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Title

The advantages of ethanol dilution method for preparing GALA-modified liposomal siRNA carrier on the in vivo gene knockdown efficiency in pulmonary endothelium

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Abstract

We previously reported that a multifunctional envelope-type nano device (MEND) modified with a GALA peptide (GALA/MEND) exerted dual functions; effective targeting the pulmonary endothelium and endosomal escape. The GALA/MEND containing encapsulated siRNA was originally prepared by the film coated hydration method (GALA/$MEND_{Hyd}$). However, an ethanol dilution method was found to be appropriate for scaling up the preparation of this liposomal nanoparticle. In this study, we report on the preparation of a GALA/MEND based on the principal of the ethanol dilution (GALA/$MEND_{EtOH}$). The gene knockdown efficacy of the $MEND_{Hyd}$ and $MEND_{EtOH}$ without GALA-modification was equivalent regardless of the preparation method. The larger sized characteristic of the GALA/$MEND_{EtOH}$ in comparison with GALA/$MEND_{Hyd}$ induced more efficient gene silencing in the pulmonary endothelium (ED_{50}; approximately 0.17 mg siRNA/kg) compared to the GALA/$MEND_{Hyd}$. The GALA/$MEND_{EtOH}$ was escaped from endosomes more rapidly than GALA/$MEND_{Hyd}$, while the pharmacokinetics and lung accumulation of GALA/$MEND_{EtOH}$ and GALA/$MEND_{Hyd}$ were comparable after *i.v.* administration. Collectively, the ethanol dilution method improves the function of the GALA/MEND as a lung-targeting siRNA carrier.

Keywords

ethanol dilution method, siRNA, lung endothelium, intracellular trafficking, lipid nanoparticles

Introduction

Specific gene silencing with RNA interference (RNAi) is a highly promising strategy for currently unmet medical needs (Aleku et al., 2008; Matsuda et al., 2007; Png et al., 2012). The pulmonary endothelium is particularly crucial target, since it is involved in a large variety of the diseases (i.e. cancer (Png et al., 2012), sepsis (Matsuda et al., 2007), acute lung injury/acute respiratory distress syndrome (McDonald et al., 2012) and pulmonary hypertension (Gaine and Rubin, 1998; McDonald et al., 2012)). However, an innovative technology for conquering tissue targeting and subsequent cytoplasmic release of the short interference RNA (siRNA) is highly required to allow the siRNA to function as a molecule for medical applications (Aleku et al., 2008; Matsuda et al., 2007; Png et al., 2012). To date, the successful delivery of the siRNA was extensively reported in cancer (Cabral et al., 2011; Peer et al., 2007; Yagi et al., 2009) or liver (Akinc et al., 2009; Semple et al., 2010), in such situations, the nanoparticles can passively gain access to the tissue parenchyma via loose junctions in the neo-vasculature or fenestrae in sinusoidal capillaries. In contrast, for the siRNA carriers targeting the continuous endothelium, ligand molecules that can strongly recognize the receptor molecules on the surface of the endothelium is prerequisite.

Traditionally, the targeting of the lung by non-viral vectors involves the use of cationic materials. However, this strategy is attended by risks, in that large aggregates with erythrocytes and/or platelets can occur (Nomoto et al., 2011), leaving them stuck in lung capillaries (Hatanaka et al., 2010; Li et al., 1999; Mahato et al., 1998). Of note, large aggregates of lipoplexes with erythrocytes (Ogris and Wagner, 2002) may cause clinical problems including microinfarctions that are caused by tissue ischemia, and/or myocardial damage (Wright et al., 1998).

We have developed a multifunctional envelope-type nano device (MEND) encapsulating siRNA for use as a non-viral carrier for the siRNA, in which siRNA was compacted with a polycation (i.e. polyethyleneimine), and then encapsulated in a lipid envelope (Hatakeyama et al., 2011). More recently, we reported that the GALA peptide (WEAALAEALAEALAEHLAEALAEALEALAA), a negatively charged peptide that was originally developed as an inducer of the disruption of endosomes (Subbarao et al., 1987) also functions as a ligand for the sialic acid-terminated oligosaccharides that are expressed on the lung endothelium (Kusumoto et al., 2013). To display the GALA-peptide outward from the surface of the liposomes, GALA peptide was conjugated with cholesterol (chol-GALA) as a lipid anchor (Kakudo et al., 2004). After *i.v.* administration, GALA-modified MEND (GALA/MEND) flows in the blood stream without aggregation, and then rapidly binds to the lung endothelium within 30 min. With the aid of the original function of the GALA as an inducer of endosomal escape, the GALA-MEND exhibited lung specific gene knockdown by a single *i.v.* administration at a dose of 0.5 mg/Kg body weight. In the previous report, the GALA/MEND was prepared using a film coated hydration method (Kusumoto et al., 2013), in which the lipid film formed by the evaporation of a lipid solution in ethanol was hydrated with a

siRNA/polycation core solution in water, followed by sonication. However, mass production is hampered by the flask size used for preparing the lipid film and heterogeneous irradiation of the sonication energy. In order to manufacture the GALA/MEND in quantities needed for preclinical and clinical development, a preparation based on the principal of ethanol dilution (Jeffs et al., 2005) is more simple, robust and potent, in terms of scaling up.

The first effort to achieve this was focused on preparing the GALA-MEND by the ethanol dilution method. As described below, we found that the gene knockdown efficacy of the GALA-MEND prepared by the ethanol dilution method (GALA/MEND$_{EtOH}$) was higher than that for the GALA-MEND prepared by the lipid hydration method (GALA/MEND$_{Hyd}$). Thus, we gained insights into the mechanism for the preferred gene knockdown efficacy of the GALA/MEND$_{EtOH}$ by comparing the pharmacokinetics and intracellular trafficking between it and the original GALA/MEND$_{Hyd}$.

Materials and methods

Preparation of MENDs by the lipid hydration method

The sequences of the siRNA and primers used in quantitative RT-PCR were reported in a previous article (Kusumoto et al., 2013). siRNA and PEI were first dissolved in a 10 mM HEPES buffer (pH 7.4) containing 5% glucose (HBG). 200 μL of PEI (0.125 mg/mL) was added to 300 μL of siRNA (0.33 mg/mL) to form a complex at a nitrogen/phosphate ratio of 1.8. A lipid film was formed by the evaporation of an ethanol solution containing 2.64 μmol of total lipids of DOTMA/Chol/EPC/STR-mPEG (30:40:30:5). To prepare the GALA/MEND$_{Hyd}$, 2 mol% of Chol-GALA was added to the lipid composition. The siRNA/PEI complex was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. To encapsulate the siRNA/PEI complex in the lipid, the lipid film was sonicated for approximately 1 min in a bath-type sonicator.

Preparation of MENDs with ethanol dilution method

An 1.2 mL of ethanol solution containing 4.4 mM of total lipids of DOTMA/Chol/EPC/STR-mPEG (30:40:30:5) was rapidly diluted with 2.8 mL of the siRNA/PEI core particle solution (0.2 mg of siRNA, 30 vol% ethanol). To prepare the GALA/MEND$_{EtOH}$ 2 mol% Chol-GALA was added to the lipid solution. The solution was further diluted by adding 1.8 mL of HBG to give 15 vol% ethanol. The diluted solution was concentrated by ultrafiltration using an Amicon Ultra 4 (Millipore Corp. Billerica, MA) by centrifugation at 1,000g for 30 min at room temperature. The particle solution remaining on the upper column was diluted with 4 mL of HBS, and again concentrated by centrifugation at 1,000g for 30 min at room temperature. The diameter and zeta potential of the MENDs were determined using an electrophoretic light-scattering spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR, UK). Materials and any other methods can be found in the supplementary section.

Result and Discussion

In the present report, we encapsulated the siRNA/polyethyleneimine (PEI) complex within a lipid envelope composed of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammunium chloride (DOTMA)/Cholesterol (Chol)/Egg phosphatidylcholine (EPC)/Polyethyleneglycol monostearate (45E.O.) (STR-mPEG) (DOTMA:Chol:EPC:STR-mPEG = 30:40:30:5, total lipid amount: 26.4 nmol for 1 ug of siRNA). For the GALA-modification, 2 mol% of chol-GALA was added to the lipid composition.

The physicochemical characteristics of the MENDs are listed in **Table 1**. Without modification by GALA, the particle size of the MENDs prepared by the lipid hydration method ($MEND_{Hyd}$) and the ethanol dilution method ($MEND_{EtOH}$) were quite comparable (approximately 150 nm in size and +30-35 mV in ξ-potential). In contrast, when chol-GALA was incorporated in the lipid composition, the size of the GALA/$MEND_{EtOH}$ (approximately 150 nm) was found to be significantly larger than that of GALA/$MEND_{Hyd}$ (approximately 100 nm). The ξ-potentials of the GALA/$MEND_{Hyd}$ and GALA/$MEND_{EtOH}$ were comparable, but were slightly decreased (approximately +20 mV) in comparison with GALA-unmodified MENDs, most probably because the GALA peptide inherently includes anionic amino acids.

The gene knockdown effects of the MENDs were evaluated using CD31 as an endothelial cell-specific key gene. **Figure 1** shows the dose response curves for the relative mRNA expression levels of CD31 to CD34 in lungs at 24 h after a single *i.v.* administration of the MENDs at a dose of 0.05-4 mg siRNA/kg. First, the gene knockdown efficacy of the MENDs without GALA-modification was comparable regardless of the preparation method (ED_{50}; approximately 1.5 mg siRNA/kg in both $MEND_{Hyd}$ and $MEND_{EtOH}$). As reported previously (Kusumoto et al., 2013), GALA modification drastically improved the gene knockdown efficacy of the $MEND_{Hyd}$ (ED_{50}; approximately 0.34 mg siRNA/kg). The most significant finding in this figure is that GALA/$MEND_{EtOH}$ (ED_{50}; approximately 0.17 mg siRNA/kg) exhibited a higher gene silencing effect than the GALA/$MEND_{Hyd}$. Therefore, the function of the GALA was unexpectedly potentiated when it is incorporated in MENDs that are prepared by the ethanol dilution method.

To investigate the mechanism for the preferred gene knockdown effect in the GALA/$MEND_{EtOH}$ above GALA/$MEND_{Hyd}$, the tissue accumulation of [^{3}H]Cholesteryl hexadecyl ether ([^{3}H]CHE)-incorporated lipid envelopes and [γ-^{32}P]-adenosine 5′-triphosphate (ATP)-labeled siRNA ([^{32}P]siRNA), or those remaining in the blood circulation was quantitatively evaluated at 1 h after the *i.v.* administration of the preparation at a dose of 2 mg siRNA/kg (**Fig. 2**). Consistent with previous work, the lipid component ([^{3}H]CHE) or siRNA ([^{32}P]siRNA) in the GALA/$MEND_{Hyd}$ accumulated in lung to a greater extent than those in the $MEND_{Hyd}$, while the accumulation of these compounds in the liver and spleen, the major organ for the clearance of liposomes was reduced. Unexpectedly, the tissue distribution of the [^{3}H]CHE and [^{32}P]siRNA in lung, liver and spleen, as

well as the level remaining in the blood was quite comparable between GALA/MEND$_{Hyd}$ and GALA/MEND$_{EtOH}$. In addition, to estimate the siRNA entrap efficacy in GALA/MENDs, we calculated the ratio of [^{32}P]siRNA to [^{3}H]CHE in blood pharmacokinetics data (Fig.2D). The entrap efficacy were about 70% regardless of preparation methods. Therefore, the improved gene knockdown function of GALA/MEND$_{EtOH}$ cannot be explained from the pharmacokinetic and siRNA efficacy points of view.

The efficacy of siRNA is also rate-limited by intracellular processing, including endosomal escape, as well as the cellular uptake process (Khalil et al., 2006). Thus, we evaluated the endosomal escape process in HeLa cell between GALA/MEND$_{Hyd}$ and GALA/MEND$_{EtOH}$ by confocal laser scanning microscopy at 6 h after the transfection. As shown in **Figure 3**, the most significant finding is that the GALA/MEND$_{EtOH}$ was dominantly observed free from the co-localization of endosome/lysosome fraction. In contrast, the intracellular signals of GALA/MEND$_{Hyd}$ are significantly poor in comparison with GALA/MEND$_{EtOH}$ prepared with ethanol dilution method. One of the possible explanation is that the GALA/MEND$_{Hyd}$ is subject to the rapid degradation. This hypothesis is also supported by the quantitative analysis of endosomal escape efficiency: the endosomal escape in GALA/MEND$_{Hyd}$ was significantly lower than GALA/MEND$_{EtOH}$. Collectively, the more prominent gene knockdown efficiency for the GALA/MEND$_{EtOH}$ prior to the GALA/MEND$_{Hyd}$ can be attributed to extensive endosome escape.

In conclusion, the GALA/MEND could be prepared by the ethanol dilution method, which is desirably in terms of scaling up the preparation of the material. Also, the larger size of the GALA/MEND$_{EtOH}$ resulted in a higher gene knockdown efficacy in comparison with GALA/MEND$_{Hyd}$ owing to its rapid endosomal escape properties. Collectively, the ethanol dilution method can be considered to be a promising technology for the mass-production and for upgrading the function of the GALA/MEND as a lung-targeting siRNA carrier.

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Figures

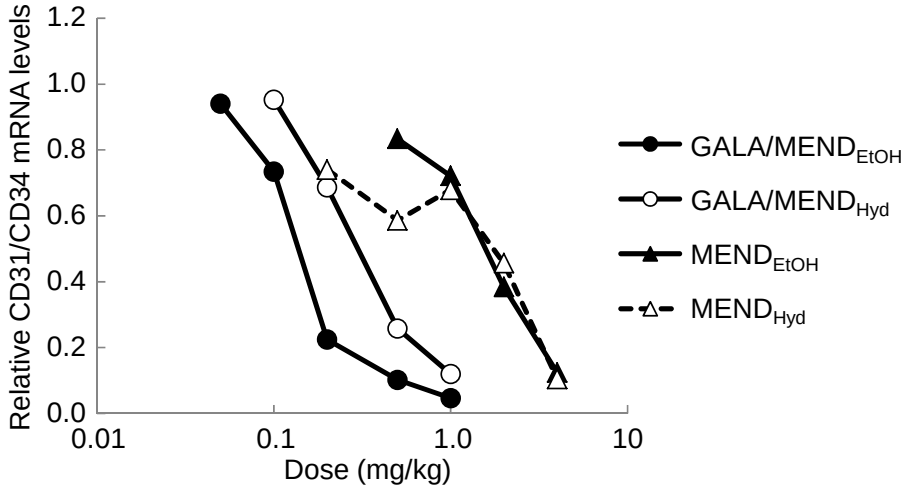
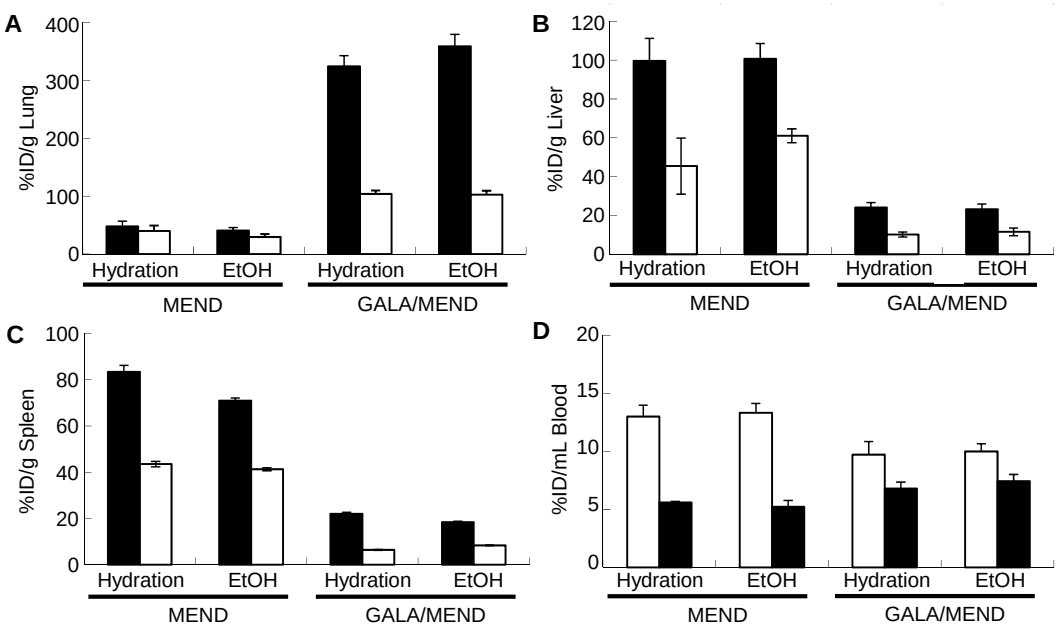


Fig. 1

Dose dependent gene silencing activity of MENDs in mice. MENDs and GALA/MENDs containing encapsulated anti-CD31 siRNA which were prepared by the lipid hydration method (MEND$_{Hyd}$) and the ethanol dilution method (MEND$_{EtOH}$) were i.v. administered at 0.05 – 4 mg/kg siRNA. At 24 h after injection, CD31 mRNA levels in lungs were determined by TaqMan real-time PCR. CD31 mRNA levels in lungs were determined by TaqMan real-time PCR. As an internal control, the mRNA expression of CD34 was also determined. Relative expression CD31/CD34 mRNA levels are shown as the mean ± S.D. (n = 3).



271

272 **Fig. 2**

273 Distribution of MENDs in the lung (A), liver (B), spleen (C) and blood (D) at 1h after
274 administration. [^{3}H]CHE (open column) and [^{32}P]siRNA (closed column) labeled MENDs,
275 which were prepared by the lipid hydration method ($MEND_{Hyd}$) and the ethanol dilution method
276 ($MEND_{EtOH}$) were i.v. administered to the mice. The distribution of MENDs is
277 represented as the percentage of the injected dose administered per g of tissue (%ID/g
278 tissue, mean ± S.D., n = 3).

279

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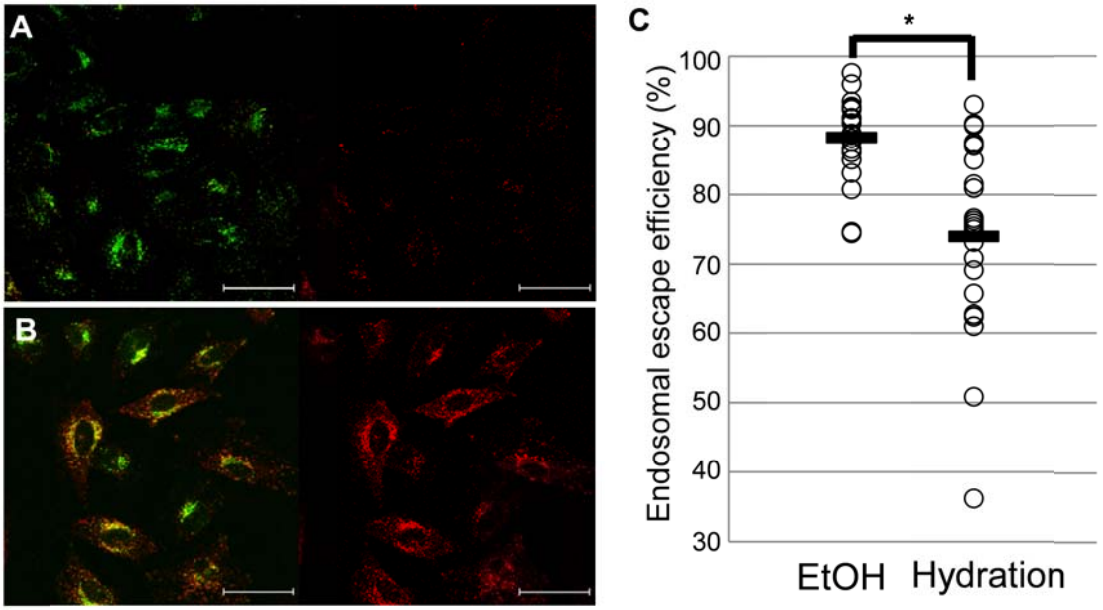


Fig. 3

Intracellular trafficking of the GALA/MENDs in HeLa cells. The fluorescence-labeled GALA/MENDs were prepared using Cy5-labeled siRNA. HeLa cells were incubated with the GALA/MENDs prepared by the lipid hydration method (A) or the ethanol dilution method (B) for 6 h. Endosomal escapes of the GALA/MENDs were analyzed by CLSM. The endosomes/lysosomes were stained with Lysotracker Blue to discriminate the siRNA in endosomes/lysosomes and the cytosol. Lysotracker Blue and Cy5 signals are colored in green and red, respectively. Bars represent 25 μm. (C) Quantitative comparison of endosome escape efficiency between GALA/$MEND_{EtOH}$ and GALA/$MEND_{Hyd}$. Statistical differences were evaluated by Mann-Whitney U-test. (*; $P < 0.01$)

Table

Table 1

Physicochemical properties of the MENDs. Data are presented as mean ± SD (n = 3)

	Size (nm)	PDI	ξ-potential (mV)
$MEND_{Hyd}$	148±5	0.222±0.016	29±2
$MEND_{EtOH}$	150±13	0.217±0.037	35±4
GALA/$MEND_{Hyd}$	103±11	0.264±0.009	22±2
GALA/$MEND_{EtOH}$	145±8	0.257±0.030	20±6