalization. In this study, cGNS were designed for MB internalization. The cGNS of positively charged permit the negatively charged MB to connect to be of a positive charge, enhancing the internalization efficiency. More than 80% of MB was incorporated into cGNS (**Table 1**). Incorporation in cGNS allowed MB to protect from the degradation by DNase I, while free MB were degraded depending on the DNase I concentration (**Figure 1**). It is highly conceivable that cGNS contribute to an increased nuclease stability of MB by avoiding the approach of DNase I to MB.

On the other hand, cGNS can be chemically crosslinked by glutaraldehyde and the degradation profile can be controlled by altering the crosslinking degree. Thus, controlled release of MB is regulated only by changing the degradation nature of cGNS. cGNS as the carrier material to internalize MB for the visualization of cell functions have been reported ^{22-25, 32-33}. Recently, it is reported that PLGA nanoparticles associated with Ki67 MB were available to detect the Ki67 mRNA. The PLGA nanoparticles are promising as the carrier of MB although the degraded products may be cytotoxic compared with cGNS ³⁴. In this study, the cytotoxicity of cGNS_{MB} and GMS was evaluated. Around 100% of cell viability was observed after culturing with cGNS_{MB} or GMS 1, 3, and 5 days . In addition, co-culturing with cGNS_{MB} or GMS- cGNS_{MB} did not affect the cell proliferation . This indicated that both cGNS and GMS- cGNS_{MB} are not cytotoxic, but biocompatible materials.

In the conventional method where the transfection complex is applied to cells directly in the culture medium, the transfection efficiency was suppressed by the interaction of protein and transfection complex. As the one method to tackle this issue, this study provides the reverse transfection of a novel approach ²⁶⁻³⁰.

In the 2D cell culture, first cGNS_{MB} are immobilized on the substrate, and then cells are seeded onto them. In this study, the pre-coating conditions were optimized. The pre-coating allowed cGNS_{MB} to immobilize onto the substrate at a high efficiency. Gelatin is a biocompatible material with a cell adhesive nature. Since pI 5 gelatin is of negatively charged in the cell culture environment, it is considered that it has an affinity for cGNS_{MB} of positively charged (**Table 2**). On the other hand, for the pI 9 gelatin of a positively charged, it is also expected to have a high affinity between gelatin and cGNS_{MB}. The concentration of pre-coating gelatin is an important factor. In the

previous study, 0.1 mg/ml of gelatin solution was used for the coating purpose ²⁶. Different concentrations of gelatin solution were used to pre-coat the culture well and the coating efficiency was determined by the micro BCA assay. When the gelatin concentration was larger than 0.1 mg/ml, the coating efficiency tended to be around 50%. Based on the basic experimental trial, it is suggested that the pre-coating density of gelatin should be 100-200 $\mu\text{g}/\text{cm}^2$ of well. On the other hand, it is reported that the stiffness of pre-coated gelatin substrate decreased with the increasing concentration of pre-coated gelatin solution ³⁵, and cells cultured on the high stiffness substrate tend to internalize more nanospheres ³⁶⁻³⁷. In other words, higher concentration of gelatin solution for pre-coating might result in relatively lower internalization efficiency of nanospheres. Considering these findings, 0.1, 1, and 2 mg/ml of gelatin solution were used in this study for further comparison. The immobilization efficiency of cGNS became around 50%, irrespective of the gelatin pre-coating condition, and was significantly low in the group without any pre-coating. This is because the gelatin has a stronger affinity to each other than the affinity between plastic and gelatin, which emphasizes the importance of gelatin pre-coating.

To confirm the internalization of cGNS to cells, the internalization efficiency was evaluated by a flow cytometry machine by using FITC-labeled cGNS. The cells without incubation with FITC-cGNS were prepared as the negative control group. The FITC fluorescent-positive cells were determined by referring to the cells without internalization of FITC-cGNS. The two peaks of FITC fluorescent-positive cells were observed for the pI 5 gelatin pre-coating group, indicating the heterogeneous internalization of FITC-cGNS. On the other hand, the pI 9 gelatin pre-coating group showed significantly higher internalization efficiency than other groups (**Figure 5**). It is highly conceivable that the electrostatic interaction between pI 5 gelatin pre-coated and cGNS_{MB} is too strong to allow cells to internalize cGNS_{MB}.

It is conceivable that cGNS_{MB} are internalized into cells via clathrin-mediated endocytosis and micropinocytosis ^{25, 33}. Because of the pH buffering effect of secondary amino group of spermine, cGNS_{MB} were endosomally escaped after internalization into cells ³⁸. With the degradation of gelatin, the MB were released into the cell cytosol, hybridized with the target sequence to emit the fluorescence ^{18, 39} (**Figure 1**) It is apparent from **Figure 6A** that the fluorescence signal of cGNS_{MB} experimentally confirmed the feasibility of reverse transfection system (**Figure 6A**). A significantly higher fluorescence intensity compared with the no-coating group indicates that the pre-coating treatment was important to enhance the transfection efficiency of reverse transfection system

(**Figure 6B**). Because for the non-precoating group, the amount of cGNS_{MB} immobilized on the dish was too low to show the fluorescence similar to other groups (**Figure 4**). In addition, the higher fluorescence intensity demonstrates the higher transfection efficiency. The significantly highest fluorescence intensity observed in the pre-coating group of 0.1 mg/ml pI 9 gelatin implies the highest transfection efficiency. It is likely that a strong electrostatic interaction between pI 5 gelatin pre-coated and cGNS_{MB} contributes to be more difficult for cells to internalize cGNS_{MB}, resulting in the low fluorescence intensity of cGNS_{MB}. However, there is no pre-coating gelatin concentration-dependent fluorescence confirmed. The relationship between the pre-coating gelatin concentration and transfection of cGNS_{MB} should be investigated. Taken together, 0.1 mg/ml of pI 9 gelatin coating was the condition optimal for the reverse transfection of cGNS.

As one trial to experimentally confirm the difference between the reverse transfection and the conventional transfection systems, the fluorescence of cGNS_{MB} was observed for all the groups. It is apparent that the transfection of cGNS_{MB} was achieved by both the two transfection systems. As the concentration of cGNS_{MB} increased, the fluorescence intensity increased (**Figure 7**). This finding can be explained in terms of the first essential step of cell internalization⁴⁰. The higher amount of cGNS_{MB} immobilized on the substrate, the higher possibility of cGNS_{MB} internalization into cells (**Figure 4**). It is likely that the higher amount of cGNS_{MB} internalized results in a higher fluorescence intensity of cGNS_{MB}. Interestingly, the increasing slope of fluorescence intensity was much lower in the case of direct transfection. It is demonstrated that the adhesion between cells and substrate minimizes the influence of serum on the gene transfection, facilitating the transfection process^{26, 28}. Based on the points, we can say with certainty, that compared in the same concentration of cGNS_{MB}, a higher fluorescence intensity of reverse transfection system would be observed than that of direct transfection system.

3D cell aggregates have been widely used as a tool to mimic the in vivo environment^{31, 41-47}. However, due to the lack of oxygen and nutrition, the cells located in the center of aggregates tend to die. To break through this problem, GMS are effective in functioning as a cell scaffold which allows cells to supply oxygen and nutrition^{31, 45-49}. On the other hand, GMS are a good carrier for biomolecules, such as low molecular-weight drug, protein, and siRNA to achieve the controlled release¹²⁻¹⁸. Increasing biological functions in cell aggregates would be achieved by preparing cell aggregate with biomolecules loaded GMS.

The visualization of cells in the aggregates is important to evaluate the biological functions. So

far, the imaging of cells in 3D constructs is technically limited. It is reported that the confocal microscopy, light sheet fluorescence microscopy, and optical sectioning structured illumination microscopy are available for the visualization of cells in 3D constructs⁵⁰. In general, the 3D sample need to be stained for the cell visualization. For the staining of cells, the cell fixation always requires, which is not allow to image cells in the live condition. Although using GFP is used to achieve the live imaging of 3D cell constructs, it is reported that the fluorescence lifetime decreases when GFP is in a close position to the cell membrane or to the cell-glass interface⁵¹⁻⁵². In addition, several fluorophores have been designed to achieve the living imaging of 3D cell constructs. Compared with the MB design, the fluorophore design is relatively of time-consuming and low efficient.

One of the challenges to visualize the cell function in cell aggregates is that the imaging probes are not easy to permeate into the center of aggregate due to the dense structure of cell aggregates. As a result, the homogeneous visualization is not always achieved. As one trial to tackle this issue, we applied the reverse transfection system combining the GMS and cGNS_{MB} to form 3D cell aggregates. GMS are used as a scaffold to allow cGNS_{MB} to immobilize on them and cells to readily internalize cGNS_{MB} from the surface of GMS. In the 2D reverse transfection system, it is confirmed that pI 9 gelatin pre-coating as substrate showed better homogeneous internalization of cGNS_{MB} compared with those of pI 5 gelatin pre-coating. Thus, pI 9 gelatin was used to prepare GMS for 3D reverse transfection system. For the detection limitation of confocal microscopy, we tried to control the size of cell aggregates smaller than 200 μm by altering the size of GMS and the ratio of cells to GMS⁵³. As expected, the size of cell aggregates increase with the increasing size of GMS and the ratio of cells to GMS. Only when the GMS with size around 18 μm were used at the GMS/cells ratio of 4/1 and 8/1, cell aggregates of size smaller than 200 μm were prepared (**Figure 8A**). On the other hand, the distribution of GMS is important to achieve homogeneously imaging the cell aggregate. So we compared the distribution of GMS in the cell aggregates prepared by 18 μm GMS with the cells to GMS ratio of 4/1 and 8/1. The result of **Figure 9** and **Table 3** indicate that GMS were distributed more homogeneously at the cells to GMS ratio of 4/1. The reason of this phenomenon might be relatively higher number of cells at the ratio of 8/1 interacting to each other earlier than those with GMS and small aggregates formed first due to strong cell-cell interaction. Based on the findings, GMS of 18 μm in diameter and the cells/GMS ratio of 4/1 were used for the following experiments. For the optimization of GMS-cGNS_{MB}, different concentrations of cGNS_{MB} were mixed with GMS to prepare GMS-cGNS_{MB}. To confirm whether MB can work or not, the target sequence solution

was added to the GMS-cGNS_{MB}. Significantly increased fluorescence intensity of MB on the GMS surface suggests that the successful preparation of GMS-cGNS_{MB} and MB function effectively (**Figure 10A**). The S/N ratio is often used as a standard to evaluate an imaging probe. The significantly highest S/N ratio was observed for the group of 20 µg/ml cGNS_{MB} (**Figure 10B**). The reason may be due to too high concentration of cGNS_{MB} introduced an extra amount of cGNS_{MB} into the system. Although the washing step was proceeded, it is possible that relatively higher amount of extra cGNS_{MB} remained in the GMS-cGNS_{MB} solution, which resulted in the high noise signal in the background. Therefore, the condition of 20 µg/ml cGNS_{MB} was chosen to prepare GMS-cGNS_{MB}.

After the preparation of GMS-cGNS_{MB}, cells were mixing with them and seeded into PVA pre-coated U bottom well to form cell aggregate. On the other hand, the conventional transfection in cell aggregates was achieved first by preparing cell aggregates, and then adding the cGNS_{MB} into the culture medium. As shown in **Figure 11A**, 3D cell aggregates were formed while the fluorescence of cGNS_{MB} was detected in every group. This indicates that cGNS_{MB} functioned well by the transfection methods. Apparently, the distribution of fluorescence in the 3D cell aggregates was homogeneous in the reverse transfection system, while the fluorescence was observed only around the surface of cell aggregates (**Figure 11B**). This can be explained in terms of the cGNS_{MB} permeation. A dense cell-cell interaction in 3D cell aggregates would prevent cGNS_{MB} from penetrating into the inside of cell aggregates, resulting in the internalization only in surface area by the conventional transfection. On the contrary, in the reverse transfection system, cGNS_{MB} immobilized on the surface of GMS allowed cells to continuously internalize the transfection complex even during the formation process of cell aggregates. It is concluded that the 3D reverse transfection combined with GMS is promising to visualize the cells situation even inside 3D cell aggregates. It is also noticed that the error bar of the fluorescence intensity was relatively big. This phenomenon may be explained in terms of different extents of cells internalizing cGNS_{MB} internalization into cells. Although the distribution of GMS-cGNS_{MB} inside cell aggregates is optimized in this study, the distribution is not always achieved in an ideal homogeneity. Although the average size of GMS-cGNS_{MB} was 18 µm, the size distribution was still large from 10 to 30 µm. It is highly conceivable that this error might result in the different extents of cells to contact with GMS-cGNS_{MB} and different extents of cGNS_{MB} internalization consequently, leading to a relatively big error bar. In the future experiment, the technique to prepare a uniform size of GMS should be investigated, and the distribution of GMS inside cell aggregates should be further optimized.

Some experiments were performed to evaluate the biocompatibility of the reverse transfection system. In the 2D cell culture system, after culturing in the cGNS_{MB} immobilized gelatin pre-coating plate, the cell viability remained around 100% and the time profile of cell proliferation was similar with that of cells cultured in the polystyrene culture plate. In the 3D cell culture system, the live/dead staining was performed. Few number of dead cells was observed in the cell aggregate cultured with GMS-cGNS_{MB}. We can say with certainty that gelatin gives cells a safety environment for the proliferation.

This study demonstrates the feasibility of reverse transfection system with cGNS_{MB} not only in the 2D cell culture, but also in the 3D cell culture. To our knowledge, this is the first report to apply the reverse transfection system for cell function visualization both in the 2D and 3D cell culture system. The reverse transfection system in the 2D cell culture to increase the transfection efficiency of siRNA²⁹ and plasmid DNA^{26, 28}, has been reported. By varying the design of molecular beacon, this visualization system has been applied in many fields. For example, in the research field of tumors, the MB system has been extensively used. On the other hand, MB is also available to be immobilized on the surface of microparticles to detect RNA⁵⁴, miRNA⁵⁵⁻⁵⁶, and DNA⁵⁷ with an advanced stability. On the contrary, little has been applied to the tissue regeneration field. The reverse transfection system will allow cell aggregates transplanted to visualize and monitor cell behaviors even in vivo. In addition, the reverse transfection system in 3D cell culture can be applied for any type of 3D cell constructs in drug discovery. It is concluded that the reverse transfection system is a promising tool for the basic cell research, drug screening, and the cell transplantation.

Conclusions

A reverse transfection system of cGNS_{MB} was established for cell visualization both in the 2D and the 3D cell culture systems. In the 2D cell culture system, the gelatin pre-coating provided a high gelatin-affinity environment for cGNS_{MB} immobilization. With continuously exposing cells to cGNS_{MB} and the decreasing interaction of cGNS_{MB} and serum, the transfection efficiency was enhanced. In the 3D cell culture, cGNS_{MB} association on the GMS surface allowed cells to internalize cGNS_{MB} and form their aggregates simultaneously. The reverse transfection system was effective in visualizing cells in 3D aggregates in a homogeneous manner. In conclusion, the reverse transfection system is a potential tool applicable basic cell research and regenerative therapy.

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SUMMARY

Chapter 1

The objective of this study is to confirm the methodological feasibility of cationized gelatin nanospheres (cGNS) to enhance the internalization efficiency of mitochondria (Mt) isolated to cells for their increasing functions. The cGNS were simply associated on the surface of Mt by the electrostatic interaction. Different sizes of cGNS were used to allow Mt to associate on the Mt surface (Mt-cGNS). As a control, cationized gelatin (cG) was used to modify the Mt surface (Mt-cG). The Mt-cG and Mt-cGNS prepared were cultured with H9c2 cells to examine their internalization. The internalization efficiency significantly increased by utilizing cGNS. However, there was no significant difference in the internalization efficiency among cGNS with different sizes. After incubation of Mt, Mt-cG, and Mt-cGNS, the superoxide amount and ATP generation were evaluated. Significantly lower superoxide amount and higher ATP amount were observed for the Mt-cGNS group compared with those of non-modified Mt group. It is conceivable that cGNS enhance the cellular internalization of Mt, leading to an improve mitochondrial functions in the recipient cells. In conclusion, cGNS are promising to improve the efficacy in mitochondria internalization.

Chapter 2

The objective of this study is to investigate the influence of exogenous mitochondria (Mt) internalization on the Mt membrane potential of cells. Cationized gelatin nanospheres (cGNS) were prepared to mix Mt at different ratios to prepare Mt associated with cGNS (Mt-cGNS). The Mt internalization depended on the Mt/cGNS mixing ratio to achieve the maximum at the ratio of 3/1. Rho 0 cells of a Mt function-deficient line were prepared to evaluate the enhancement of Mt membrane potential of rho 0 cells after the internalization of Mt-cGNS. When evaluated by using tetramethylrhodamine methyl ester (TMRM) reagent, the mitochondrial membrane potential of rho 0 cells after incubation with Mt-cGNS enhanced compared with that incubated with Mt only and maintain at a significantly higher level even for 6 days. The Mt-cGNS were internalized into rho 0 cells by an actin-dependent pathway, followed by fused with endogenous Mt. It is concluded that association with the cGNS enabled Mt to enhance the cellular internalization, followed by the fusion

with endogenous Mt to maintain an enhanced Mt membrane potential.

Chapter 3

The objective of this research is to develop an imaging method with cationized gelatin nanospheres incorporating molecular beacon (cGNSMB) to visualize an autophagy activity in living cells. Cationized gelatin nanospheres (cGNS) was prepared by the conventional coacervation method, and then molecular beacon (MB) was incorporated into them. The cGNSMB prepared were internalized into cells at a high efficiency. In this study, a starvation medium of serum and amino acids-free was used to induce autophagy. The autophagy activity was confirmed by an immunofluorescence staining for microtubule-associated proteins light chain 3B (LC3B) of an autophagy specific protein. With the autophagy induction time, the number of LC3 fluorescent dots increased, indicating an increased autophagy activity. As the autophagy-related genes, sequestosome 1 (SQSTM1) and cathepsin F (CTSF) which up-regulate after autophagy induction, were chosen as the targets of cGNSMB. The fluorescence intensity of cGNSMB targeting to SQSTM1 and CTSF increased with the starvation treatment time, which well corresponding with the gene expression results. When applied to cells in different autophagy conditions, the cGNSMB visualized the autophagy activity corresponding with the autophagy condition of cells. From the results obtained, it is concluded that the cGNSMB are a promising method to visualize the autophagy of cells. The advantage of cGNSMB visualization is to obtain the temporal and spatial information without destroying sample cells.

Chapter 4

The objective of this research is to design a reverse transfection system with cationized gelatin nanospheres (cGNS) incorporating a molecular beacon (MB) to visualize a cell function. The cGNS were prepared by the conventional coacervation method. The MB as an imaging probe was incorporated into the cGNS to prepare imaging complexes (cGNSMB). The conventional transfection of 2D culture was performed by incubating MC3T3 cells in the medium containing cGNSMB. The reverse transfection was done by incubating cells on the substrate which had been pre-coated with both gelatin and cGNSMB. Significantly higher internalization efficiency and fluorescence intensity of cGNSMB were observed in the reverse transfection system than the conventional one. To apply this system for visualization of 3D cell aggregate, gelatin microspheres

(GMS) were prepared, while cGNSMB were bound on the GMS to prepare the GMS-cGNSMB of a cell scaffold. Then, the cells were incubated with GMS-cGNSMB to form 3D cell aggregates. On the other hand, as a control, the conventional transfection of 3D culture was performed by incubating the cell aggregates formed with the medium containing cGNSMB. Homogeneous fluorescence of MB from the inside to the outside of aggregates was observed for the reverse transfection group. However, for the conventional transfection, the fluorescence was observed only around the surface of cell aggregates. It is concluded that the reverse transfection system with cGNS incorporating MB is promising to visualize the cell function of a higher transfection efficiency for the 2D culture and in a homogeneous manner for the 3D culture.

LIST OF PUBLICATIONS

Chapter 1

Yang, W., Abe, S., & Tabata, Y. (2023). Association with cationized gelatin nanospheres enhances cell internalization of mitochondria efficiency. *Regenerative Therapy*, 24, 190-200.

Chapter 2

Yang, W., Abe, S., Ando, M., & Tabata, Y. (2025). Association with Cationized Gelatin Nanospheres Enhances Mitochondria Uptake and Membrane Potential. *Tissue Engineering Part A*, 31(15-16), 1098-1108.

Chapter 3

Takehana, S., **Yang, W.,** & Tabata, Y. (2022). Potential method of autophagy imaging with cationized gelatin nanospheres incorporating molecular beacon. *ACS Applied Bio Materials*, 5(6), 2965-2975.

Chapter 4

Yang, W., Jo, J. I., & Tabata, Y. (2023). A reverse transfection system with Cationized Gelatin Nanospheres incorporating molecular beacon as a tool to visualize cell function. *ACS Applied Bio Materials*, 6(9), 3363-3375.

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