



Title	Advantages of ethanol dilution method for preparing GALA-modified liposomal siRNA carriers on the in vivo gene knockdown efficiency in pulmonary endothelium
Author(s)	Kusumoto, Kenji; Akita, Hidetaka; Santiwarangkool, Sarochin; Harashima, Hideyoshi
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1 **Title**

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

5 **Author**

6 Kenji Kusumoto¹, Hidetaka Akita², Sarochin Santiwarangkool², Hideyoshi Harashima²

8 ¹Laboratory for Formulation research, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno,
9 Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan

10 ²Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences,
11 Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

13 **Corresponding author**

14 Hidetaka Akita, E-mail: akita@pharm.hokudai.ac.jp

15 Hideyoshi Harashima, E-mail: harasima@pharm.hokudai.ac.jp

16 Phone: +81-11-706-3735

17 Fax: +81-11-706-4879

19 **Abstract**

20 We previously reported that a multifunctional envelope-type nano device (MEND) modified with a
21 GALA peptide (GALA/MEND) exerted dual functions; effective targeting the pulmonary
22 endothelium and endosomal escape. The GALA/MEND containing encapsulated siRNA was originally
23 prepared by the film coated hydration method (GALA/MEND_{Hyd}). However, an ethanol dilution method
24 was found to be appropriate for scaling up the preparation of this liposomal nanoparticle. In this
25 study, we report on the preparation of a GALA/MEND based on the principal of the ethanol dilution
26 (GALA/MEND_{EtOH}). The gene knockdown efficacy of the MEND_{Hyd} and MEND_{EtOH} without
27 GALA-modification was equivalent regardless of the preparation method. The larger sized
28 characteristic of the GALA/MEND_{EtOH} in comparison with GALA/MEND_{Hyd} induced more efficient
29 gene silencing in the pulmonary endothelium (ED₅₀; approximately 0.17 mg siRNA/kg) compared to
30 the GALA/MEND_{Hyd}. The GALA/MEND_{EtOH} was escaped from endosomes more rapidly than
31 GALA/MEND_{Hyd}, while the pharmacokinetics and lung accumulation of GALA/MEND_{EtOH} and
32 GALA/MEND_{Hyd} were comparable after *i.v.* administration. Collectively, the ethanol dilution
33 method improves the function of the GALA/MEND as a lung-targeting siRNA carrier.

35 **Keywords**

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

37

38 **Introduction**

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

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

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

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

79 The first effort to achieve this was focused on preparing the GALA-MEND by the ethanol dilution
80 method. As described below, we found that the gene knockdown efficacy of the GALA-MEND
81 prepared by the ethanol dilution method ($\text{GALA/MEND}_{\text{EtOH}}$) was higher than that for the
82 GALA-MEND prepared by the lipid hydration method ($\text{GALA/MEND}_{\text{Hyd}}$). Thus, we gained insights
83 into the mechanism for the preferred gene knockdown efficacy of the $\text{GALA/MEND}_{\text{EtOH}}$ by
84 comparing the pharmacokinetics and intracellular trafficking between it and the original
85 $\text{GALA/MEND}_{\text{Hyd}}$.

86

87 Materials and methods

88 **Preparation of MENDs by the lipid hydration method**

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

98 **Preparation of MENDs with ethanol dilution method**

99 An 1.2 mL of ethanol solution containing 4.4 mM of total lipids of DOTMA/Chol/EPC/STR-mPEG
100 (30:40:30:5) was rapidly diluted with 2.8 mL of the siRNA/PEI core particle solution (0.2 mg of siRNA,
101 30 vol% ethanol). To prepare the $\text{GALA/MEND}_{\text{EtOH}}$ 2 mol% Chol-GALA was added to the lipid solution.
102 The solution was further diluted by adding 1.8 mL of HBG to give 15 vol% ethanol. The diluted solution
103 was concentrated by ultrafiltration using an Amicon Ultra 4 (Millipore Corp. Billerica, MA) by
104 centrifugation at 1,000g for 30 min at room temperature. The particle solution remaining on the upper
105 column was diluted with 4 mL of HBS, and again concentrated by centrifugation at 1,000g for 30 min at
106 room temperature. The diameter and zeta potential of the MENDs were determined using an
107 electrophoretic light-scattering spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR,
108 UK). Materials and any other methods can be found in the supplementary section.

109

110 **Result and Discussion**

111 In the present report, we encapsulated the siRNA/polyethyleneimine (PEI) complex within a lipid
112 envelope composed of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammunium chloride
113 (DOTMA)/Cholesterol (Chol)/Egg phosphatidylcholine (EPC)/Polyethyleneglycol monostearate
114 (45E.O.) (STR-mPEG) (DOTMA:Chol:EPC:STR-mPEG = 30:40:30:5, total lipid amount: 26.4
115 nmol for 1 ug of siRNA). For the GALA-modification, 2 mol% of chol-GALA was added to the
116 lipid composition.

117 The physicochemical characteristics of the MENDs are listed in **Table 1**. Without modification by
118 GALA, the particle size of the MENDs prepared by the lipid hydration method ($MEND_{Hyd}$) and the
119 ethanol dilution method ($MEND_{EtOH}$) were quite comparable (approximately 150 nm in size and
120 +30-35 mV in ξ -potential). In contrast, when chol-GALA was incorporated in the lipid composition,
121 the size of the GALA/ $MEND_{EtOH}$ (approximately 150 nm) was found to be significantly larger than
122 that of GALA/ $MEND_{Hyd}$ (approximately 100 nm). The ξ -potentials of the GALA/ $MEND_{Hyd}$ and
123 GALA/ $MEND_{EtOH}$ were comparable, but were slightly decreased (approximately +20 mV) in
124 comparison with GALA-unmodified MENDs, most probably because the GALA peptide inherently
125 includes anionic amino acids.

126 The gene knockdown effects of the MENDs were evaluated using CD31 as an endothelial
127 cell-specific key gene. **Figure 1** shows the dose response curves for the relative mRNA expression
128 levels of CD31 to CD34 in lungs at 24 h after a single *i.v.* administration of the MENDs at a dose of
129 0.05-4 mg siRNA/kg. First, the gene knockdown efficacy of the MENDs without
130 GALA-modification was comparable regardless of the preparation method (ED_{50} ; approximately 1.5
131 mg siRNA/kg in both $MEND_{Hyd}$ and $MEND_{EtOH}$). As reported previously (Kusumoto et al., 2013),
132 GALA modification drastically improved the gene knockdown efficacy of the $MEND_{Hyd}$ (ED_{50} ;
133 approximately 0.34 mg siRNA/kg). The most significant finding in this figure is that
134 GALA/ $MEND_{EtOH}$ (ED_{50} ; approximately 0.17 mg siRNA/kg) exhibited a higher gene silencing
135 effect than the GALA/ $MEND_{Hyd}$. Therefore, the function of the GALA was unexpectedly potentiated
136 when it is incorporated in MENDs that are prepared by the ethanol dilution method.

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

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

152 The efficacy of siRNA is also rate-limited by intracellular processing, including endosomal escape,
153 as well as the cellular uptake process (Khalil et al., 2006). Thus, we evaluated the endosomal escape
154 process in HeLa cell between GALA/MEND_{Hyd} and GALA/MEND_{EtOH} by confocal laser scanning
155 microscopy at 6 h after the transfection. As shown in **Figure 3**, the most significant finding is that
156 the GALA/MEND_{EtOH} was dominantly observed free from the co-localization of
157 endosome/lysosome fraction. In contrast, the intracellular signals of GALA/MEND_{Hyd} are
158 significantly poor in comparison with GALA/MEND_{EtOH} prepared with ethanol dilution method.
159 One of the possible explanation is that the GALA/MEND_{Hyd} is subject to the rapid degradation. This
160 hypothesis is also supported by the quantitative analysis of endosomal escape efficiency: the
161 endosomal escape in GALA/MEND_{Hyd} was significantly lower than GALA/MEND_{EtOH}.
162 Collectively, the more prominent gene knockdown efficiency for the GALA/MEND_{EtOH} prior to the
163 GALA/MEND_{Hyd} can be attributed to extensive endosome escape.
164 In conclusion, the GALA/MEND could be prepared by the ethanol dilution method, which is
165 desirably in terms of scaling up the preparation of the material. Also, the larger size of the
166 GALA/MEND_{EtOH} resulted in a higher gene knockdown efficacy in comparison with
167 GALA/MEND_{Hyd} owing to its rapid endosomal escape properties. Collectively, the ethanol dilution
168 method can be considered to be a promising technology for the mass-production and for upgrading
169 the function of the GALA/MEND as a lung-targeting siRNA carrier.

170

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177

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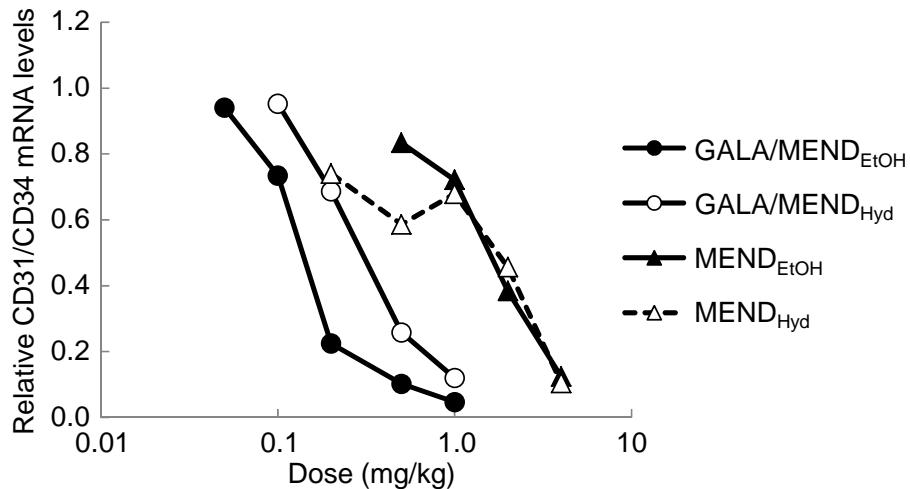
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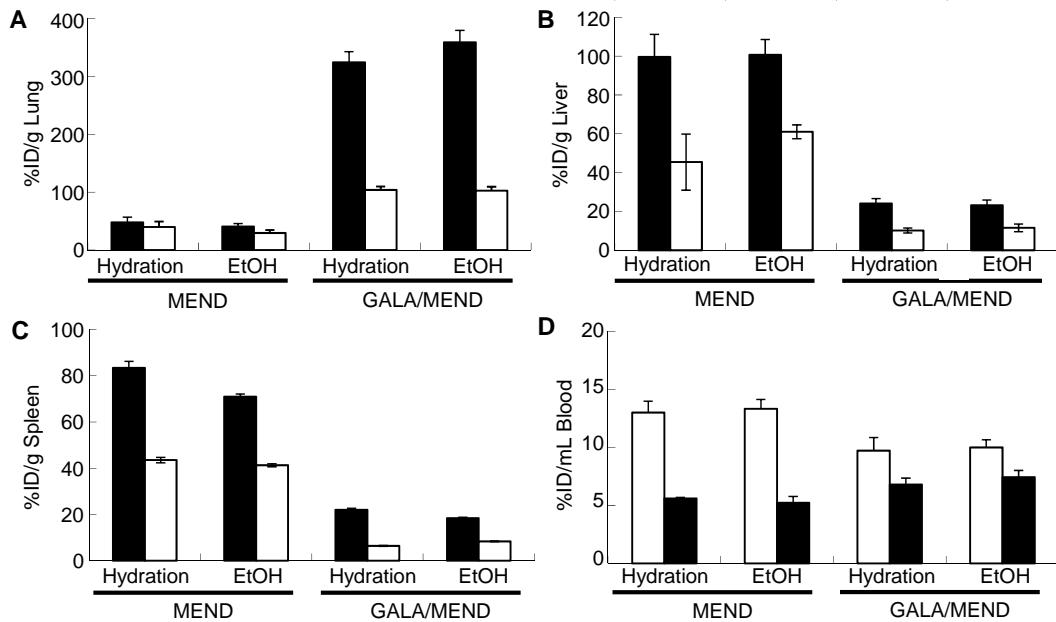
259

260 **Figures**

261

262 **Fig. 1**

263 Dose dependent gene silencing activity of MENDs in mice. MENDs and GALA/MENDs
264 containing encapsulated anti-CD31 siRNA which were prepared by the lipid hydration
265 method (MEND_{Hyd}) and the ethanol dilution method (MEND_{EtOH}) were i.v. administered at 0.05
266 – 4 mg/kg siRNA. [At 24 h after injection, CD31 mRNA levels in lungs were determined by](#)
267 [TaqMan real-time PCR.](#) CD31 mRNA levels in lungs were determined by TaqMan
268 real-time PCR. As an internal control, the mRNA expression of CD34 was also
269 determined. Relative expression CD31/CD34 mRNA levels are shown as the mean ± S.D.
270 (n = 3).



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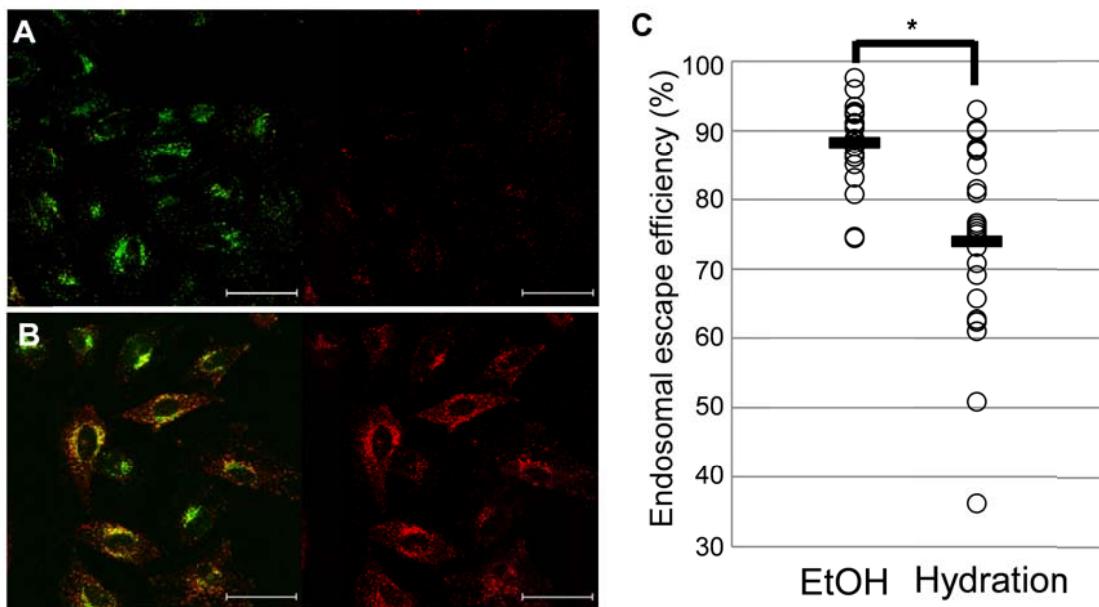
Fig. 2

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

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282

283 **Fig. 3**

291 Intracellular trafficking of the GALA/MENDs in HeLa cells. The fluorescence-labeled
 292 GALA/MENDs were prepared using Cy5-labeled siRNA. HeLa cells were incubated with the
 293 GALA/MENDs prepared by the lipid hydration method (A) or the ethanol dilution method (B) for 6
 294 h. Endosomal escapes of the GALA/MENDs were analyzed by CLSM. The endosomes/lysosomes
 295 were stained with Lysotracker Blue to discriminate the siRNA in endosomes/lysosomes and the
 296 cytosol. Lysotracker Blue and Cy5 signals are colored in green and red, respectively. Bars represent
 297 25 µm. (C) Quantitative comparison of endosome escape efficiency between GALA/MEND_{EtOH} and
 298 GALA/MEND_{Hyd}. Statistical differences were evaluated by Mann-Whitney U-test. (*; P < 0.01)

292

293 **Table**294 **Table 1**

295 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

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