Title: An mRNA vaccine against SARS-CoV-2: Lyophilized, liposome-based vaccine candidate EG-COVID induces high levels of virus neutralizing antibodies

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Abstract

In addition to the traditional method of vaccine development, the mRNA coronavirus vaccine, which is attractive as a challenging vaccination, recently opened a new era in vaccinology. Here we describe the EG-COVID which is a novel liposome-based mRNA candidate vaccine that encodes the spike (S) protein of SARS-CoV-2 with 2P-3Q substitution in European variant. We developed the mRNA vaccine platform that can be lyophilized using liposome-based technology. Intramuscular injection of the EG-COVID elicited robust humoral and cellular immune response to SARS-CoV-2. Furthermore, sera obtained from mice successfully inhibited SARS-CoV-2 viral infection into Vero cells. We developed EG-COVID and found it to be effective based on *in vitro* data, and we plan to initiate a clinical trial soon. Since EG-COVID is a lyophilized mRNA vaccine that is convenient for transportation and storage, accessibility to vaccines will be significantly improved.

Introduction

COVID-19 is caused by the coronavirus, which is an enveloped RNA virus that is widely distributed in humans, other mammals, and birds and causes respiratory, intestinal, liver, and nerve diseases. (Weiss et al., 2011). In addition to the very common coronavirus responsible for the human common cold, there are three coronaviruses that cause serious illness in humans: SARS-CoV, MERS-CoV, and SARS-CoV-2 (Cox et al., 2020). In December 2019, an outbreak of severe acute respiratory syndrome coronavirus 2 (now namely, SARS-CoV-2) infection occurred at seafood market in Wuhan, Hubei Province, China, and spread across China (Zhu et al., 2020). Considering the rapid spread of this virus and its impact on an international scale, COVID-19 was declared a pandemic by the World Health Organization on March 11, 2020 (WHO,1 DEC 2020). The urgent development of safe and effective vaccines in the global coronavirus pandemic has become a common goal for humanity, where numerous multinational pharmaceutical companies and biotechnology companies are immersed in the development of COVID-19 vaccines around the world simultaneously.

The mRNA-based vaccines are in the limelight because of their potential for rapid development than the conventional vaccines at the COVID-19 pandemics. The history of mRNA-based vaccines is relatively short, and its first application was introduced in the 1990s, using mice to intramuscularly inject mRNA to locally produce an encoded reporter protein (Wolff et a.,1990). The mRNA vaccine combines the benefits of the subunit vaccine and the live attenuated vaccine, and there is no risk associated with the live attenuated vaccine or the DNA vaccine (Reichmuth et al., 2016). Of all the viral antigens studied, the spike (S) and nucleocapsid (N) proteins are considered to be important immunogenic antigens for SARS-CoV-2 (Assadiasl,

70 2020). Among these, most vaccine candidates use the coronavirus glycosylated spike
71 protein (S) as the primary antigenic target.

72 Liposomes are defined as phospholipid vesicles consisting of one or more
73 concentric lipid bilayers enclosing discrete aqueous spaces and its use as
74 pharmaceutical applications are well known (Ickenstein et al., 2019, Torchilin, 2005,
75 Sercombe et al., 2015). In 1970, liposomes were proposed as a drug carrier for altering
76 the therapeutic index of a drug by reducing toxicity or increasing the efficacy of the
77 parent drug (Lian et al., 2001). Vartak and colleagues suggested the use of liposome
78 as antigen or immunomodulatory molecules carrier (Vartak et al., 2016). Furthermore,
79 cationic liposome is considered as an effective adjuvant for vaccine delivery as well
80 as enhancing both cellular and humoral immune reactions (Mai et al., 2020). As a
81 reference, Herpes zoster vaccines are reported to use neutral or cationic liposome as
82 antigen delivery systems (Lal et al., 2015, Cunningham et al., 2016, Wui et al, 2019,
83 and 2021). Cationic liposomes have many important safety advantages, especially
84 when making viral vectors, are less expensive, have no restrictions on the size of
85 nucleic acids, and are easily accessible without special experience in their use and
86 handling (Karmali et al., 2007). Since the cationic liposome is positively charged, it is
87 able to hold nucleic acids such as mRNA that is negatively charged and deliver mRNAs
88 to cells and tissues efficiently. When prepared under proper conditions, these
89 lipoplexes (lipid-nucleic acid complex) retain their full positive charge, allowing them
90 to efficiently bind to negatively charged cell membranes, enter the cells, protects
91 nucleic acids from nucleases in the serum or cytoplasm (Karmail et al., 2007).

92 By the way, all currently developed mRNA-based vaccines employ the lipid
93 nanoparticles (LNP) as their delivery vehicles for mRNA. LNPs are composed of

ionizable lipid, helper lipid, cholesterol, and PEG-lipid (Kim, et al., 2021). Although LNPs are very efficient tools for delivery of mRNA into human body, all the LNP based vaccines are distributed in liquid form only, and require special cold chain system to maintain their efficacy.

In this report, we established cationic liposome based mRNA delivery system and developed a lyophilizable SARS-CoV2 candidate vaccine (EG-COVID) with mRNA encoding the full spike glycoprotein (S) of the European mutant strain (D614G) with 2P-3Q substitution (Bangaru et al., 2020). We found that EG-COVID could elicit robust humoral and cellular immune response to SARS-CoV-2, furthermore, sera obtained from mice successfully inhibited SARS-CoV-2 viral infection into Vero cells. So here we want to describe the immunogenicity and neutralizing antibody activity of the EG-COVID.

Author's contributions

1. Conception or design of the work: Y.J. C., S.H.K., K.S.K., H.C.H.
2. Data collection: Y.U.H., M.J.C., S.A.P.
3. Data analysis and interpretation: Y.U.H., M.J.C., S.A.P., S.W.C.
4. Writing the article: H.C.H., S.H-K.
5. Critical revision of the article: Y.J. C., S.H-K.
6. Funding acquisition: Y.J.C
7. Final approval of the version to be published: All authors reviewed and approved the final article.

Competing interests

The authors declare no conflict of interest.

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Results

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

It was published that the substitution of K986P and K987P (2P) increase stability of the protein, and the substitution of Prolines to Glutamate in S1/S2 polybasic cleavage site (3Q) confers the protease resistance (Corbett et al., 2020, Torchilin, 2005). Based on these findings, we used the mRNA sequence encoding the SARS-CoV-2 Spike protein with 2P-3Q substitutions in European variant (D614G) and named CoV2-F004. The translated Spike protein from CoV2-F004 was predicted to be superior to Wuhan variant (614D) in structural stability and antigen presentation ability through AI-modeling simulation.

In vitro transcription of CoV2-F004 mRNA

CoV2-F004 mRNA was produced by TriLink biotechnologies with Cleancap® technology to increase translation efficiency. 5-Methoxyuridine was used to reduce immune response of cells against mRNA. Produced mRNA demonstrated an expected size of single band in agarose gel electrophoresis (Figure 1C), demonstrating the functionality of CoV-2-F004 structure.

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).

In vitro Expression of EG-COVID

We have generated a complex with cationic liposomes as a delivery system of Cov2-F004 naming them as EG-COVID and verified the ability to deliver Cov2-F004 mRNA

and expression of SARS-CoV2 spike protein *in vitro* (Figure 4). Re-hydrated lyophilized form of EG-COVID 001, 002, 003 and 004, which were prepared differently in their lipid composition, were treated in the culture medium of 293T cells, and expression of SARS-Cov2 protein was confirmed by western blot after 24-hour incubation. It was confirmed that there was some difference in the expression of protein depending on the lipid composition of EG-COVID.

Immunogenicity of EG-COVID

Female B6C3F1/slc mice were immunized by intramuscular administration of L-EG-COVID and F-EG-COVID twice at 3-week interval. After 2 weeks of last administration, mice were sacrificed to evaluate immunogenicity of EG-COVID. We used both liquid (L-EG-COVID) and lyophilized (F-EG-COVID) form of EG-COVID in the experiment.

Humoral immunity was confirmed by anti-RBG antibody titer, and cellular immunity was confirmed by the amount of IFN-γ secreted from splenocytes stimulated with S1 and S2 peptides. We found that both liquid and lyophilized form of EG-COVID induced humoral and cellular immunity successfully (Figure 5A and 5B).

We also verified that the antibody formed by EG-COVID actually has neutralizing ability against SARS-CoV2 virus. The result obtained using surrogate virus neutralization tests which assess RBD binding ability of antibodies showed that EG-COVID immunization successfully generated RBD binding antibodies in serum. Furthermore, EG-COVID immunized serum significantly inhibited plaque formation induced by Wuhan variant of SARS-CoV2 virus infection into VERO cells. There was no difference in EG-COVID immunization ability between liquid and lyophilized formulations (Figure 5C and 5D).

Therefore, we can conclude that EG-COVID induced a sufficient level of humoral and cellular immunity against SARS-CoV-2 virus and is a first-class candidate for mRNA vaccine that can be easily lyophilized.

215 **Materials and Methods**

216

217 ***Lipids and chemicals***

218 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and Cholesterol were
219 purchased from Merck Millipore (Darmstadt, Germany) or Avanti Polar lipid (AL, USA).
220 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,1'-((2-(4-(2-((2-(bis(2-
221 hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-
222 yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200) were from Corden Pharma (Plankstadt,
223 Germany). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-
224 rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were obtained from
225 Avanti Polar Lipid (AL, USA). Hydroxyethyl piperazine Ethane Sulfonic acid (HEPES)
226 solution was purchased from Sigma Aldrich (MO, USA). Sucrose was obtained from
227 Merck Millipore (Darmstadt, Germany). 1 M Sodium acetate solution (pH 4.6) were
228 from T&I (Gangwon-do, Korea).

229

230 ***mRNAs***

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

233

234 ***Preparation of EG-COVID and mRNA-encapsulated LNP***

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

240 solution containing the DOTAP: DOPE: Cholesterol mixture was dried in a round
241 bottom flask using a rotary evaporator (IKA, Germany) to form a thin-lipid film. The
242 lipid film was rehydrated with a nuclease free- 4% (w/v) sucrose solution (Sigma
243 Aldrich, MO, USA) in 20 mM HEPES (pH7.4; Sigma Aldrich, MO, USA) and
244 homogenized using a microfluidizer (Avestin, ON, Canada). The mRNA CoV2-F004
245 were coated to the cationic liposome. The EG-COVID was lyophilized using a freeze-
246 dryer (Lyoph-Pride, Ilshin BioBase, Gyeonggi-do, Korea).
247 LNP were prepared using microfluidic mixer Ignite™ (Precision NanoSystems,
248 Vancouver, BC, Canada) as previously described by Sabnis and colleagues (Sabnis.
249 et al. 2018) In brief, 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-
250 hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-
251 200; Coden Pharma, Germany), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC;
252 Avanti Polar Lipid, AL, USA), Cholesterol (Merck Millipore, Germany) and 1,2-
253 dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000; Avanti
254 Polar Lipid, AL, USA) were resolved in ethanol at molar ratios of 50:10:38.5:1.5. The
255 lipid mixture was obtained with 25 mM sodium acetate buffer (pH 3; T&I, Gangwon-do,
256 Korea) containing *Renilla* luciferase mRNA (TriLink, CA, USA) at a N/P ratio of 5.6.
257 using microfluidic mixer Ignite™. Formulations were dialyzed against nuclease free-
258 4% (w/v) sucrose solution in 20 mM HEPES (pH7.4). The EG-COVID and mRNA-
259 encapsulated LNP were stored at 4℃ and used within a week.

260

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

262 The particle size and polydispersity index (PDI) of liposomes were determined by
263 dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments,

264 Worcestershire, UK). Malvern Zetasizer DTS software (version 7.12) was used for
265 data acquisition and analysis. All measurements were carried out in triplicate.
266
267 ***Morphology of EG-COVID***
268 The cryogenic transmission electron microscopy (Cryo-TEM) was used for
269 determination of morphology of EG-COVID. Liquid form of EG-COVID or lyophilized
270 EG-COVID powder was rehydrated with deionized water were used for Cryo-TEM
271 analysis. Three microliters of the liposome preparation were placed on a 200 mesh
272 copper grid of lace carbon film and semi-automatically vitrified using a Vitrobot ™ (FEI,
273 OR, USA). Vitrified samples were maintained at liquid nitrogen temperatures during
274 sample transfer and imaged with a Tecnai F20 G2 (FEI, OR, USA) at 120 keV.
275
276 ***Cell culture and mRNA transfection***
277 HEK-293T cell was obtained from ATCC (Manasass, VA, USA). Cell was cultured in
278 Dulbecco's modified Eagle media (DMEM) (Corning, NY, USA) containing 1x
279 penicillin/streptomycin (Thermo Scientific, MA, USA) and 10 % fetal bovine serum
280 (FBS) (Hyclone, Marlborough, IL, USA) in a humidified atmosphere containing 5%
281 CO2 at 37 °C. Transfection of CoV2-F004 mRNA was performed using Lipofectamin
282 messengerMax RNA (Invitrogen, thermo Scientific, MA, USA) in accordance with the
283 manufacturer's instructions. EG-COVID 001, 002, 003 and 004 (10 ug mRNA containg
284 complex each) were introduced into 293T cells by 18 hour incubation in OPTi-MEM
285 (Thermo Scientific, MA, USA).
286
287 ***Western blotting***
288 Cells were washed twice in phosphate-buffered saline (PBS) (WELGENE, Kyunggi-

289 do, Korea) and lysed in lysis buffer (Cell signaling, Danvers, MA, USA) with protease
290 inhibitor (Cell signaling, Danvers, MA, USA). Lysates were cleared by centrifugation
291 and supernatants were obtained. Equal amounts of proteins were resolved on 8% Bis-
292 Tris page gel (Invitrogen, Thermo Scientific, Waltham, MA, USA). Proteins were
293 resolved eletro-trasferred onto polyvinylidene fluoride (PVDF) membranes (GE
294 Healthcare, Chicago, IL, USA). The membranes incubated with the appropriate
295 antibody in Tris buffered with Tween-20 (TTBS). The primary and secondary
296 antibodies were SARS-CoV-2-S1 (Novus, Centennial, CO, USA), beta-actin (Cell
297 signaling, Danvers, MA, USA) and anti-rabbit (Invitrogen, Thermo Scientific, Waltham,
298 MA, USA).
299
300 ***Bioluminescence imaging***
301 For bioluminescence imaging studies, 6-week-old female Balb/c nude or C57BL/6N
302 mice (SLC, Japan) were used. The mice received *Renilla* luciferase mRNA-liposome
303 (or lipid nanoparticle) complex intramuscularly. 6 hours after the administration, mice
304 were anesthetized with intraperitoneal injection of Avertin working solution (Sigma
305 Aldrich, MO, USA), then, 0.15 mg/mL ViviRen luciferase substrate (Promega, WI, USA)
306 were injected intravenously as 1mg/kg. The bioluminescence imaging was obtained
307 with Ami HTX (Spectral Instruments Imaging, AZ, USA) immediately after the injection
308 of substrate. All images were taken under 60 seconds of exposure time and analyzed
309 with Aura software (Spectral Instruments Imaging, AZ, USA). The regions of interest
310 (ROIs, photons/s) were manually selected based on the signal intensity.
311
312 ***Immunization of mice***
313 For immunogenicity studies, 6-week-old female B6C3F1 mice (SLC, Japan) were

314 randomly assigned into experimental groups (n = 6). The Mice were immunized
315 intramuscularly two doses at 3-week intervals with an EG-COVID (L-EG-COVID, F-
316 EG-COVID). Two weeks after the final immunization, blood was obtained by cardiac
317 puncture and spleen were isolated from the immunized mice. Sera were obtained by
318 centrifugation at 3,000 RPM for 5 minutes at room temperature and kept at -70°C deep
319 freezer. After extracting the spleen from the immunized mice, the spleens of each
320 group were pooled and washed in PBS (T&I, Gangwon-do, Korea) with 1%
321 Penicillin/Streptomycin (Gibco, MA, USA). A 70 μm cell strainer (VWR life science, PA,
322 USA) was used to homogenize the tissue. Homogenized tissues were transferred to
323 a 15 mL tube (SPL, Gyeonggi-do, Korea), followed by centrifugation at 4° C, 3,000
324 rpm for 5 minutes, and then the supernatant was removed. The cells were suspended
325 in 3 mL of RBC lysis buffer (Biolegend, CA, USA), allowed to stand at room
326 temperature for 3 minutes, and then centrifuged at 4° C, 3,000 rpm for 5 minutes, and
327 then the supernatant was removed. Cells were suspended by adding 3 mL of PBS with
328 1% Penicillin/Streptomycin, followed by centrifugation at 4° C, 3,000 rpm for 5 minutes
329 to wash the cells. Discard the supernatant and RPMI1640 (Gibco, MA, USA) [10%
330 FBS (Hyclone, MA, USA), 100 μ/mL penicillin (Gibco, MA, USA), 100 μg/mL
331 streptomycin (Gibco, MA, USA), 0.5 ng/mL recombinant mouse IL-2 (R&D system, MN,
332 USA), and 50 μM β-mercaptoethanol (Sigma Aldrich, MO, USA)] was added at 2 x 10^7
333 cells/mL.
334
335 ***Enzyme-linked Immunosorbent Assay (ELISA)***
336 Antigen-specific antibody titer was determined by end-point dilution ELISA. A 96 well
337 immunoplate (Thermo Fisher, MA, USA) was coated with 100 ng/well of SARS-CoV-2

338 RBD protein (mybiosource, CA, USA) for 16 hours at 4℃. The plates were blocked
339 with 1% bovine serum albumin (MP Biomedicals, CA, USA) in phosphate buffered
340 saline (PBS) for 1hr at 37 ℃, and incubated with sera that was serial diluted with 1%
341 bovine serum albumin in PBS for 1 hour at 37℃. Bound antibodies were detected with
342 HRP-conjugated goat anti-mouse total IgG antibody (Jackson, PA, USA) diluted
343 1:5000 for 1 hour at 37℃. The color reaction was developed by adding KPL sureblue
344 TMB (3,3',5,5'-tetramethylbenzidine) microwell peroxidase substrate (Seracare, MA,
345 USA) and 1N Sulfuric acid (Daejung, Busan, Korea) was added to stop the reaction.
346 Absorbance was measured on an Epoch Microplate Spectrophotometer (Biotek, VT,
347 USA) at 450 nm (Ryu, et al., 2016).
348
349 ***In vitro re-stimulation and cytokine analysis***
350 Splenocytes were resuspended in RPMI1640 (Gibco, MA, USA) supplemented with
351 10% FBS (Hyclone, MA, USA), 100 u/mL penicillin (Gibco, MA, USA),100 μg/mL
352 streptomycin (Gibco, MA, USA), 0.5 ng/mL recombinant mouse IL-2(R&D system, MN,
353 USA), and 50 μM β-mercaptoethanol (Sigma Aldrich, MO, USA). Cells were seeded
354 at 2 x 10^6 cells/well in a 96 well culture plate (SPL, Gyeonggi-do, Korea) and stimulated
355 with two pools of SARS-CoV-2 spike peptide (JPT, Germany) or media. Each peptide
356 pool (JPT1, S1 peptide pool; JPT2, S2 peptide pool) was used at 1 μg/mL (Corbett, et
357 al, 2020). After 72 hours of incubation at 37℃, culture media were collected for assay.
358 IFN- γ secreted from splenocytes were measured using mouse IFN-γ Duoset ELISA
359 kit (R&D system, MN, USA).

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

361 The surrogate virus neutralization test (sVNT) assay was performed using the SARS-
362 CoV-2 surrogate virus neutralization test kit (GenScript, NJ, USA). Briefly, Sera
363 diluted(1:10) with sample dilution buffer were mixed with HRP-conjugated RBD
364 solution at a 1:1 volume, and incubated for 30 min at 37℃. The mixture were added
365 into the plate pre-coated with hACE2 receptor, and incubated for 15 min at 37℃.
366 Absorbance was measured on an Epoch Microplate Spectrophotometer (Biotek, VT,
367 USA) at 450 nm. The percentage of inhibition the interaction of RBD and hACE2 was
368 calculated as neutralization (%) = (1-OD value of sera/OD value of negative control) x
369 100 % (Tan, et al, 2020).

370

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

372 PRNT was performed at Clinical Research Center of Masan National Tuberculosis
373 Hospital, an affiliate of Korea Disease Control and Prevention Agenecy (KDCA). All
374 the procedures were conducted in the biosafety level 3 (BL-3) laboratory.

375 Vero cells were seeded at 8 x 10^5 cells/well in 6 well plates and cultured overnight.
376 After 2-fold dilution of the serum from 1:10 to 1:10, 240, the diluted serum was mixed
377 1:1 with 400 Plaque forming unit (PFU)/100 μL of SARS-CoV2 viruses and incubated
378 at 37°C for 1 hour. Vero cells were washed with PBS, and then a serum-virus mixture
379 was added. After incubation for 90 minutes to allow viral infection, the culture medium
380 was removed and washed with PBS. Then, DMEM (Gibco, MA, USA) medium
381 containing 2% FBS and 1.5% Agarose was added, solidified at room temperature for
382 20 minutes, and cultured at 37° C for 72 hours. Then, after 4% formalin was added

383 and fixed at room temperature for 1 hour, the medium was removed, and the cells
384 were stained with 0.4% crystal violet in 20% methanol solution. After washing three
385 times with distilled water, the plate was completely dried. Plaque number was visually
386 counted and the IC_{50} titer was calculated.
387

388 **Discussion**

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

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

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

413 analysis and vaccine development research. Currently available vaccines are mostly
414 developed based on the Wuhan strain and all of them have been reported to be less
415 effective against the South African variants (Wu et al., 2021). Our vaccine was
416 developed based on a European variant to which the 2P-3Q substitution was applied
417 and is expected to demonstrate the efficacy against the South African variant.
418 Interestingly, Novavax's vaccine targeting the same European variant (D614G) with
419 2P-3Q substitution as our EG-COVID, showed 95.6% effectiveness against the
420 original variants and also for the newer variant (85.6% for the UK variant B.1.1.7 and
421 60% for the South Africa variant B.1.351) (Mahase, 2021). With this in mind, we
422 anticipated that EG-COVID would have a similar effect against the original variant and
423 new variants as the Novavax's vaccine.

424 The human immune system has an effective defense system when attacked by
425 viruses or bacteria. In this sense, IFN-γ plays an important role in the recognition and
426 elimination of pathogens (Kak et al, 2018). We demonstrated that immunization with
427 EG-COVID in mice induced anti-RBD (Receptor Binding Domain) antibodies and
428 SARS-CoV-2 neutralizing antibodies with two doses. Also, we found that EG-COVID
429 induced cellular immune response. Therefore, we concluded that EG-COVID
430 successfully induced virus specific adaptive immune response, which could play an
431 effective antiviral role against SARS-CoV-2. Spike protein and RBDs are the primary
432 targets of neutralizing antibodies because they prevent the virus from binding to airway
433 epithelial cells through invasion of the ACE 2 receptor (Cox et al., 2020). We
434 demonstrated EG-COVID generated neutralizing antibodies against recombinant RBD
435 protein. Since there was an approach whereby neutralizing antibodies directed
436 primarily to RBD of spike protein could be applied as a complementary option to treat
437 severe forms of COVID-19 (Assadiasl et al., 2020), the neutralizing antibody titer

438 induced by EG-COVID could be an important indicator of an effective vaccine against
439 SARS-CoV-2 infection.

440 All currently available mRNA COVID-19 vaccines are supplied in liquid form and
441 requires special cold chain for storage and transportation of the vaccines. This is a
442 factor that increases the cost of supplying the vaccines and at the same time makes it
443 difficult to distribute the vaccines. This results in the low accessibility of mRNA
444 vaccines, especially for developing countries. To overcome this, it is necessary to
445 prepare the mRNA vaccine in a form that can be stored at room temperature or
446 refrigerated at 4°C, and lyophilization is essential for this purpose. As we observed in
447 our study, EG-COVID is being developed in a form that can be lyophilized and the
448 success of our vaccine will promote equitable access to mRNA vaccines worldwide,
449 as well as establish a system that can deliver vaccines to everyone who needs it in a
450 timely manner.

451 In conclusion, EG-COVID is a potent and safe vaccine against SARS-CoV-2 that
452 can be stored for a prolonged time in a conventional refrigerator.

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556 **Figures**

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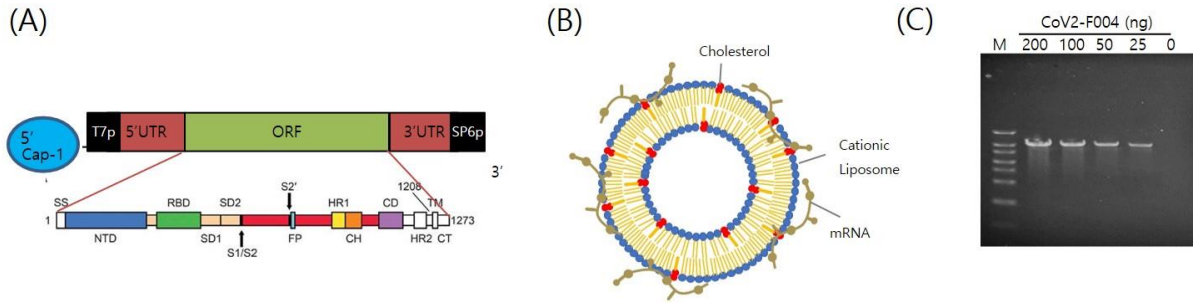

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559 ***Figure 1. The structure of EG-COVID vaccine*** *(A) A schematic illustration of*

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

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

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

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

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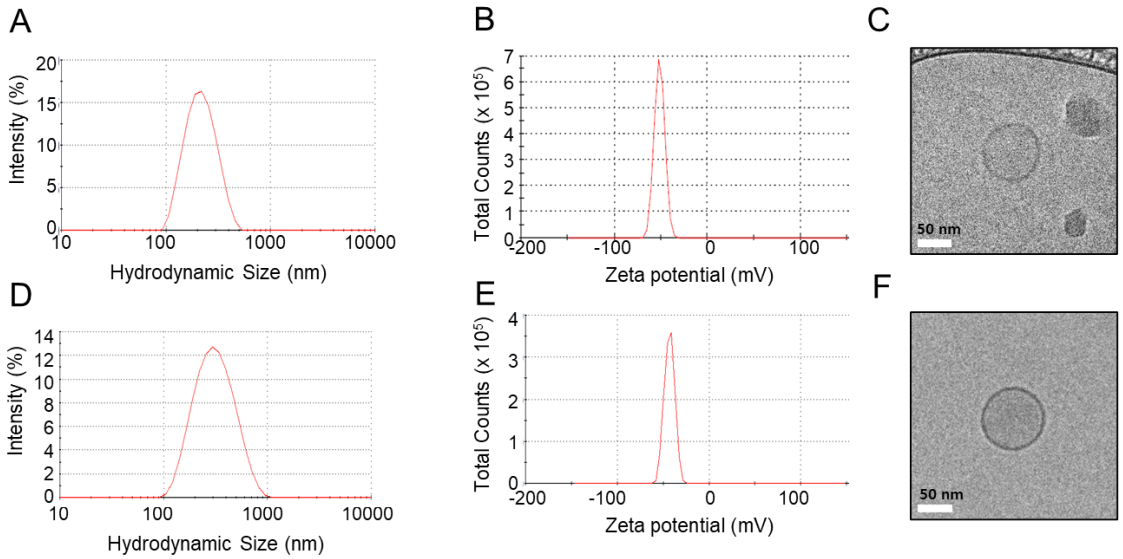
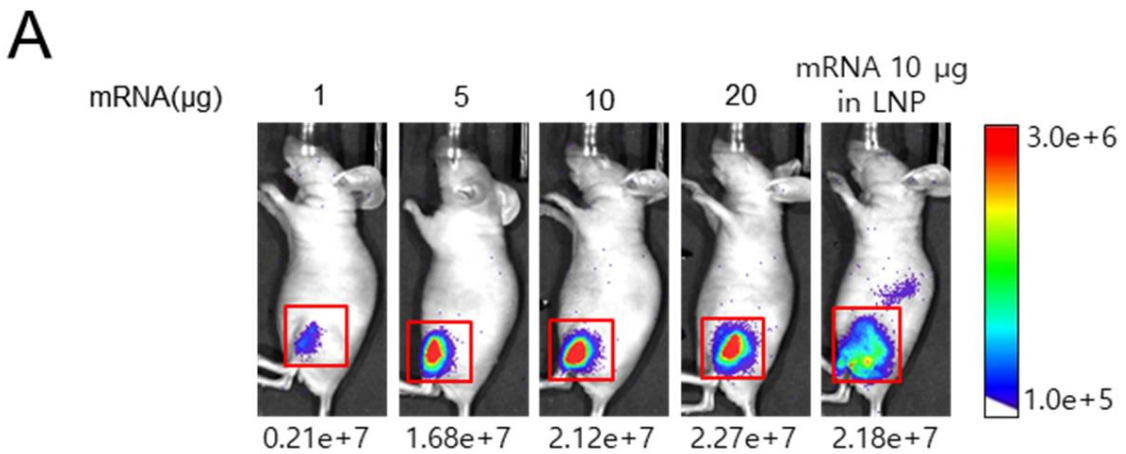


Fig 2. Physical characteristics of the EG-COVID. *Representative DLS size distribution (A), zeta-potential (B), and Cryo-TEM image (C) of EG-COVID; Representative DLS size distribution (D), zeta-potential (E), and Cryo-TEM image (F) of F-EG-COVID. Scale bar = 50 nm.*



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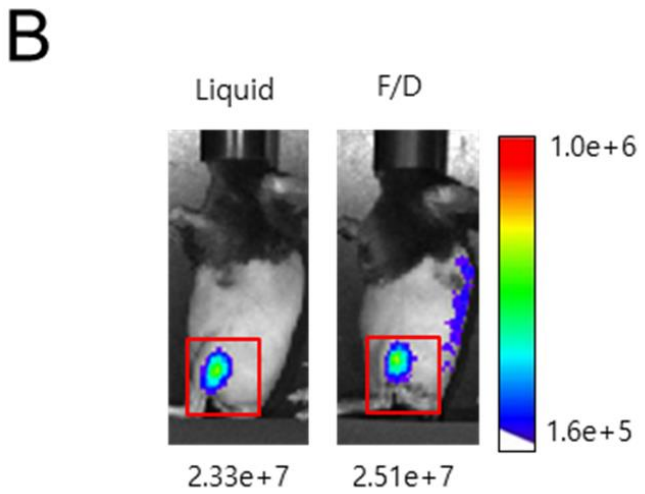

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586 ***Figure 3. Cationic Liposomes as Efficient mRNA Carriers.*** *(A) 1, 5, 10, and 20 μg of*
587 *Renilla luciferase mRNAs were complexed with cationic liposomes and intramuscularly*
588 *injected into mice and bioluminescence was measured for 60 seconds after administration*
589 *of Renilla luciferase substrate via tail vein. 10 μg of Renilla luciferase mRNA complexed*
590 *in Lipid nano particle was used as a control (B) Liquid and lyophilized (F/D) forms of Renilla*
591 *luciferase mRNA-cationic liposomes were injected and bioluminescence was measured.*
592 *10 μg of Renilla luciferase mRNA was used in each form.*

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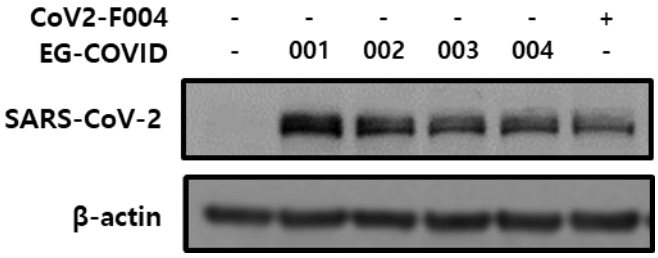
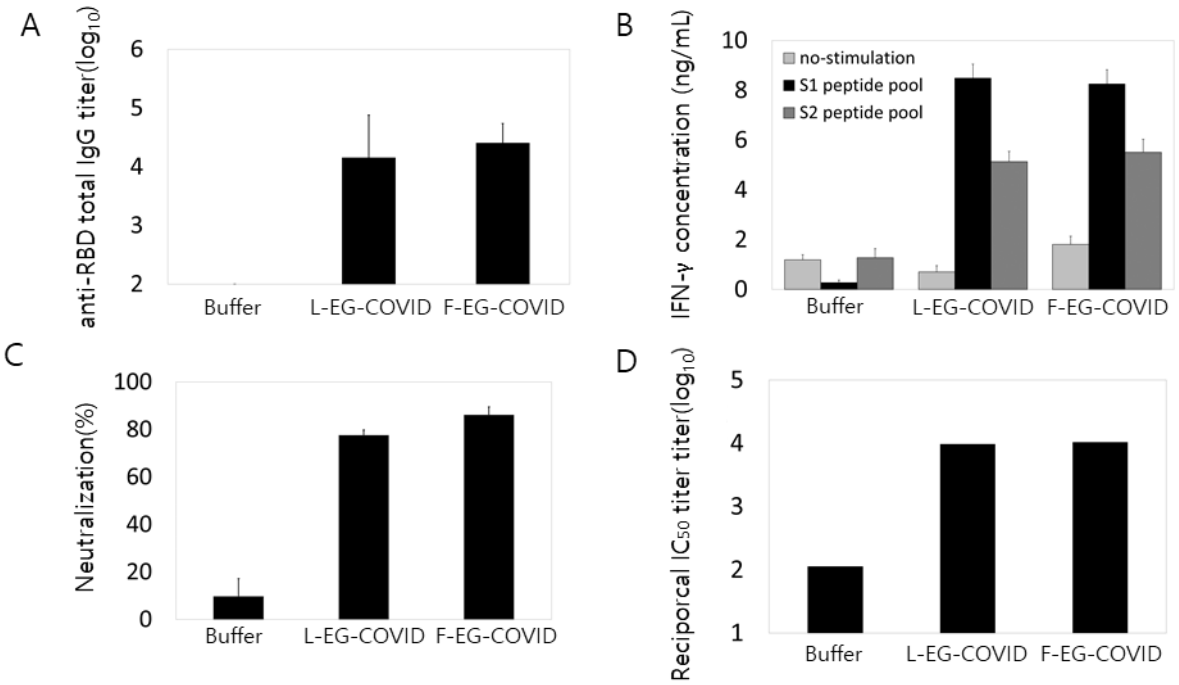


Figure 4. Expression of SARS-CoV-2 antigen by EG-COVID*. Four EG-COVID formulations generated with different lipid compositions were introduced into 293T cells and the expression of CoV2-F004 was confirmed by western blot using antibody against SARS-CoV2 protein. All EG-COVID formulations were re-hydrated 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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608 ***Figure 5. Immunogenicity of EG-COVID and neutralizing antibody reaction***
609 ***induced by EG-COVID immunization.*** *(A) Anti-RBD-specific total IgG antibody titers in*
610 *sera were measured by an end-point dilution ELISA method. (B) Amount of IFN-γ secreted*
611 *into the culture medium from stimulated splenocytes were measured by sandwich ELISA*
612 *method after 72 hours. (C) sVNT (surrogate virus neutralization test) was used to assess*
613 *the neutralizing ability of the RBD protein ($p < 0.001$, compared with the buffer by Student's*
614 *t test). (D) Viral neutralizing activity of immunized sera was measured by PRNT (plaque*
615 *reduction neutralization test) and IC_{50} was calculated.*

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