1   **Title:** An mRNA vaccine against SARS-CoV-2: Lyophilized, liposome-based vaccine

2   candidate EG-COVID induces high levels of virus neutralizing antibodies

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24

25   **Abstract**

26

27   In addition to the traditional method of vaccine development, the mRNA coronavirus

28   vaccine, which is attractive as a challenging vaccination, recently opened a new era

29   in vaccinology. Here we describe the EG-COVID which is a novel liposome-based

30   mRNA candidate vaccine that encodes the spike (S) protein of SARS-CoV-2 with 2P-

31   3Q substitution in European variant. We developed the mRNA vaccine platform that

32   can be lyophilized using liposome-based technology. Intramuscular injection of the

33   EG-COVID elicited robust humoral and cellular immune response to SARS-CoV-2.

34   Furthermore, sera obtained from mice successfully inhibited SARS-CoV-2 viral

35   infection into Vero cells. We developed EG-COVID and found it to be effective based

36   on *in vitro* data, and we plan to initiate a clinical trial soon. Since EG-COVID is a

37   lyophilized mRNA vaccine that is convenient for transportation and storage,

38   accessibility to vaccines will be significantly improved.

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46 **Introduction**

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48 COVID-19 is caused by the coronavirus, which is an enveloped RNA virus that is

49 widely distributed in humans, other mammals, and birds and causes respiratory,

50 intestinal, liver, and nerve diseases. (Weiss et al., 2011). In addition to the very

51 common coronavirus responsible for the human common cold, there are three

52 coronaviruses that cause serious illness in humans: SARS-CoV, MERS-CoV, and

53 SARS-CoV-2 (Cox et al., 2020). In December 2019, an outbreak of severe acute

54 respiratory syndrome coronavirus 2 (now namely, SARS-CoV-2) infection occurred at

55 seafood market in Wuhan, Hubei Province, China, and spread across China (Zhu et

56 al., 2020). Considering the rapid spread of this virus and its impact on an

57 international scale, COVID-19 was declared a pandemic by the World Health

58 Organization on March 11, 2020 (WHO,1 DEC 2020). The urgent development of

59 safe and effective vaccines in the global coronavirus pandemic has become a

60 common goal for humanity, where numerous multinational pharmaceutical

61 companies and biotechnology companies are immersed in the development of

62 COVID-19 vaccines around the world simultaneously.

63 The mRNA-based vaccines are in the limelight because of their potential for

64 rapid development than the conventional vaccines at the COVID-19 pandemics. The

65 history of mRNA-based vaccines is relatively short, and its first application was

66 introduced in the 1990s, using mice to intramuscularly inject mRNA to locally

67 produce an encoded reporter protein (Wolff et a.,1990). The mRNA vaccine

68 combines the benefits of the subunit vaccine and the live attenuated vaccine, and

69 there is no risk associated with the live attenuated vaccine or the DNA vaccine

70 (Reichmuth et al., 2016). Of all the viral antigens studied, the spike (S) and

71 nucleocapsid (N) proteins are considered to be important immunogenic antigens for

72 SARS-CoV-2 (Assadiasl, 2020). Among these, most vaccine candidates use the

73 coronavirus glycosylated spike protein (S) as the primary antigenic target.

74   Liposomes are defined as phospholipid vesicles consisting of one or more

75 concentric lipid bilayers enclosing discrete aqueous spaces and its use as

76 pharmaceutical applications are well known (Ickenstein et al., 2019, Torchilin, 2005,

77 Sercombe et al., 2015). In 1970, liposomes were proposed as a drug carrier for

78 altering the therapeutic index of a drug by reducing toxicity or increasing the efficacy

79 of the parent drug (Lian et al., 2001). Vartak and colleagues suggested the use of

80 liposome as antigen or immunomodulatory molecules carrier (Vartak et al., 2016).

81 Furthermore, cationic liposome is considered as an effective adjuvant for vaccine

82 delivery as well as enhancing both cellular and humoral immune reactions (Mai et al.,

83 2020). As a reference, Herpes zoster vaccines are reported to use neutral or cationic

84 liposome as antigen delivery systems (Lal et al., 2015, Cunningham et al., 2016, Wui

85 et al, 2019, and 2021). Cationic liposomes have many important safety advantages,

86 especially when making viral vectors, are less expensive, have no restrictions on the

87 size of nucleic acids, and are easily accessible without special experience in their

88 use and handling (Karmali et al., 2007). Since the cationic liposome is positively

89 charged, it is able to hold nucleic acids such as mRNA that is negatively charged and

90 deliver mRNAs to cells and tissues efficiently. When prepared under proper

91 conditions, these lipoplexes (lipid-nucleic acid complex) retain their full positive

92 charge, allowing them to efficiently bind to negatively charged cell membranes, enter

93 the cells, protects nucleic acids from nucleases in the serum or cytoplasm (Karmail

94 et al., 2007).

95   By the way, all currently developed mRNA-based vaccines employ the lipid

96   nanoparticles (LNP) as their delivery vehicles for mRNA. LNPs are composed of

97   ionizable lipid, helper lipid, cholesterol, and PEG-lipid (Kim, et al., 2021). Although

98   LNPs are very efficient tools for delivery of mRNA into human body, all the LNP

99   based vaccines are distributed in liquid form only, and require special cold chain

100   system to maintain their efficacy.

101   In this report, we established cationic liposome based mRNA delivery system

102   and developed a lyophilizable SARS-CoV2 candidate vaccine (EG-COVID) with

103   mRNA encoding the full spike glycoprotein (S) of the European mutant strain (D614G)

104   with 2P-3Q substitution (Bangaru et al., 2020). We found that EG-COVID could elicit

105   robust humoral and cellular immune response to SARS-CoV-2, furthermore, sera

106   obtained from mice successfully inhibited SARS-CoV-2 viral infection into Vero cells.

107   So here we want to describe the immunogenicity and neutralizing antibody activity of

108   the EG-COVID.

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Author's contributions

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130

Competing interests

132   The authors declare no conflict of interest.

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144

145   **Results**

146   ***Design and optimization of CoV2-F004 (SARS-CoV-2-2P-3Q full-length spike)***

147   It was published that the substitution of K986P and K987P (2P) increase stability of

148   the protein, and the substitution of Prolines to Glutamate in S1/S2 polybasic

149   cleavage site (3Q) confers the protease resistance (Corbett et al., 2020, Torchilin,

150   2005). Based on these findings, we used the mRNA sequence encoding the SARS-

151   CoV-2 Spike protein with 2P-3Q substitutions in European variant (D614G) and

152   named CoV2-F004. The translated Spike protein from CoV2-F004 was predicted to

153   be superior to Wuhan variant (614D) in structural stability and antigen presentation

154   ability through AI-modeling simulation.

155

156   ***In vitro transcription of CoV2-F004 mRNA***

157   CoV2-F004 mRNA was produced by TriLink biotechnologies with Cleancap®

158   technology to increase translation efficiency. 5-Methoxyuridine was used to reduce

159   immune response of cells against mRNA. Produced mRNA demonstrated an

160   expected size of single band in agarose gel electrophoresis (Figure 1C),

161   demonstrating the functionality of CoV-2-F004 structure.

162

Characterization of EG-COVID

Liquid form of EG-COVID (L-EG-COVID) and reconstituted lyophilized form of EG-COVID (F-EG-COVID) were analyzed for hydrodynamic size and zeta potential. L-EG-COVID and F-EG-COVID had a Z-average diameter of 191.7 ± 8.5 nm and 266.7±12.2 nm with a polydispersity index (PDI) of 0.2 and a zeta potential of –54.5 ± 3 mV and –44.4 ± 2.1 mV, respectively (Figure 2A, 2B, 2D and 2E). Cryo-transmission electron microscopy (Cryo-TEM) of L-EG-COVID and F-EG-COVID revealed that both of EG-COVID mostly forms small unilamellar vesicles (SUV) with diameter of 80-100 nm (Figure 2C and Figure 2F).

Cationic liposomes as mRNA delivery carriers

In order to confirm the ability of cationic liposomes as mRNA delivery carriers, complexes of cationic liposomes and *Renilla* luciferase mRNA were prepared and intramuscularly injected into mice to confirm the mRNA delivery ability of cationic liposomes. We used 1 to 20 μg of mRNA complexed with cationic liposomes and observed that the expression of *Renilla* luciferase was successfully achieved. The level of expression of 10 ug of mRNA with cationic liposome was similar to the level of 10 μg of mRNA delivered using lipid nano particle. Therefore, we concluded that our liposome complex is nicely functional as mRNA delivery carriers for mRNA vaccines (Figure 3A). In addition, we prepared a lyophilized form of the *Renilla* luciferase mRNA-cationic liposome complex and confirmed that the lyophilized form of the complex has similar mRNA delivery capability to that of the liquid form (Figure 3B).

186   *In vitro Expression of EG-COVID*

187   We have generated a complex with cationic liposomes as a delivery system of Cov2-

188   F004 naming them as EG-COVID and verified the ability to deliver Cov2-F004

189   mRNA and expression of SARS-CoV2 spike protein *in vitro* (Figure 4). Re-hydrated

190   lyophilized form of EG-COVID 001, 002, 003 and 004, which were prepared

191   differently in their lipid composition, were treated in the culture medium of 293T cells,

192   and expression of SARS-Cov2 protein was confirmed by western blot after 24-hour

193   incubation. It was confirmed that there was some difference in the expression of

194   protein depending on the lipid composition of EG-COVID.

195

196   *Immunogenicity of EG-COVID*

197   Female B6C3F1/slc mice were immunized by intramuscular administration of L-EG-

198   COVID and F-EG-COVID twice at 3-week interval. After 2 weeks of last administration,

199   mice were sacrificed to evaluate immunogenicity of EG-COVID. We used both liquid (L-

200   EG-COVID) and lyophilized (F-EG-COVID) form of EG-COVID in the experiment.

201   Humoral immunity was confirmed by anti-RBG antibody titer, and cellular immunity

202   was confirmed by the amount of IFN-γ secreted from splenocytes stimulated with S1 and

203   S2 peptides. We found that both liquid and lyophilized form of EG-COVID induced

204   humoral and cellular immunity successfully (Figure 5A and 5B).

205   We also verified that the antibody formed by EG-COVID actually has neutralizing

206   ability against SARS-CoV2 virus. The result obtained using surrogate virus neutralization

207   tests which assess RBD binding ability of antibodies showed that EG-COVID

208   immunization successfully generated RBD binding antibodies in serum. Furthermore,

209   EG-COVID immunized serum significantly inhibited plaque formation induced by Wuhan

210   variant of SARS-CoV2 virus infection into VERO cells. There was no difference in EG-

211   COVID immunization ability between liquid and lyophilized formulations (Figure 5C and

212   5D). Therefore, we can conclude that EG-COVID induced a sufficient level of humoral

213   and cellular immunity against SARS-CoV-2 virus and is a first-class candidate for mRNA

214   vaccine that can be easily lyophilized.

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Materials and Methods

Lipids and chemicals

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and Cholesterol were purchased from Merck Millipore (Darmstadt, Germany) or Avanti Polar lipid (AL, USA). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200) were from Corden Pharma (Plankstadt, Germany). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were obtained from Avanti Polar Lipid (AL, USA). Hydroxyethyl piperazine Ethane Sulfonic acid (HEPES) solution was purchased from Sigma Aldrich (MO, USA). Sucrose was obtained from Merck Millipore (Darmstadt, Germany). 1 M Sodium acetate solution (pH 4.6) were from T&I (Gangwon-do, Korea).

mRNAs

Renilla luciferase mRNA was purchased from TriLink (CA, USA). CoV2-F004 mRNA was produced by TriLink (CA, USA)

Preparation of EG-COVID and mRNA-encapsulated LNP

Cationic liposomes were prepared with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Merck Millipore, Darmstadt, Germany), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Corden Pharma, Plankstadt Germany) and Cholesterol (Merck Millipore, Germany) by a thin-film method modified from previously described by Wui (Wui et al., 2019). Briefly, for the thin-film method, a

245   chloroform solution containing the DOTAP: DOPE: Cholesterol mixture was dried in

246   a round bottom flask using a rotary evaporator (IKA, Germany) to form a thin-lipid

247   film. The lipid film was rehydrated with a nuclease free- 4% (w/v) sucrose solution

248   (Sigma Aldrich, MO, USA) in 20 mM HEPES (pH7.4; Sigma Aldrich, MO, USA) and

249   homogenized using a microfluidizer (Avestin, ON, Canada). The mRNA CoV2-F004

250   were coated to the cationic liposome. The EG-COVID was lyophilized using a freeze-

251   dryer (Lyoph-Pride, Ilshin BioBase, Gyeonggi-do, Korea).

252   LNP were prepared using microfluidic mixer Ignite™ (Precision NanoSystems,

253   Vancouver, BC, Canada) as previously described by Sabnis and colleagues (Sabnis.

254   et al. 2018) In brief, 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-

255   hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-

256   200; Coden Pharma, Germany), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC;

257   Avanti Polar Lipid, AL, USA), Cholesterol (Merck Millipore, Germany) and 1,2-

258   dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000; Avanti

259   Polar Lipid, AL, USA) were resolved in ethanol at molar ratios of 50:10:38.5:1.5. The

260   lipid mixture was obtained with 25 mM sodium acetate buffer (pH 3; T&I, Gangwon-

261   do, Korea) containing *Renilla* luciferase mRNA (TriLink, CA, USA) at a N/P ratio of

262   5.6. using microfluidic mixer Ignite™. Formulations were dialyzed against nuclease

263   free- 4% (w/v) sucrose solution in 20 mM HEPES (pH7.4). The EG-COVID and

264   mRNA-encapsulated LNP were stored at 4℃ and used within a week.

265

266   ***Measurement of particle size and zeta potential of EG-COVID***

267   The particle size and polydispersity index (PDI) of liposomes were determined by

268   dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments,

269 Worcestershire, UK). Malvern Zetasizer DTS software (version 7.12) was used for

270 data acquisition and analysis. All measurements were carried out in triplicate.

271

Morphology of EG-COVID

273 The cryogenic transmission electron microscopy (Cryo-TEM) was used for

274 determination of morphology of EG-COVID. Liquid form of EG-COVID or lyophilized

275 EG-COVID powder was rehydrated with deionized water were used for Cryo-TEM

276 analysis. Three microliters of the liposome preparation were placed on a 200 mesh

277 copper grid of lace carbon film and semi-automatically vitrified using a Vitrobot ™

278 (FEI, OR, USA). Vitrified samples were maintained at liquid nitrogen temperatures

279 during sample transfer and imaged with a Tecnai F20 G2 (FEI, OR, USA) at 120 keV.

280

Cell culture and mRNA transfection

282 HEK-293T cell was obtained from ATCC (Manasass, VA, USA). Cell was cultured in

283 Dulbecco's modified Eagle media (DMEM) (Corning, NY, USA) containing 1x

284 penicillin/streptomycin (Thermo Scientific, MA, USA) and 10 % fetal bovine serum

285 (FBS) (Hyclone, Marlborough, IL, USA) in a humidified atmosphere containing 5%

286 CO_2 at 37 °C. Transfection of CoV2-F004 mRNA was performed using Lipofectamin

287 messengerMax RNA (Invitrogen, thermo Scientific, MA, USA) in accordance with the

288 manufacturer's instructions. EG-COVID 001, 002, 003 and 004 (10 ug mRNA

289 containg complex each) were introduced into 293T cells by 18 hour incubation in

290 OPTi-MEM (Thermo Scientific, MA, USA).

291

Western blotting

293 Cells were washed twice in phosphate-buffered saline (PBS) (WELGENE, Kyunggi-

294   do, Korea) and lysed in lysis buffer (Cell signaling, Danvers, MA, USA) with protease

295   inhibitor (Cell signaling, Danvers, MA, USA). Lysates were cleared by centrifugation

296   and supernatants were obtained. Equal amounts of proteins were resolved on 8%

297   Bis-Tris page gel (Invitrogen, Thermo Scientific, Waltham, MA, USA). Proteins were

298   resolved eletro-trasferred onto polyvinylidene fluoride (PVDF) membranes (GE

299   Healthcare, Chicago, IL, USA). The membranes incubated with the appropriate

300   antibody in Tris buffered with Tween-20 (TTBS). The primary and secondary

301   antibodies were SARS-CoV-2-S1 (Novus, Centennial, CO, USA), beta-actin (Cell

302   signaling, Danvers, MA, USA) and anti-rabbit (Invitrogen, Thermo Scientific,

303   Waltham, MA, USA).

304

305   ***Bioluminescence imaging***

306   For bioluminescence imaging studies, 6-week-old female Balb/c nude or C57BL/6N

307   mice (SLC, Japan) were used. The mice received *Renilla* luciferase mRNA-liposome

308   (or lipid nanoparticle) complex intramuscularly. 6 hours after the administration, mice

309   were anesthetized with intraperitoneal injection of Avertin working solution (Sigma

310   Aldrich, MO, USA), then, 0.15 mg/mL ViviRen luciferase substrate (Promega, WI,

311   USA) were injected intravenously as 1mg/kg. The bioluminescence imaging was

312   obtained with Ami HTX (Spectral Instruments Imaging, AZ, USA) immediately after

313   the injection of substrate. All images were taken under 60 seconds of exposure time

314   and analyzed with Aura software (Spectral Instruments Imaging, AZ, USA). The

315   regions of interest (ROIs, photons/s) were manually selected based on the signal

316   intensity.

317

318   ***Immunization of mice***

319   For immunogenicity studies, 6-week-old female B6C3F1 mice (SLC, Japan) were

320   randomly assigned into experimental groups (n = 6). The Mice were immunized

321   intramuscularly two doses at 3-week intervals with an EG-COVID (L-EG-COVID, F-

322   EG-COVID). Two weeks after the final immunization, blood was obtained by cardiac

323   puncture and spleen were isolated from the immunized mice. Sera were obtained by

324   centrifugation at 3,000 RPM for 5 minutes at room temperature and kept at -70°C

325   deep freezer. After extracting the spleen from the immunized mice, the spleens of

326   each group were pooled and washed in PBS (T&I, Gangwon-do, Korea) with 1%

327   Penicillin/Streptomycin (Gibco, MA, USA). A 70 μm cell strainer (VWR life science,

328   PA, USA) was used to homogenize the tissue. Homogenized tissues were

329   transferred to a 15 mL tube (SPL, Gyeonggi-do, Korea), followed by centrifugation at

330   4° C, 3,000 rpm for 5 minutes, and then the supernatant was removed. The cells

331   were suspended in 3 mL of RBC lysis buffer (Biolegend, CA, USA), allowed to stand

332   at room temperature for 3 minutes, and then centrifuged at 4° C, 3,000 rpm for 5

333   minutes, and then the supernatant was removed. Cells were suspended by adding 3

334   mL of PBS with 1% Penicillin/Streptomycin, followed by centrifugation at 4° C, 3,000

335   rpm for 5 minutes to wash the cells. Discard the supernatant and RPMI1640 (Gibco,

336   MA, USA) [10% FBS (Hyclone, MA, USA), 100 μ/mL penicillin (Gibco, MA, USA),

337   100 μg/mL streptomycin (Gibco, MA, USA), 0.5 ng/mL recombinant mouse IL-2

338   (R&D system, MN, USA), and 50 μM β-mercaptoethanol (Sigma Aldrich, MO, USA)]

339   was added at 2 x 10^7 cells/mL.

340

341   ***Enzyme-linked Immunosorbent Assay (ELISA)***

342   Antigen-specific antibody titer was determined by end-point dilution ELISA. A 96 well

343   immunoplate (Thermo Fisher, MA, USA) was coated with 100 ng/well of SARS-CoV-

344   2 RBD protein (mybiosource, CA, USA) for 16 hours at 4℃. The plates were blocked

345   with 1% bovine serum albumin (MP Biomedicals, CA, USA) in phosphate buffered

346   saline (PBS) for 1hr at 37 ℃, and incubated with sera that was serial diluted with 1%

347   bovine serum albumin in PBS for 1 hour at 37℃. Bound antibodies were detected

348   with HRP-conjugated goat anti-mouse total IgG antibody (Jackson, PA, USA) diluted

349   1:5000 for 1 hour at 37℃. The color reaction was developed by adding KPL sureblue

350   TMB (3,3',5,5'-tetramethylbenzidine) microwell peroxidase substrate (Seracare, MA,

351   USA) and 1N Sulfuric acid (Daejung, Busan, Korea) was added to stop the reaction.

352   Absorbance was measured on an Epoch Microplate Spectrophotometer (Biotek, VT,

353   USA) at 450 nm (Ryu, et al., 2016).

354

355   ***In vitro re-stimulation and cytokine analysis***

356   Splenocytes were resuspended in RPMI1640 (Gibco, MA, USA) supplemented with

357   10% FBS (Hyclone, MA, USA), 100 u/mL penicillin (Gibco, MA, USA),100 µg/mL

358   streptomycin (Gibco, MA, USA), 0.5 ng/mL recombinant mouse IL-2(R&D system,

359   MN, USA), and 50 µM β-mercaptoethanol (Sigma Aldrich, MO, USA). Cells were

360   seeded at 2 x 10^6 cells/well in a 96 well culture plate (SPL, Gyeonggi-do, Korea) and

361   stimulated with two pools of SARS-CoV-2 spike peptide (JPT, Germany) or media.

362   Each peptide pool (JPT1, S1 peptide pool; JPT2, S2 peptide pool) was used at 1

363   µg/mL (Corbett, et al, 2020). After 72 hours of incubation at 37℃, culture media were

364   collected for assay. IFN- γ secreted from splenocytes were measured using mouse

365   IFN-γ Duoset ELISA kit (R&D system, MN, USA).

366 ***Surrogate Virus Neutralization Test (sVNT)***

367 The surrogate virus neutralization test (sVNT) assay was performed using the

368 SARS-CoV-2 surrogate virus neutralization test kit (GenScript, NJ, USA). Briefly,

369 Sera diluted(1:10) with sample dilution buffer were mixed with HRP-conjugated RBD

370 solution at a 1:1 volume, and incubated for 30 min at 37℃. The mixture were added

371 into the plate pre-coated with hACE2 receptor, and incubated for 15 min at 37℃.

372 Absorbance was measured on an Epoch Microplate Spectrophotometer (Biotek, VT,

373 USA) at 450 nm. The percentage of inhibition the interaction of RBD and hACE2 was

374 calculated as neutralization (%) = (1-OD value of sera/OD value of negative control)

375 x 100 % (Tan, et al, 2020).

376

377 ***Plaque reduction neutralization test (PRNT)***

378 PRNT was performed at Clinical Research Center of Masan National Tuberculosis

379 Hospital, an affiliate of Korea Disease Control and Prevention Agenecy (KDCA). All

380 the procedures were conducted in the biosafety level 3 (BL-3) laboratory.

381 Vero cells were seeded at 8 x 10^5 cells/well in 6 well plates and cultured

382 overnight. After 2-fold dilution of the serum from 1:10 to 1:10, 240, the diluted serum

383 was mixed 1:1 with 400 Plaque forming unit (PFU)/100 μL of SARS-CoV2 viruses

384 and incubated at 37°C for 1 hour. Vero cells were washed with PBS, and then a

385 serum-virus mixture was added. After incubation for 90 minutes to allow viral

386 infection, the culture medium was removed and washed with PBS. Then, DMEM

387 (Gibco, MA, USA) medium containing 2% FBS and 1.5% Agarose was added,

388 solidified at room temperature for 20 minutes, and cultured at 37° C for 72 hours.

389 Then, after 4% formalin was added and fixed at room temperature for 1 hour, the

390   medium was removed, and the cells were stained with 0.4% crystal violet in 20%

391   methanol solution. After washing three times with distilled water, the plate was

392   completely dried. Plaque number was visually counted and the IC_{50} titer was

393   calculated.

394

Discussion

Even in the current situation, we are experiencing the profound health threat of COVID-19 to humanity on a daily basis, hearing news of new infections and unfortunate victims of COVID-19 caused by SARS-CoV-2.

Although several types of COVID-19 vaccines have been developed and used, it is worth noting these three types of vaccines: mRNA vaccine (Moderna, Pfizer-BioNTech, and Curevac), adenovirus vaccine (Oxford-AstraZeneca and Johnson & Johnson) and recombinant protein vaccine (Novavax), and the governments of each country are actively carrying out the vaccination. The current supply of the COVID-19 vaccine is limited compared to demand, and as new variants such as strains B.1.1.7, P.1, B.1.427 / B.1.429 and B.1.351 (Wu et al., 2021) continue to emerge, the effectiveness of existing vaccines being developed against the Wuhan strain of COVID-19 is questioned. Therefore, it is necessary to develop new vaccines to meet the demand for vaccines and ensure efficient vaccines against numerous variants of COVID-19.

Since the mRNA vaccine can be developed in a relatively short time compared to inactivated or recombinant protein vaccines, we initiated the development of an mRNA vaccine against COVID-19 to respond to the urgent demand for vaccines and the future needs of vaccines against emerging variants. In this report, we generated an mRNA vaccine candidate coded for a full-length spike glycoprotein, EG-COVID, European mutant strain (D614G) with 2P-3Q substitution (Bangaru et al., 2020). When we initiated the development of the COVID-19 vaccine, European strains started to emerge and became prevalent, so we predicted that it would be appropriate to develop using the European strain rather than the Wuhan variant. Furthermore, the 2P-3Q substitution is expected to show better efficacy in our study

420 using artificial intelligence analysis and vaccine development research. Currently

421 available vaccines are mostly developed based on the Wuhan strain and all of them

422 have been reported to be less effective against the South African variants (Wu et al.,

423 2021). Our vaccine was developed based on a European variant to which the 2P-3Q

424 substitution was applied and is expected to demonstrate the efficacy against the

425 South African variant. Interestingly, Novavax's vaccine targeting the same European

426 variant (D614G) with 2P-3Q substitution as our EG-COVID, showed 95.6%

427 effectiveness against the original variants and also for the newer variant (85.6% for

428 the UK variant B.1.1.7 and 60% for the South Africa variant B.1.351) (Mahase, 2021).

429 With this in mind, we anticipated that EG-COVID would have a similar effect against

430 the original variant and new variants as the Novavax's vaccine.

431 The human immune system has an effective defense system when attacked by

432 viruses or bacteria. In this sense, IFN-γ plays an important role in the recognition and

433 elimination of pathogens (Kak et al, 2018). We demonstrated that immunization with

434 EG-COVID in mice induced anti-RBD (Receptor Binding Domain) antibodies and

435 SARS-CoV-2 neutralizing antibodies with two doses. Also, we found that EG-COVID

436 induced cellular immune response. Therefore, we concluded that EG-COVID

437 successfully induced virus specific adaptive immune response, which could play an

438 effective antiviral role against SARS-CoV-2. Spike protein and RBDs are the primary

439 targets of neutralizing antibodies because they prevent the virus from binding to

440 airway epithelial cells through invasion of the ACE 2 receptor (Cox et al., 2020). We

441 demonstrated EG-COVID generated neutralizing antibodies against recombinant

442 RBD protein. Since there was an approach whereby neutralizing antibodies directed

443 primarily to RBD of spike protein could be applied as a complementary option to treat

444 severe forms of COVID-19 (Assadiasl et al., 2020), the neutralizing antibody titer

445   induced by EG-COVID could be an important indicator of an effective vaccine

446   against SARS-CoV-2 infection.

447   All currently available mRNA COVID-19 vaccines are supplied in liquid form

448   and requires special cold chain for storage and transportation of the vaccines. This is

449   a factor that increases the cost of supplying the vaccines and at the same time

450   makes it difficult to distribute the vaccines. This results in the low accessibility of

451   mRNA vaccines, especially for developing countries. To overcome this, it is

452   necessary to prepare the mRNA vaccine in a form that can be stored at room

453   temperature or refrigerated at 4°C, and lyophilization is essential for this purpose. As

454   we observed in our study, EG-COVID is being developed in a form that can be

455   lyophilized and the success of our vaccine will promote equitable access to mRNA

456   vaccines worldwide, as well as establish a system that can deliver vaccines to

457   everyone who needs it in a timely manner.

458   In conclusion, EG-COVID is a potent and safe vaccine against SARS-CoV-2

459   that can be stored for a prolonged time in a conventional refrigerator.

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564   **Figures**
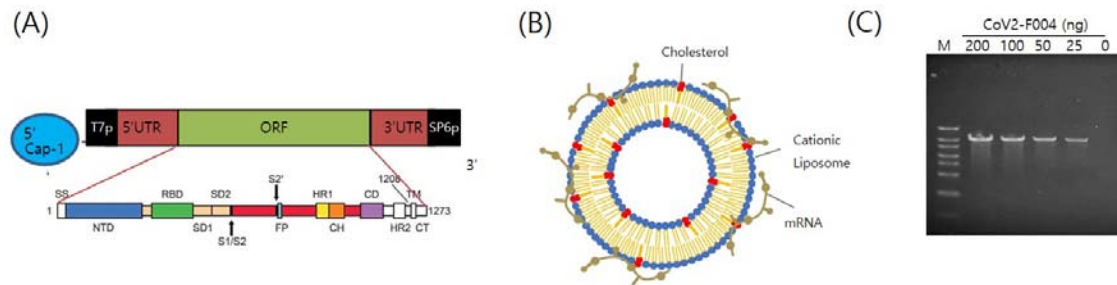
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567   *Figure 1. The structure of EG-COVID vaccine (A) A schematic illustration of*

568   *antigen coding mRNA Cov2-F004 structure. (B) Schematic illustration of the*

569   *structure of EG-COVID. (C) The functionality of CoV2-F004 Structure. In vitro*

570   *transcription of CoV2-F004 showed the increase of transcript according to CoV2-*

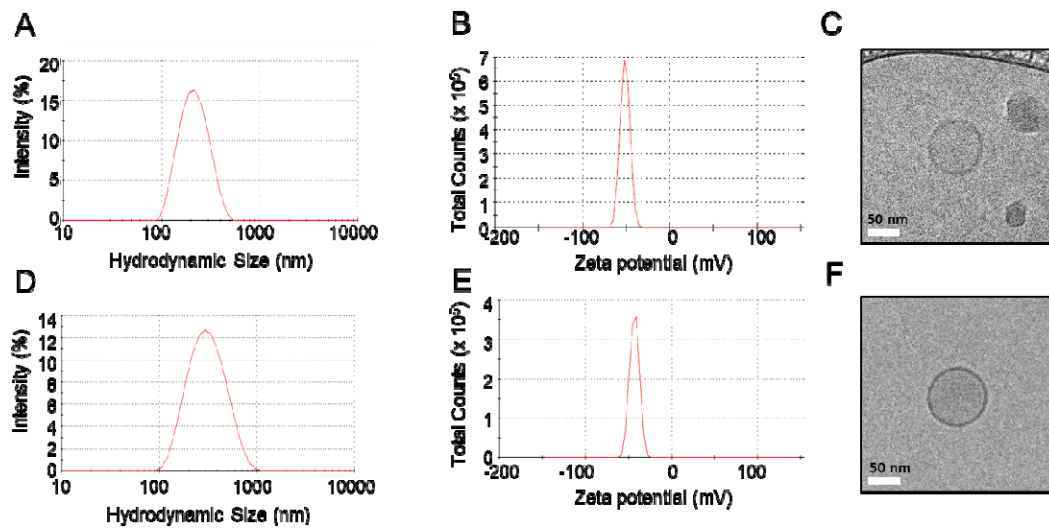571   *F004 amount used in the transcription. M; size marker.*

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Fig 2. Physical characteristics of the EG-COVID. *Representative DLS size distribution (A), zeta-potential (B), and Cryo-TEM image (C) of EG-COVID; Representative DLS size distribution (D), zeta-potential (E), and Cryo-TEM image (F) of F-EG-COVID. Scale bar = 50 nm.*
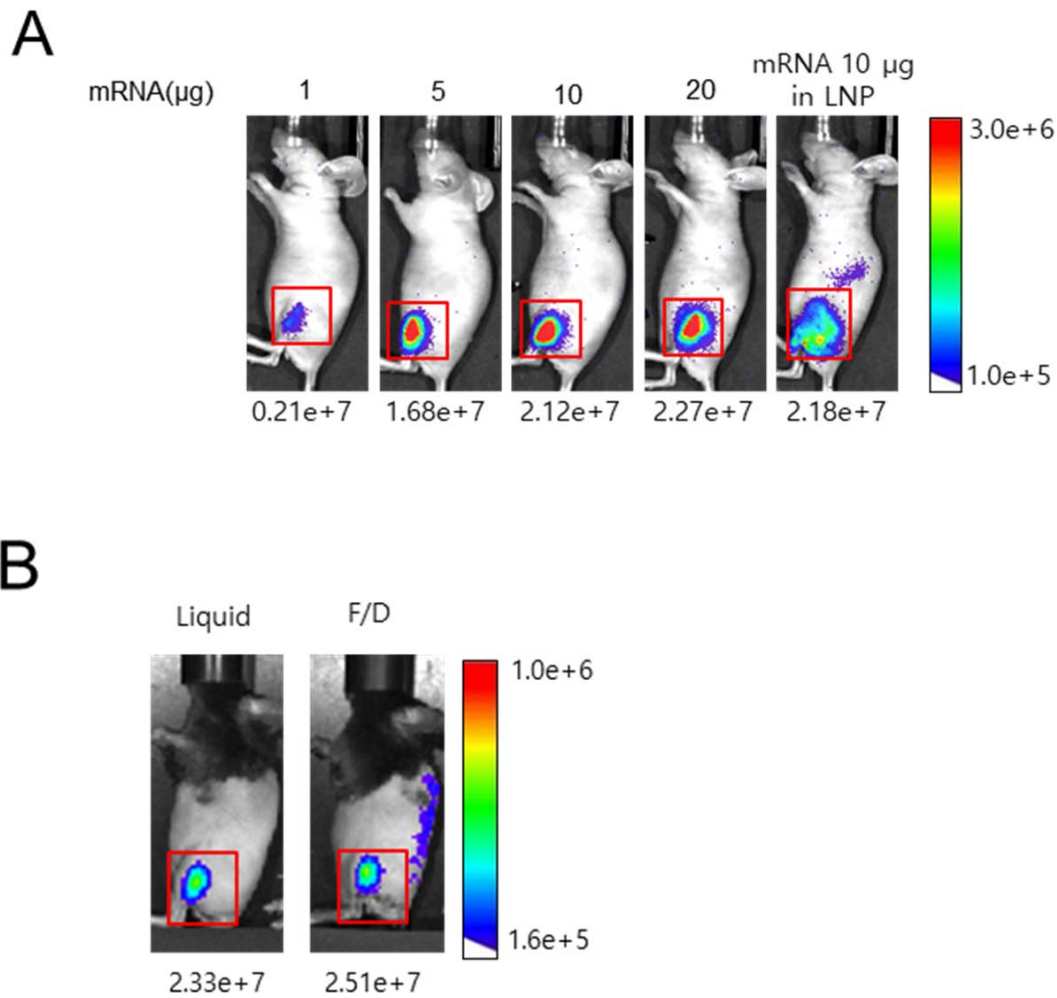
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Figure 3. Cationic Liposomes as Efficient mRNA Carriers. *(A) 1, 5, 10, and 20 μg of Renilla luciferase mRNAs were complexed with cationic liposomes and intramuscularly injected into mice and bioluminescence was measured for 60 seconds after administration of Renilla luciferase substrate via tail vein. 10 μg of Renilla luciferase mRNA complexed in Lipid nano particle was used as a control (B) Liquid and lyophilized (F/D) forms of Renilla luciferase mRNA-cationic liposomes were injected and bioluminescence was measured. 10 μg of Renilla luciferase mRNA was used in each form.*
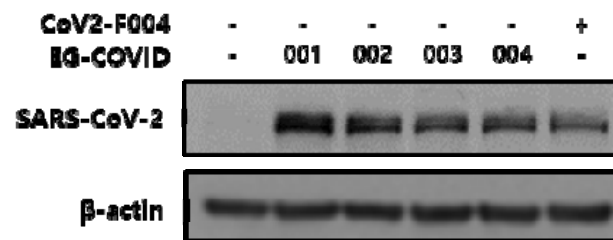
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Figure 4. Expression of SARS-CoV-2 antigen by EG-COVID. *Four EG-COVID formulations generated with different lipid compositions were introduced into 293T cells and the expression of CoV2-F004 was confirmed by western blot using antibody against SARS-CoV2 protein. All EG-COVID formulations were re-hydrated from lyophilized forms. CoV2-F004 mRNA alone was introduced to 293T cells using Lipofectamine messengerMax RNA kit and used as a control.*
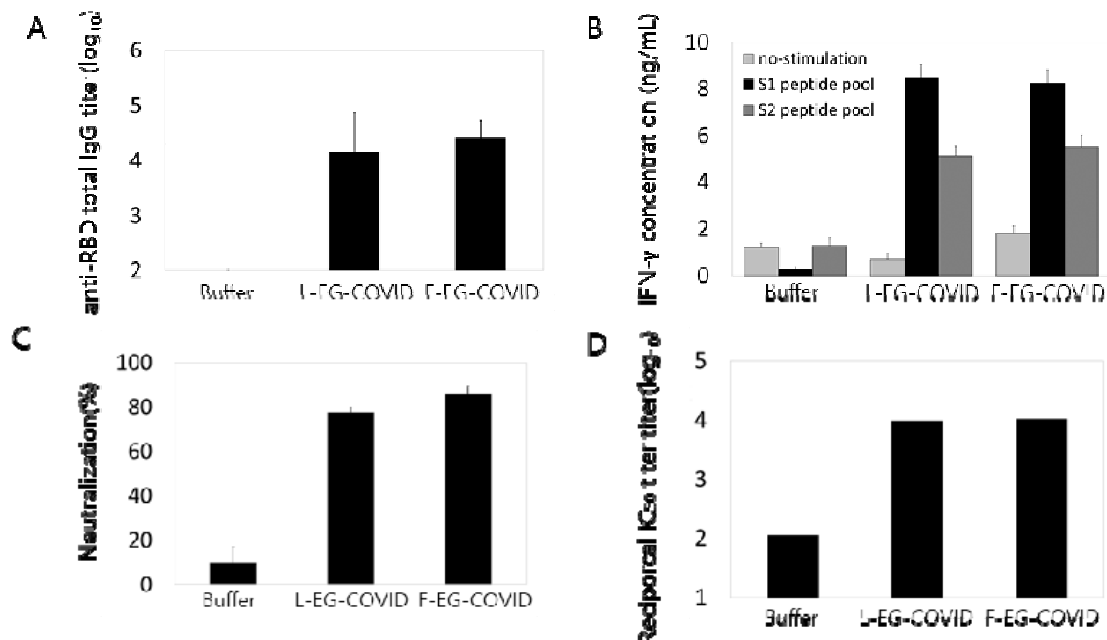
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Figure 5. Immunogenicity of EG-COVID and neutralizing antibody reaction induced by EG-COVID immunization. *(A) Anti-RBD-specific total IgG antibody titers in sera were measured by an end-point dilution ELISA method. (B) Amount of IFN-γ secreted into the culture medium from stimulated splenocytes were measured by sandwich ELISA method after 72 hours. (C) sVNT (surrogate virus neutralization test) was used to assess the neutralizing ability of the RBD protein (p <0.001, compared with the buffer by Student's t test). (D) Viral neutralizing activity of immunized sera was measured by PRNT (plaque reduction neutralization test) and IC$_{50}$ was calculated.*