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Scalable preparation of poly(ethylene glycol)-grafted siRNA-loaded lipid nanoparticles using a commercially available fluidic device and tangential flow filtration

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Abstract

While a number of siRNA delivery systems have been developed, the methods used in their preparation are not suitable for large-scale production. We herein report on methodology for the large-scale preparation of liposomal siRNA using a fluidic device and tangential flow filtration. A number of studies have appeared on the use of fluidic devices for preparing and purifying liposomes, but no systematic information regarding appropriate membrane type of commercially available apparatus is available. The findings reported herein indicate that, under optimized conditions, a fluidic device and tangential flow filtration can be used to produce siRNA lipid nanoparticles with the same characteristics as traditional ones'. The *in vivo* silencing efficiency of these lipid nanoparticles in the liver was comparable to laboratory-produced nanoparticles. In addition, con-focal laser scanning microscopy analyses revealed that they accumulated in the liver accumulation at the same levels as particles produced by batch-type and continuous-type procedures. This methodology has the potential to contribute to the advancement of this process from basic research to clinical studies of liposomal DDS.

Keywords: Liposomes; Nanoparticles; DNA/oligonucleotide delivery; siRNA; hetatocytes; liver; large-scale preparation

1. Introduction

Since siRNA can inhibit a gene of interest in mammalian cells without severe toxicity, the delivery of siRNA delivery to a target tissue or a cell has been a subject of intense interest [1-3]. Liposomes are one of the most highly developed carriers for use in this area, since they are highly efficient and are biocompatible [4, 5]. However, it is difficult to prepare liposomes on a large-scale using traditional preparation methods, such as the reverse phase evaporation method, the lipid hydration method, freeze-thawing and extrusion technique [6]. The use of microfluidic devices combined with the ethanol dilution method have been reported to be useful for preparing liposomes. Such methodology includes staggered herringbone structures [7], microfluidic devices with on-chip micro dialysis [8], multi-channel fluids [9], thermoplastic microfluidic devices [10], an ultra-sound assisted microfludic device [11] for the large-scale preparation of liposomes.

The objective of this study was to develop a simple, robust method for the large-scale preparation of liposomes using commercially available instruments. Liposome formulation generally consists of two procedures namely, a mixing step and a

purification step. In the mixing step, an organic solvent containing lipid molecules and a buffer, which may include therapeutics such as an anti-cancer drug or siRNA, are rapidly mixed in a microfluidic device. A sudden decrease in the concentration of the organic solvent results in the formation of liposomes. The rapid mixing produces lipid nano-particles with a homogeneous particle size distribution and is highly reproducible. Specifically the formulation of size-limited lipid nano-particles requires very rapid mixing within milliseconds [12]. To remove organic solvent in the mixture, lipid nano-particles (LNPs) are then subjected to a removal procedure, such as dialysis, ultrafiltration and ultracentrifugation. As liposomes are composed of hydrophobic molecules, the membrane material used in these operations are thought to be critical. Lastly, almost all therapeutics must be sterilized by filtration, if they are to be used in clinical procedures. Previous reports indicate that liposomes can be sterilized by gamma irradiation [13, 14]. However, such instrumentation is not commonly available in laboratories, readily available semi-large-scale preparation methods are generally used to achieve further progress in pre-clinical studies dealing with developing liposomal therapeutics.

For the efficient siRNA delivery, we developed an LNP-type siRNA delivery system, a multi-functional envelop-type nano device (MEND), which enables nucleic acids and other macromolecules to be assembled into LNPs [15]. We previously reported w MEND containing a pH-responsive cationic lipid, YSK05, (YSK-MEND) could deliver therapeutic nucleic acids to target tissues, such as liver [16-18], cancer [19, 20] and tumor endothelial cells [21]. Using the YSK-MEND as a model carrier, we mixed the lipid and siRNA solution, removed the organic solvent by tangential flow filtration (TFF) and sterilized the final liposome preparation by filtration. We report herein on the effect of flow velocity, flow rate at the mixing step, the type TFF membrane and sterilization by filtration on the characteristics of the YSK-MEND.

2. Materials and Methods

2.1 Materials

YSK05 was synthesized as previously reported [22]. Anti-coagulation-factor 7 siRNA (si-FVII) was purchased from Hokkaido System Science (Sapporo, Japan). si-FVII sequences; sense 5’-gga ucs ucu caa guc uua cTT-3’ anti-sense 5’-gua aga cuu gag aug auc cTT-3’ (lower cases and upper cases denote RNA and DNA, respectively). Cholesterol (chol) was purchased from SIGMA Aldrich (St. Louis, MO). 1,2-dimiristoyl-*rac*-grylerol methoxypolyethylene $glycol_{2,000}$ (PEG-DMG), egg phosphatidyl choline (EPC) were purchased from the NOF CORPORATION (Tokyo, Japan). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine (DiD) were purchased from PromoKine (Heidelberg, Germany). Phosphate buffered saline without Mg^{2+} and Ca^{2+} (PBS (–)) was purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Preparation of EPC/chol liposomes

To preliminarily optimize the preparation conditions for LNPs, we first prepared

EPC/chol liposomes with a fluidic device. A Deneb-type micromixer (KC-M-H-SUS; YMC CO., Kyoto, Japan; volume in mixing 32 μL, minimum diameter 0.2 mm × 0.2 mm (width × depth)) with a Harvard 33 Twin Syringe Pump (Harvard, Holliston, MA, USA) were used for the fluidic mixing. First, 10 mM EPC in ethanol and 10 mM chol in ethanol were diluted to a concentration of 0.1 – 16.0 mM (total) with ethanol in a tube. The PBS (–) and lipid solution were then allowed to flow through the micromixer at the indicated velocity with a fixed velocity ratio of lipid and PBS (–). The ethanol in the mixture was then diluted 10-fold with PBS (–), and the final produce characterized with Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worchestershire, UK).

2.3 Preparation of YSK-MEND

The YSK-MEND was prepared by a batch method, as previously reported [19-22]. Briefly, lipids (YSK05/chol 70/30 (total 3,000 nmol), 3 mol% PEG-DMG) dissolved in tertiary-butanol were gradually added to citrate buffer (pH 4.0). After diluting the mixture with phosphate buffered saline (PBS), the resulting solvent was subjected to ultrafiltration with an Amicon Ultra-15 (Millipore, Billerica, MA).

For the continuous, large-scale manipulation of the YSK-MEND, we used a Deneb-type micromixer (KC-M-H-SUS; YMC CO., Kyoto, Japan; volume in mixing 32 μL, minimum diameter 0.2 mm × 0.2 mm (width × depth)) with a Harvard 33 Twin Syringe Pump (Harvard, Holliston, MA, USA). Lipids (YSK05, chol and PEG-DMG were 5,600 nmol, 2,400 nmol, 72 nmol (1 mol% of total lipid), respectively) in 3.6 mL of ethanol and siRNA (2.7 μM) in acetate buffer (25 mM, pH 4.0) were added to two syringes. The lipid mixture and diluted siRNA solvent were then injected into the micromixer at 4.0 mL/min and 12.0 mL/min, respectively. The ratio of flow rates was maintained at 1:3. The formulated liposomes were then subjected to ultrafiltration or TFF. TFF was performed with MicroKros modules (SPECTRUM, Gardena, CA, USA) using a membrane that was composed of modified polyethersulfone (PES) or polysulfone (PS) with a 500 kDa pore. After adding PBS to a volume of 5.0 mL, the solution was concentrated to a volume of 500 μL and 5.0 mL of PBS was then added to the MicroKros module with a cylinder. The procedure was repeated 3-times until the ethanol concentration was sufficiently diluted. For sterilization, the concentrate was passed through 0.20 μm-filters composed of cellulose acetate (CA, ADVANTEC

DISMIC-25 AS), a mixed cellulose ester (CE, DISMIC-25 CS), polycarbonate (PC, Millipore ISOPORE®) or polyestersulfone (PES, PALL Acrodisc®). DiI or DiD was added to the lipid mixture at 0.5 mol% of the total lipid when a fluorescent label was required. The characteristics of the MENDs were determined using a Zetasizer Nano ZS ZEN360. A siRNA encapsulation efficiency and recovery ratio were calculated using RiboGreen, as previously reported [20].

2.4 Animal study

Male, 4-week-old ICR mice were purchased from SLC Japan (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

2.5 Determination of an accumulation of YSK05-MEND in the liver using fluorescence and knockdown effect of factor VII

To fluorescently label YSK-MEND, DiI, a fluorescent hydrophobic molecule, was

added to the lipid mixture at 0.5 mol% of total lipid used in the preparation procedure. The DiI-labeled YSK-MEND was administered via the tail vein of male, 4-week-old ICR mice. After collecting the liver deeply anesthetized mice at the indicated times, 100 mg samples of the liver were homogenized, in TRIzol (Invitrogen, Carlsbad, CA, USA) for RNA extraction, in 1× Passive Lysis Buffer (Promega, Madison, WI, USA) for measuring fluorescent intensity. RNA extraction and quantitative reverse transcription – the polymerase chain reaction, (qRT-PCR) was performed according to the manufacture's protocol [21]. Primer sets for FVII and *Gapdh* were used for PCR; FVII forward: TCG AAT CCA TGT CAG AAC GGA GGT, FVII reverse: CCG ACC CTC AAA GTC TAG GAG GCA, *Gapdh* forward: AGC AAG GAC ACT GAG CAA G, *Gapdh* reverse: TAG GCC CCT CCT GTT ATT ATG. To determine the amount of systemically injected YSK-MEND that had accumulated, the fluorescent intensity of DiD derived from the YSK-MEND was measured (excitation 630 nm, emission 675 nm, band width 25 nm).

2.6 Observation of the accumulation of MENDs in the liver by con-focal laser

scanning microscopy

Livers were removed from ICR mice under ether anesthesia 30 min after the MENDs labeled with DiI were injected into the tail vein. The collect liver tissue was immersed in PBS containing 10 μg/mL of Hoechst33342 (Dojindo, Tokyo, Japan) for 30 min. The liver was observed by Nikon A1 microscopy.

2.7 Statistical Analysis

Comparisons between multiple treatments were made using the one way analysis of variance (ANOVA), followed by the Bonferroni or SNK test. Pair-wise comparisons between treatments were made using the student's *t*-test. A P-value of <0.05 was considered to be significant.

3. Results and Discussion

3.1 Alteration of mixing method for siRNA and lipid solution

Prior to the MEND preparation, EPC and cholesterol liposomes were formulated by microfluidic mixing. At low flow rate (lipid; 0.1 – 1.0 mL/min), particle size was very large and the polydispersity index (PdI) of the liposomes indicated a heterogeneous particle size distribution (**Figure 1A**). On the other hand, small liposomes with a homogenous particle distribution could be assembled when a higher flow rate (lipid; 2.0 – 4.0 mL/min) was used. When the concentration of lipid was too low (<0.5 mM), it was not possible to obtain single dispersed liposomes (**Figure 1B**). Accordingly, the flow rate was fixed at 3.0/12.0 (lipid/buffer) mL/min in subsequent experiments.

We also prepared an siRNA-loaded MEND. Based on the above result, the lipid and siRNA flow rate was set to 4.0 and 12.0 mL/min, respectively. Moreover, since very rapid mixing resulted in the formation of smaller particles, the amount of PEG-DMG used was decreased to 1 mol% from 3 mol% for preparing a MEND with the same particle size using the microfluidic device (data not shown). To compare the efficiency of siRNA delivery of the MENDs by handling ($MEND_{handling}$) and the microfluidic device ($MEND_{fluidic}$), ICR mice were administered MENDs encapsulating siRNA against factor VII (FVII)at a dose of 0.5 and 0.1 mg/kg, and the expression of FVII was

then measured by qRT-PCR. The properties of the $MEND_{handling}$ and $MEND_{fluidic}$ are shown in **Table 1**. The size, PdI, zeta-potential (ZP), siRNA encapsulation efficiency (EE), and siRNA and lipid recovery rate (RR) were almost the same between two carriers. As FVII is a liver specific gene [23], FVII is often used as a marker gene to evaluate a efficiency of liver-specific *in vivo* silencing [24, 25]. When carriers formulated with anti-FVII siRNA (si-F7) were systemically administered into ICR mice at doses of 0.5 or 0.1 mg/kg, the silencing effect of the $MEND_{handling}$ was found to be equal to that of the $MEND_{batch}$ at both doses (**Figure 2**).

	Size (nm)	PdI	ZP (mV)	siRNA EE (%)	siRNA RR (%)	Lipid RR (%)
Batch	94±3	0.23±0.03	-5±4	90±2	65±26	51±13
Fluidic	85±11	0.25±0.04	-6±6	89±4	74±19	73±6

3.2 Purification by tangential flow filtration (TFF)

The alcohol and siRNA that was not encapsulated were removed by the TFF system using a membrane that was composed of polyether sulfone (PS) or polyethersulfone (PES), instead of a spin column. The MEND prepared by manual mixing was subjected to TFF purification, and its physiological properties and siRNA and lipid RR examined

(**Table 2**). In addition, lipid absorption per surface area was calculated from the value in the datasheet for the TFF device. The partitioning coefficient (logP) calculated by ChemBioDraw Ultra software of PS and PES monomer is 7.811 and 1.103, respectively. The higher hydrophobicity of PS appeared to contribute to the absorption of MENDs. As a result, mPES was found to be superior to PS in the siRNA and lipid RR. The absorption of lipid to the filter membrane of the TFF system with mPES and the spin column were similar. Likewise, altering the purification method had no effect on silencing effect after the systemic injection of the MEND (**Figure 3A**).

Table 2 The effect of purification method on MEND formulation

	Size (nm)	PdI	ZP (mV)	siRNA EE (%)	siRNA RR (%)	Lipid RR (%)	Absorption per surface area (nmol/cm^2)
PS	94±3	0.23±0.03	-5±4	90±2	65±26	51±13	140
mPES	85±11	0.25±0.04	-6±6	89±4	74±19	73±6	41
Spin colmun	87±10	0.15±0.02	-9±3	98±1	98±12	88±8	47

PdI; polydispersity index, ZP; zeta potential, EE; encapsulation efficiency, RR; recovery ratio

For a more strict comparison, the distribution of MENDs in the liver was evaluated

by confocal laser scanning microscopy (CLSM). Fluorescence derived from MENDs were detected throughout the liver in the both the spin column and the TFF system with PES groups (**Figure 3B**).

3.3 Sterilization of MEND by filtration

To validate the method used to sterilize the MEND, we evaluated four kinds of membranes, polycarbonate (PC), cellulose acetate (CA), cellulose ester (CE) and PES. After passing the fluorescently labeled MEND through 0.2 μm-pore membranes, the RR value was drastically decreased by the filtration using CE (**Figure 4**). On the other hand, 90% of the lipid and siRNA of the MEND were recovered in the case of CA, PC and PES. Thus, all of the materials, except for CE, could be used for sterilization. The exact reason for why only CE failed result in sterilization is not clear. Further study of membrane materials and the shape of the opening in the membrane should be required for the efficent filtration of LNPs.

3.4 Serial preparation of MEND using a fluidic device, TFF and filter sterilization.

Finally, we assembled the MEND by a continuous procedure, that is, combining the micromixer, TFF purification and filter sterilization. The MENDs were prepared by a

previously described manual method ($MEND_{batch}$) or by fluidic mixing, TFF and sterilization ($MEND_{con}$) on a one mg siRNA scale and FVII mRNA expression levels were then compared. The silencing efficiency (**Figure 5A**) and liver delivery (**Figure 5B**) of these MENDs were similar. On the other hand, the distribution of $MEND_{batch}$ was a bit more homogenous than that of the $MEND_{con}$ (**Figure 5C**). Taken together we identified the optimized conditions for preparing MENDs, and thus succeeded in preparing siRNA-loaded LNP on a large scale using commercially available devices.

4. Conclusions

Large-scale preparation is one of major obstacles in the bench-to-bed transition of nanocarriers. We herein attempted to confirm that it is possible to prepare siRNA-loaded lipid nanoparticles (LNPs) on a large scale using commercially available instruments. At the siRNA/lipid mixing step, a rapid flow rate (over 4.0/12.0 mL/min (lipid/siRNA)) and modestly high lipid concentration (>2.0 mM) was required. Moreover, it was possible to easily alter the conditions for spin column filtration with tangential flow filtration (TFF) in the purification step. Notably, the hydrophobicity of the TFF membrane appears to be a dominant factor for the absorption of LNPs to the TFF membrane. Finally, cellulose acetate, polycarbonate and polyethersulfone filters allowed us to sterilize LNPs solutions without fatal loss of lipid envelope and its cargo. Accordingly, we succeeded in preparing siRNA-loaded LNPs on a 1 mg scale. These findings provide critical information regarding the preparation of large quantities of nanocarriers.

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Figure legend

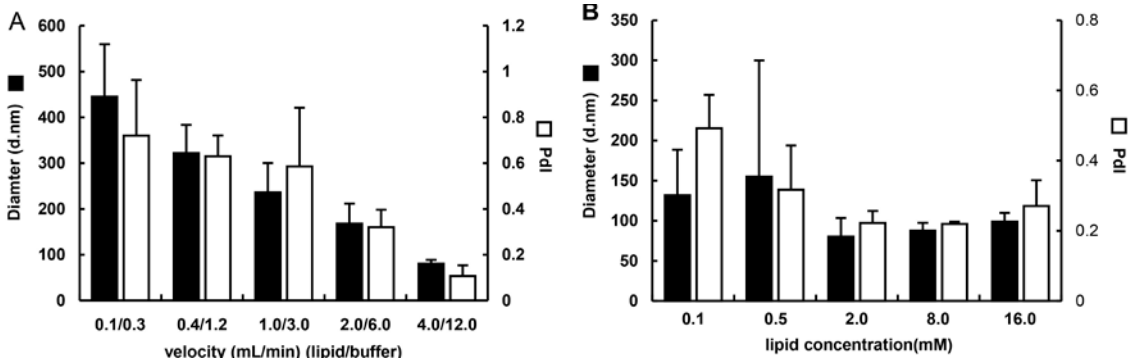

Figure 1. Preparation of PEGylated Liposomes with Fluidic Device. Liposomes (EPC/chol 50/50) were prepared by means of a microfluidic mixer. A) The effect of velocity of the lipid solution and buffer on particle size distribution. When the velocity varied from 0.1/0.3 – 4.0/12.0 mL/min (lipid/PBS (–)), size and polydispersity index (PdI) was determined by dynamic light scattering. B) The effect of lipid concentration on particle size distribution. Total lipid concentration was changed from 0.1 – 16.0 mM in a mixing step. The diameter and PdI were measured. Data represent the mean ± standard deviation (n=3).

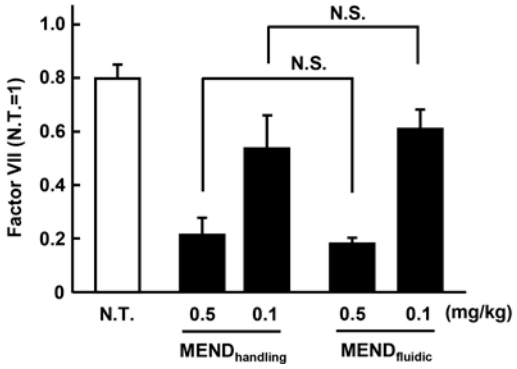

Figure 2. Comparison of the silencing efficiency on handling and fluidic preparation. To compare the silencing effect of the YSK-MEND prepared using the microfluidic device (YSK05/chol 70/30, PEG-DMG 1 mol%, $MEND_{fluidic}$) with that of YSK-MEND prepared by the handling method (YSK05/chol 70/30, PEG-DMG 3 mol%, $MEND_{handling}$), mice were treated with both MENDs at a dose of 0.1 and 0.5 mg/kg. When the P-value (Student's t-test) exceeded 0.05, the difference was considered to not be significant between MENDs at the same dose. Data represent the mean ± standard deviation (n=3).

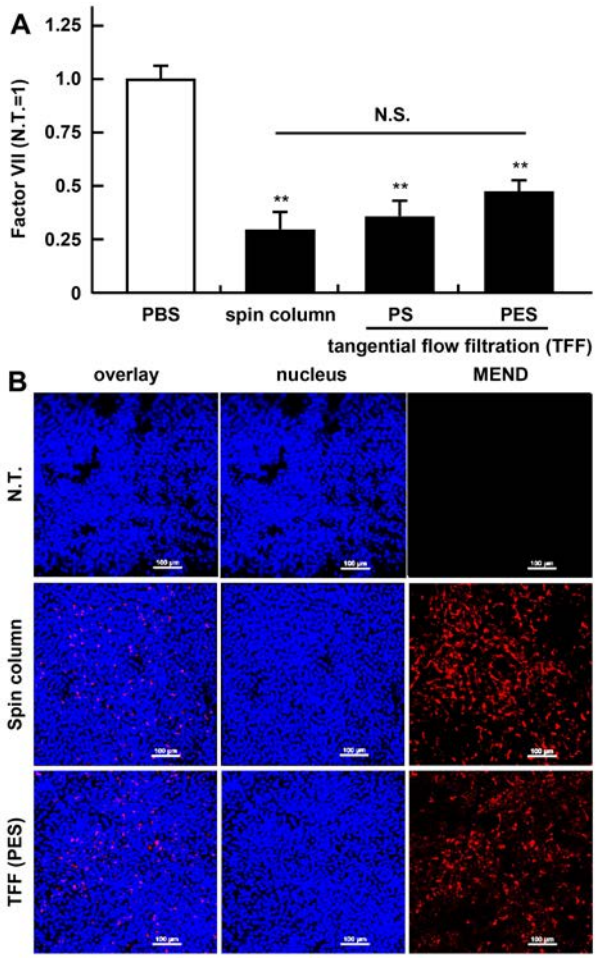

Figure 3. The effect of purification method and membrane material on the silencing effect and efficiency of delivery to liver tissue. A) Silencing effect of MENDs purified by a spin column, TFF (polysulfone) and TFF (polyethersulfone) in the liver tissue. MENDs were administered into ICR mice at a dose of 0.5 mg/kg, and the FVII expression level in the liver tissue was then determined. Data represents the mean ± standard deviation (n=3). **:P<0.01 (ANOVA, followed by SNK test). B) The distribution of MENDs in liver tissue. Mice were administered fluorescently labeled

MENDs. For staining nuclei, whole liver tissues were immersed in 10 μg/mL Hoechst33342 for 30 min, and the liver tissues were then observed by Nikon A1. The images are captured by 20× objective lens. Bars are 100 μm. Red and blue dots mean MENDs and nucleus, respectively.

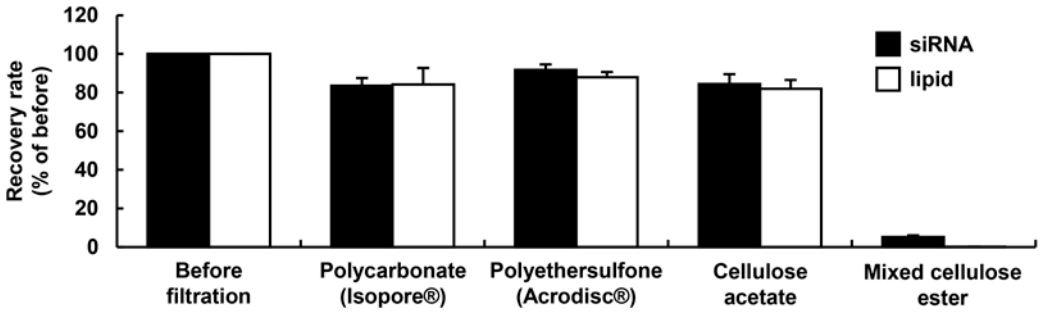

Figure 4. Sterilization of MENDs by filtration. After subjecting the MENDs to sterilization by 0.2 mm-pore filter, siRNA and the lipid envelope recovery rate was determined by comparing fluorescent intensity. Filtration using a filter consisting of polycarbonate (PC), polyethersulfone (PES), cellulose acetate (CA) and mixed cellulose ester (CE) were compared in terms of recovery rate. Data represent the mean ± standard deviation (n=3).

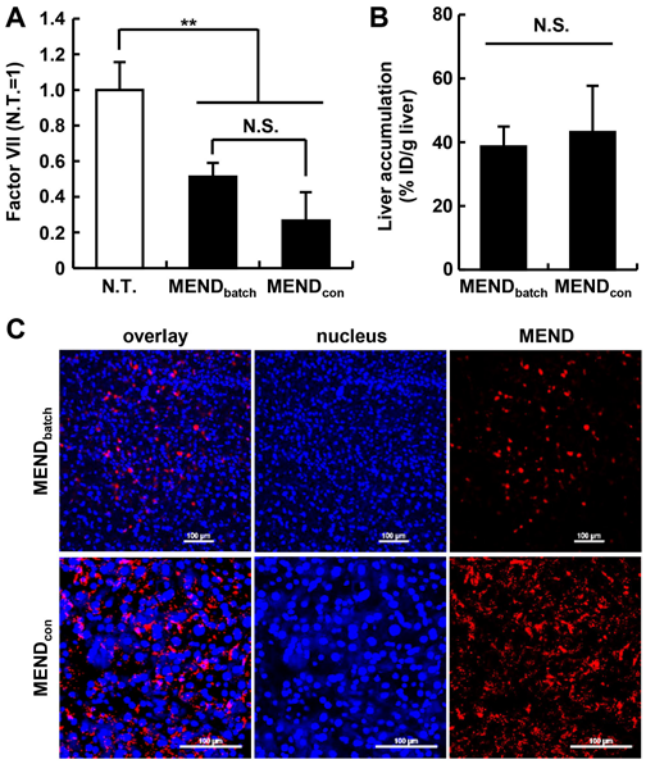

Figure 5. Evaluation of the MEND by large-scale preparation. A) Silencing effect of MEND prepared by traditional batch method ($MEND_{batch}$) and MEND prepared by fluidic mixing, TFF purification and sterilization ($MEND_{con}$). MENDs were systemically administered into ICR mice at a dose of 0.5 mg/kg, and then FVII mRNA expression level was determined by qRT-PCR. When P-value (Student's t-test) was over 0.05, we considered the difference not significant between MENDs at the same dose. Data represents mean ± standard deviation (n=3). **:P<0.01 (ANOVA followed by SNK test). B) Liver accumulation of MENDs. Accumulation amount of both MENDs after systemic injection was determined by fluorescent intensity derived from lipid

envelope. After injection, liver was excised and homogenized. Then, fluorescence in the homogenates were measured by fluorophotometer. C) Distribution of $MEND_{batch}$ and $MEND_{con}$ in the liver tissue. Nuclei was stained by immersing whole liver tissues in 10 μg/mL Hoechst33342 for 30 min. Liver tissues were observed by Nikon A1. The images are captured by 20× objective lens. Bars are 100 μm. Red and blue dots mean MENDs and nucleus, respectively.

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