EXOSOME LIKE LIPOSOME GENERATION BY CELL EXTRUSION THROUGH A MICRO CHANNEL

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ABSTRACT

Several methods for generating liposomes have been studied due to their advantages of structural stability and high delivery efficiency; however, limitations prevent their use as mass delivery compartments to cells. This study proposes a novel method to generate nano-scale diameter liposomes using hydrophilic micro channels and cells. The generated nano-liposomes have the potential to contain membrane proteins in the lipid bilayer structure and have a shape similar to cell-secreted vesicles. On varying the length and width of microfluidic channels, the size of generated liposomes changed, and the uptake amount of the cells also changed with size.

KEYWORDS
Vesicle, Liposome, microfluidic channel, Exosome.

INTRODUCTION

Exosomes are nano-scale diameter compartments enclosed by a phospholipid membrane. They are secreted from cells and involved in many physiological activities. Due to their functions, long-term structure stability and high efficiency for trans-membrane transportations, many generation methods of liposome such as natural swelling, freezing and thawing, ultrasonication, electroformation, extrusion through polymer membrane, microdroplets and microfluidic methods, have been developed to exploit advantages of cell-secreted exosomes.

However, artificially generated liposomes have limitations to be used as exosomes in research and application fields. First, the size of liposome is bigger than exosome, hindering trans-memebrane transportation. Most of them have micro-scale size, and even if nano-scale liposomes are generated, they are not generated in uniform size. Second, the structure of liposome is different from exosome. Depending on the generation methods, sometimes liposomes have multilamellar membrane structure while exosomes have unilamellar phospholipid bilayer. Third, the composition of liposome is also different from that of exosome. To mimic the composition of cell-secreted exosomes, natural phospholipids have been suggested as a base membrane material of liposome. However, these synthetic membranes do not usually have proteins tethered on them.

This study proposes a novel method to generate nano-scale diameter liposome with biological contents by extruding cells through a micro channel. Similar to cell-secreted exosomes, the generated liposomes with this method had membrane proteins in the lipid bilayer structure and contain RNAs and proteins of original cell. Depending on the surface property, hydrophilic and hydrophobic, of micro channels, liposome generation changed. In a hydrophilic micro channel, exosome like liposome was generated, but in a hydrophobic micro channel, liposome was not generated. On varying dimensions of micro channels, the size, zeta potential and contents amount of exosome like liposome also changed. There are optimized points to generate liposome which have similar size as exosome and lots of cellular contents. The fusion rate of cell and the liposomes was also instigated as varying the liposome size.

This exosome-like liposomes are expected to be used importantly in the research fields of exosome and microvesicle, and also have applications in fields like drug delivery systems.

EXPERIMENT and RESULT

The pattern of the micro channel was made by SU-8 2025, using a conventional soft lithography with a thickness of 10 μm (Fig. 1(d), (e)). These SU-8 patterns were used as the master for a micro channel made of nine parts polydimethylsiloxane (PDMS) silicon elastomer base and one part curing agent. After PDMS was bonded to glass, the bonded devices were soaked for 24 hours in phosphate buffer saline (PBS) after plasma treatment on the surface of micro channels to induce a hydrophilic property. A hydrophobic micro-channel was made separately by 2% PTFE in a solvent. The contact angles were 26.8° at the hydrophilic channel and 115.2 ° at the hydrophobic channel.

When ES cells had almost filled the culture dish, but had not formed an embryonic body, they were detached with Tryp-LE (Gibco) and suspended in a PBS with a concentration of 1.5 million cells/mL and 0.5 mM ethylenediaminetetraacetate (EDTA) and loaded in a 1 mL disposable syringe. They were then extruded through the fabricated micro-channels using a syringe pump (11 plus dual syringe, Harvard Apparatus, USA) having a 3, 5, and 7 μm channel widths and 100, 200, and 400 μm channel lengths. The flow rate was 6.5 μL/min in all cases. An acrylic vise was fabricated and assembled with the micro-channels to prevent the leakage of samples during extrusion (Fig. 1b). In addition, during extrusion, the syringe pump was shaken to prevent cell from settling down in

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a syringe. As a pre-cleaning process, extruded samples were collected and centrifuged at 1×10^3 g-force for 20 min to eliminate unbroken cells. Next, the supernatants were put into cushions consisting of 10 and 50 % optiprep solutions, which were then centrifuged at 1×10^5 g-force for 2 hrs. The centrifuged sample had several layers; the second layer from the bottom, the nano-liposome layer, was collected. The entire nano-liposome generation processes, except the cell preparation, was carried out at 4 ℃.

The product of the hydrophilic micro channel after extrusion has a mean diameter of around 90 nm (Fig.2(a)) which is in the expected range for the diameter of nano-liposomes in relation to the established size of membrane vesicles. On the other hand, the product of the hydrophobic micro channel resulted in a 1.7 nm diameter, which is the same as that just of the buffer solution. To find out what had happened at hydrophobic channel, the products were checked using microscope images after the pre-cleaning process. Figure 2(d) shows that many unbroken cells settled down at the bottom of the tube after the pre-cleaning process when the product came from the hydrophobic micro channel. However, Fig.2 (b) shows that unbroken cells were not present in the product that came from the hydrophilic micro channel.

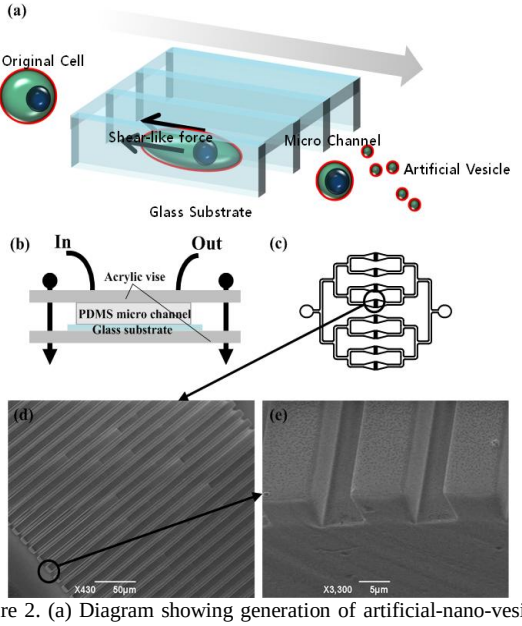

Figure 2. (a) Diagram showing generation of artificial-nano-vesicle using a simple micro channel. (d), (e) Scanning Electron Microscopic Images of extrusion channels.

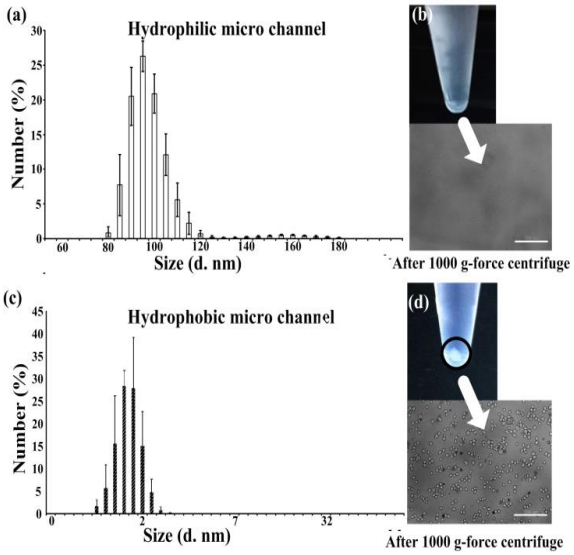

Figure 1. On the hydrophilic(a) and hydrophilic(c) surface, the sample show around 90 nanometer and around 1 nanometer size of mean diameter. Microscope images of results, from hydrophilic surface(b) and hydrophobic surface(d), of the pre-cleaning process after 1000 g-force.

Fig. 3 shows that as channel length increased, the mean diameter of nano-liposomes decreased and the deviation of mean diameter also decreased. The largest nano-liposomes are generated at a channel length of 100 μm. The nano-liposomes generated at channel length 200 and 400 μm are very similar, but the mean diameter of nano-liposomes from the 400 μm channel is smaller than for those from 200 μm. When channel width is controlled with a fixed channel length of 200 μm, the mean diameters form a reversed U shape; however, the difference of them are not large. If the type of the particle is known, the zeta potential is closely related to the diameter of the particle because it represents the surface charge of the particle. In Fig. 3, as our expectations according to concept of zeta potential, zeta potentials show the same trend with the result of mean diameter.

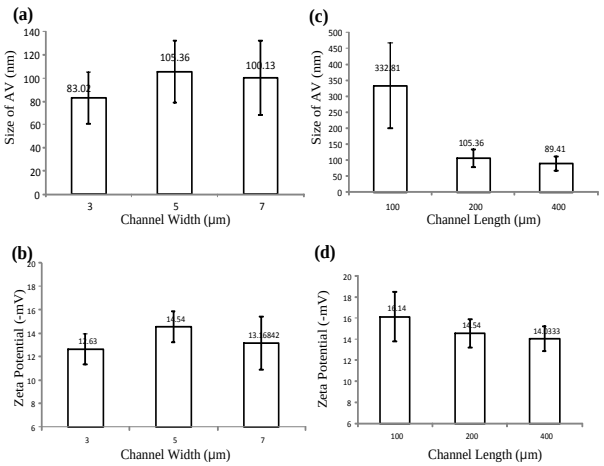

Figure 3. (a) The sizes of nano-liposomes and their zeta potential according to three micro-channel widths (3, 5, and 7 μm) with a flow rate of 6.5 μL/min. (b) the same for nano-liposomes according to the channel lengths equal to 100, 200, and 400 μm with the same flow rate.

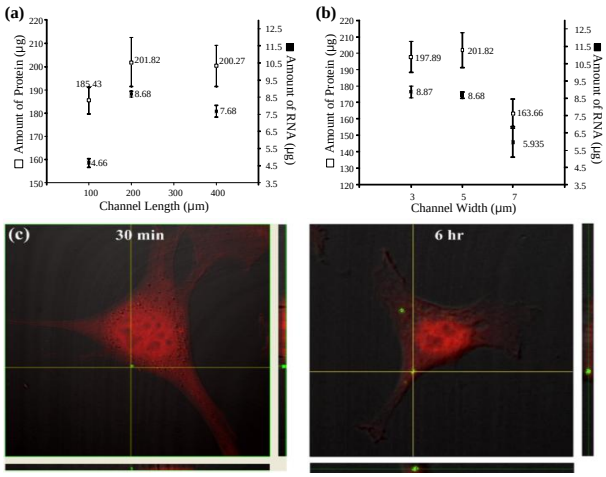

Figure 4. (a) (b) Amount of Protein of nano-liposomes and RNA came from every cases of experiments. (c) Confocal images of NIH 3T3 cells which uptake 65 nm size nano-liposomes at 30 min and 6 hours treatment time. (nano-liposomes: green, Cytosol: red)

The quantity of generated nano-liposomes was verified from the amount of RNA and proteins they contained (fig. 4 (a),(b)) because the role of the shear-like force to generate nano-liposomes cannot be interpreted from only the mean diameter data. Fewer nano-liposomes were generated when the channel width was 7 μm and channel length was 100 μm with a channel length of 200 μm and channel width of 5 μm, respectively. The reason is that the former conditions are insufficient to generate nano-liposomes because a 7 μm channel width is more than the diameter of ES cells and a 100 μm channel has less time to induce a shear-like force on the cells. In addition, comparing with mean diameter data, fewer nano-liposomes were generated when a larger deviation in mean diameter was observed. This implies that a sufficient shear-like force is necessary to generate uniform nano-liposomes. From this perspective, in this study, the main parameter to induce a shear-like force is the length of the micro channel rather than the width of that.

From confocal scanning microscopy, a position of nano-liposomes at each treatment time was verified. It could clearly define that fluorescent intensity was changed by changing amount of uptake. It was necessary because FACS cannot distinguish the real uptake from the attachment of nano-liposomes on the cell membrane. Fig. 4(c) shows that nano-liposomes, whose mean diameter was 65 nm, were on the membrane when treatment time was 30 minutes and passed through the cell membrane after 6 hours of nano-liposome treatment. The results of confocal images proved that the cellular uptake happened.

CONCLUSION

Nano-liposomes that can incorporate membrane proteins were generated by a micro channel through extrusion of cells. The shape of nano-liposomes was verified to be similar to membrane vesicles secreted by cells. The content of the nano-liposomes was also measured to be similar to secreted vesicles originating in cells; the content was identified to be proteins, RNA. The mean diameter and amount of protein and RNA of nano-liposomes showed that as channel length increased, mean diameter and its deviation of nano-liposomes decreased. In cellular uptake, according to treatment time of nano-liposomes, their position in the cell was changed membrane to inside of the cell. The results obtained show the various possible sizes, the gene, and the membrane proteins of extruded nano-liposomes. These results can be used in stem cell research and in research in the area of lipid bilayer mechanics.

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REFERENCES

[1] Théry, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nature reviews. Immunology* **2**, 569-79 (2002).

[2] Théry, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. *Nature reviews. Immunology* **9**, 581-93 (2009).

[3] Brewer, J.M., Tetley, L., Richmond, J., Liew, F.Y. & Alexander, J. Lipid vesicle size determines the Th1 or Th2 response to entrapped antigen. *Journal of immunology (Baltimore, Md. : 1950)* **161**, 4000-7 (1998).

[4] Février, B. & Raposo, G. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Current opinion in cell biology* **16**, 415-21 (2004).

[5] Felnerova, D., Viret, J.-F., Glück, R. & Moser, C. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. *Current opinion in biotechnology* **15**, 518-29 (2004)

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