Supporting information

Antiviral effects of pyrroloquinoline quinone through redox catalysis to prevent coronavirus infection

Nur Syafiqah Mohamad Ishak*, Tomoe Numaguchi and Kazuto Ikemoto*.

Niigata Research Laboratory, Mitsubishi Gas Chemical Company, Inc., 182, Tayuhama, Kita-ku, Niigata City, Niigata, Japan 950-3112.

*Corresponding author: E-mail: syafiqah@mgc.co.jp (NSMI), kazuto-ikemoto@mgc.co.jp (KI)

Materials

Pyrroloquinoline quinone (BioPQQ) was manufactured by Mitsubishi Gas Chemical Co. Inc. Ascorbic acid, glutamic acid, formaldehyde solution (35%) and glutaraldehyde solution (25%) were purchased from FujiFilm Wako Chemical Co. Inc. Reduced form PQQ (RPQQ) was produced by PQQ and VC reaction[1,2]. Imidazolopyrroloquinoline (IPQ) was produced by PQQ and glycine reaction under aerobic conditions[3]. PQQ trimethylester (PQQ-TME) was synthesized by methylation of PQQ using dimethyl sulphate[4]. Viruses were precipitated using polyethylene glycol 6000 (Tokyo Chemical Industry Co. Ltd.). Other chemical reagents were purchased from FujiFilm Wako Chemical Co.

Plaque assay

The viruses were quantified using the plaque assay with carboxymethylcellulose (CMC) liquid overlay. In a 24-well plate containing pre-cultured confluent monolayer CRKF cells (2×10^5 cells/well), viruses were added, in a dilution series, to wells and incubated for 1 h at 37 °C. Then, overlay medium was added to each well and incubated for 2 days at 37 °C and 5% CO_2 incubator. To quantify the forming plaques on cell culture, the overlay medium was discarded, the cells were fixed with 4% formaldehyde for 1 h and stained with 0.5% crystal violet for 15 min. The viruses were quantified as plaque forming units per mL (PFU/mL), as shown in Figure S1.

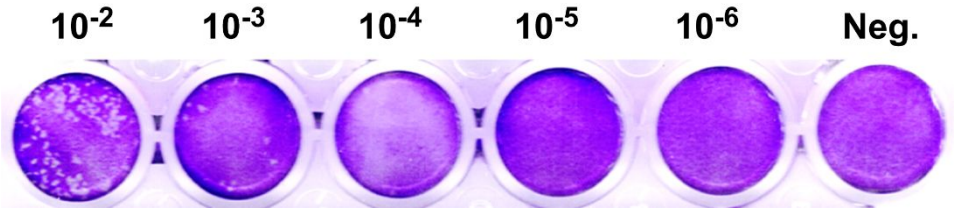


Figure S1: Plaque assay method for infective virus quantification as plaque forming units (PFU).

Cytotoxicity assay

To evaluate PQQ cytotoxicity in CRFK cell culture, the cells were pre-cultured with 100 µL EMEM culture media in a 96-well plate (1×10^4 cells/well) for 24 h. The culture medium was replaced with fresh medium and PQQ (0.25–250 µM) was added to the media in designated wells. After 24 h, cytotoxicity assays were performed using Cell-counting kit-8 (Dojindo Lab), according to the manufacturer's protocol. The concentration of 50% cytotoxicity (CC_{50}) was calculated, and a cell viability (%) graph was generated using an

online tool: "Quest Graph™ IC50 Calculator." *AAT Bioquest, Inc.*, 13 Jan. 2023, https://www.aatbio.com/tools/ic50-calculator. The CC_{50} of PQQ was 44.8 μM (Figure S2), and PQQ concentrations ≤25 μM did not exhibit significant cytotoxic effects, with less than 10% cell death.

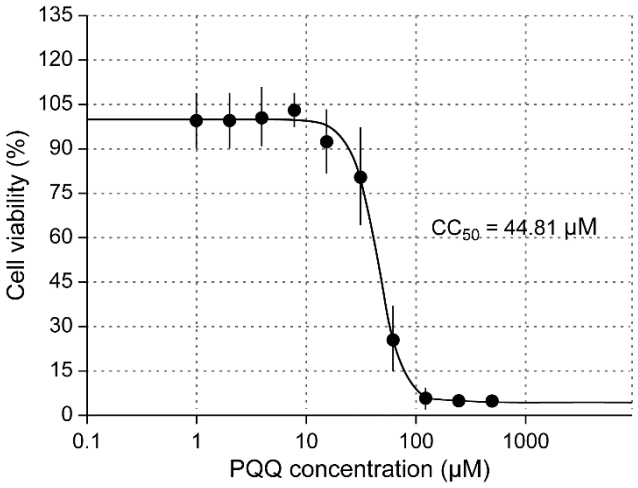


Figure S2: Cytotoxicity test, at different PQQ concentrations, in the CRFK cell line.

Infection inhibition assay (RT-qPCR)

Table S1: Sequences of primer pairs used in this study.

Target	Forward primer	Reverse primer	Ref.
FCoV (Virus genome)	GGCAACCCGATGTTTAAAACTGG	CACTAGATCCAGACGTTAGCTC	[5]
GAPDH (Mammalian cell)	AGTATGATTCCACCCACGGCA	GATCTCGCTCCTGGAAGATGGT	[6]

Viral particle size distribution measurement

To measure viral particle size, virus solution was concentrated as follows. Stock virus (25 mL) from one culture bottle was divided into four centrifugal ultrafiltration filter units (CFU100K) and centrifuged at 300 rpm for 30 min. Total concentrated virus (2 mL) was collected and combined with 18 mL PBS buffer. The virus solution was concentrated to 1.1 mL, distributed into 100 μL samples, and stored at −80˚C until further use. To prepare the

sample mixtures, PQQ stock, VC and Glu were prepared in PBS buffer. In each sample, concentrated virus was diluted 10-fold. PQQ was added to obtain a final concentration of 200 μM, whereas VC and Glu were 5 times the PQQ weight concentration. Treated samples were incubated at 37 °C for 1 h. Next, the samples were diluted 100× with PBS buffer. Particle size distribution and zeta potential were measured using the Horiba Nano Particle analyzer (SZ-100; Horiba, Japan), and the results are shown in Table S2 and Figure S3.

Table S2: Average particle size and zeta potential for each sample mixture.

Sample	(A) Virus only	(B) Virus + PQQ	(C) Virus + PQQ + VC	(D) Virus + PQQ + VC + Glu
Average particle size (nm)	85.4	148.7	178.7	1101.9
Zeta potential (mV)	-0.8	-0.1	0	-1.1

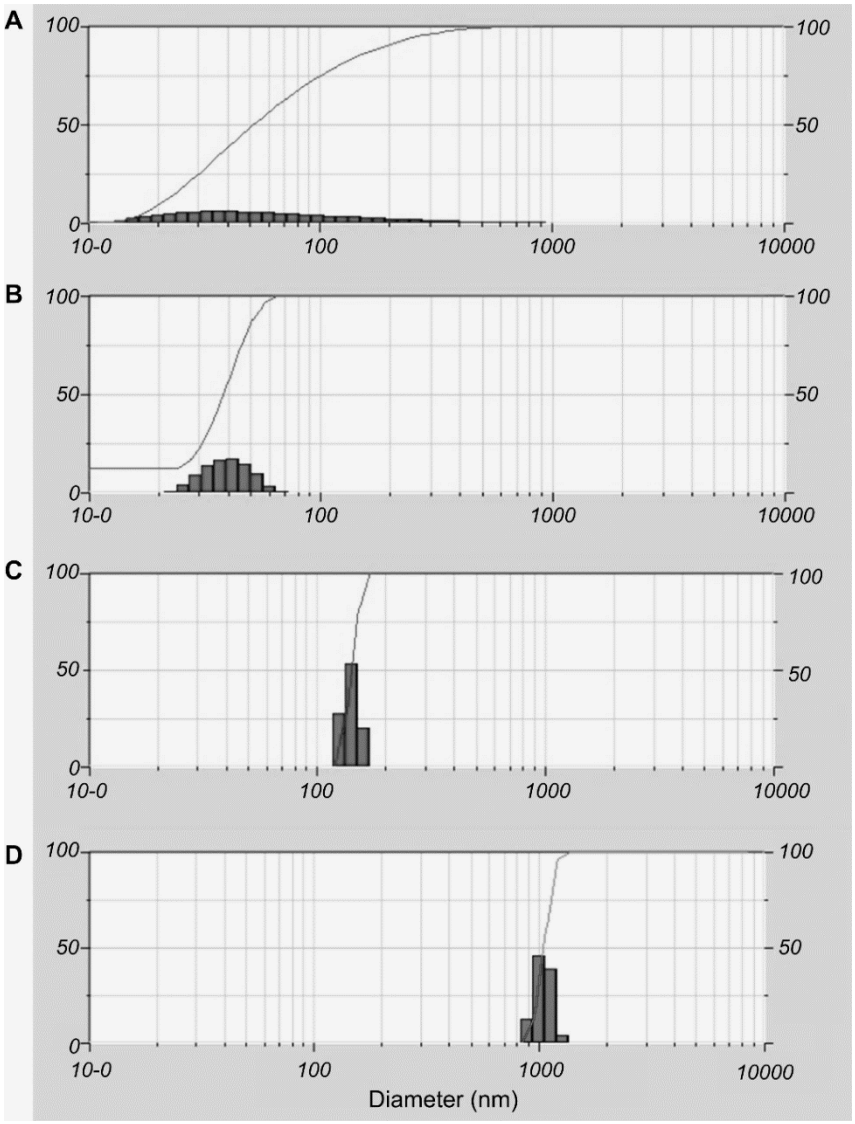


Figure S3: Particle size distributions from samples that contained only virus (a), virus and PQQ (b), virus, PQQ and VC (c) and virus, PQQ, VC and glu (d).

NMR experiment

The combination of PQQ, ascorbic acid, and glutamic acid had a higher inhibitory effect on viral infection than PQQ alone. The change in PQQ in the mixture was examined using NMR. Four peaks of PQQ were detected in water in the aromatic region owing to the equilibrium between the quinone and acetal structures. The difference in chemical shift could be attributed to the difference in counter ions; the mixture of PQQ, ascorbic acid, and glutamic acid has two peaks, indicating that PQQ is reactive. The substance produced by PQQ, ascorbic acid and glutamic acid is reduced PQQ. No peak was derived from glutamic acid (Table S3).

Solvent: Heavy water (pH 7), 100 mM phosphate buffer (Na2HPO4 75 mg, NaH2PO4 60 mg/D2O 10 mL). The spectrum data of the samples listed below are shown in Tables S3.

Sample

1) 0.66 mg PQQ + 6.6 mg VC + 6.6 mg glutamic acid/600 μL Solvent + Sodium trimethylsilyl propionate (TSP)

2) PQQ 0.13 mg/600 μL Solvent +TSP

3) VC13.3 mg/600 μL Solvent +TSP

4) Glu13.3 mg/600 μL Solvent +TSP

DMSO-d6

5) RPQQ

6) Centrifugation of PQQ+ VC+ Glutamic acid precipitate

Table S3: H-NMR (D2O (pH7), TSP) spectrum data

	SAMPLE		**AROMATIC**
1	PQQ+ VC+ Glutamic acid	2.15 m, 2.44 m, 3.77 m 4.03 m, 4.63 m	7.31(1), 8.12(1)
2	PQQ		7.15(1.2), 7.17(1), 8.16(1), 8.26(1.2)
3	VC	3.74 m, 4.03 m, 4.60 m	
4	Glutamic acid	2.15 m, 2.44 m, 3.77 m	
	DMSO-D6 (TMS)		
5	RPQQ		7.37, 8.57
6	PQQ+ VC+ Glutamic acid precipitation		7.25, 8.43

Fluorescence microscopy experiment

For fluorescence staining, duplicate virus stocks (20 mL) were concentrated into 1.2 mL using an ultrafiltration membrane (CFU100K) and centrifugation at 3000rpm for 30 min.

Both concentrated viruses were transferred into ultrafiltration membranes (CFU100K) and washed with 10 mL PBS before centrifugation at 3000 rpm for 30 min. Concentrated viruses were filtered through ultrafiltration membranes (CFU100K), washed with 10 mL PBS, and centrifuged at 3000 rpm for 30 min to obtain a final 40× concentrated virus solution (1 mL).

SYBR Gold (Thermo Fisher Scientific Inc.) (5 μL) was added into 2 mL pure water. Next, 1 mL PBS and 1 mL concentrated virus solution were added into the SYBR Gold solution and incubated for 15 min. Next, the solution was filtered through an ultrafiltration membrane (CFU100K), washed with 10 mL PBS, and centrifuged at 3000 rpm for 15 min. The flow through solution was again filtered through an ultrafiltration membrane (CFU100K), washed with 10 mL PBS, and centrifuged at 3000 rpm for 30 min. PBS was added to the flow through solution (up to 4 mL) to obtain a 10× concentrated stained virus solution. The stained viruses were stored at −80 °C.

We examined the effect of PQQ on viral attachment to the host cells. CRFK cells were pre-seeded (5×10^4 cells/well) onto 24-well plates and cultured at 37 °C for 3 days until confluent. Stained virus was mixed with 0, 50, or 500 μM PQQ and incubated at 37 °C for 1 h. Cells were infected with the virus solutions for 30 min. Following incubation, cells were washed with culture media twice and suspended in 200 μL culture media for microscope observation (Figure S4). The fluorescent images were captured under the GFP filter at 1s exposure time and 17.1 gain. Cell-attached viruses were observed as green fluorescence. As the PQQ concentration increased, the number of viral particles attached to the cells decreased (Figure S5).

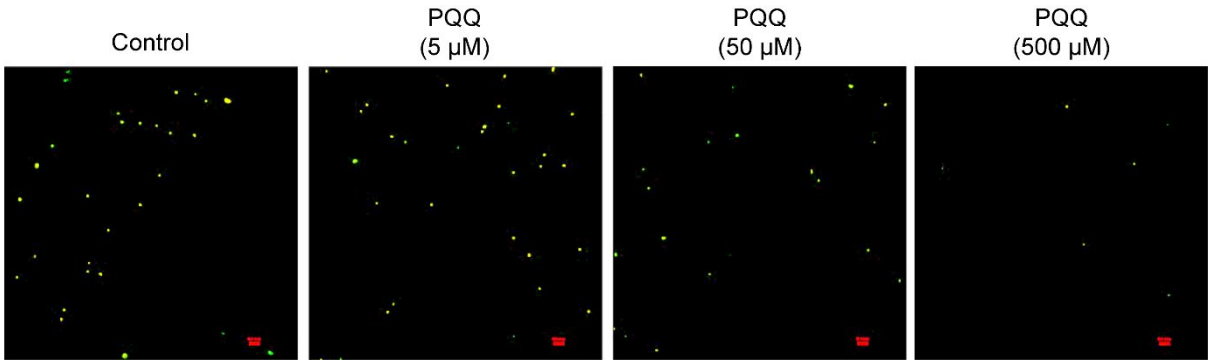


Figure S4: SYBR gold stained-viral particles attached to the host cell were observed under a fluorescence microscope.

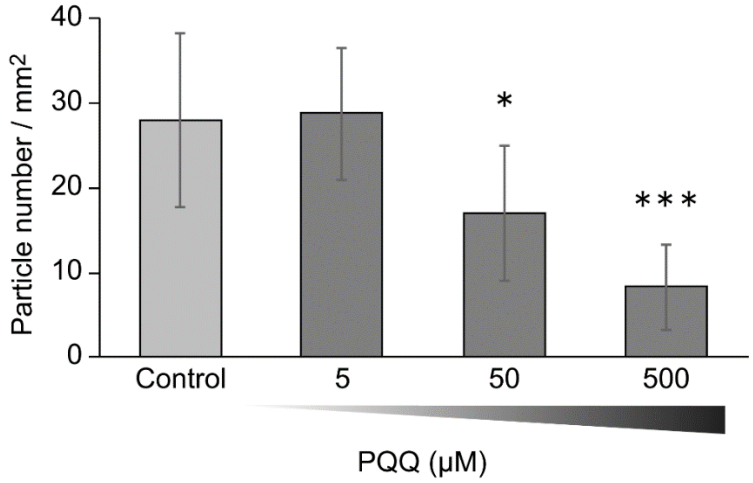


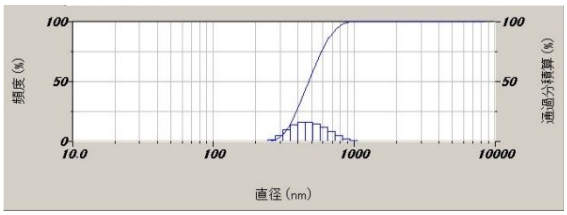
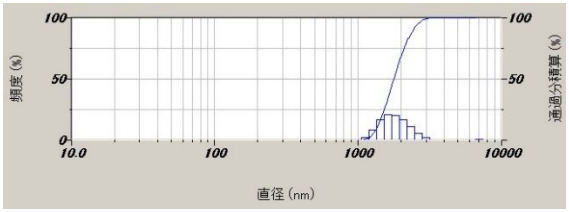
Figure S5: Number of viral particles attached to the host cell in each treatment. Asterisks denote a statistically significant difference (*$p < 0.05$; ***$p < 0.001$) when compared with the control as determined using one-way ANOVA followed by Dunnett's post hoc test.

Sample preparation for TEM, STEM and SDS-PAGE experiments

Virus suspensions were prepared using serum-free medium (LifeFactors; Rionegro, Colombia) and purified with PEG precipitation to obtain a clear imaging result. CRFK cells (8×10^6) were seeded in a 175 cm^2 bottle and incubated for 24 h. Following incubation, all medium was removed and replaced with 25 mL of fresh medium. Virus stock (400 µL) was added. The infected cells were incubated for 3 days at 37 °C and 5% CO_2. The culture was frozen and thawed 3 times using a deep freezer and then filtered through a 0.25 µm filter. The filtrate was concentrated to 200 µL by centrifugation at 3000 rpm for 30 min with two 100KCFU ultrafiltration units. PBS (10 mL) was added to the filtrate and centrifuged at 3000 rpm for 30 min with one 100 KCFU ultrafiltration unit to make 200 µL of concentrated filtrate. To every 1 mL of virus solution, 100 µL of X10 PBS and 200 µL of 40% PEG (Mw6000) were added. This solution was stored in a refrigerator for 24 h. The virus solution was centrifuged at 12,000 rpm for 15 min, the supernatant was removed, and 1 mL 1× PBS buffer was added. Then, PBS (100 µL, 10x) and 200 µL of 40% PEG (Mw6000) were added to the virus solution and stored in a refrigerator for 24 h day. The virus solution was centrifuged at 12,000 rpm for 15 min and divided into 100 µL samples. Virus activity was measured by infected CRFK cell viability, after 24 h, using cell counting kit (Dojindo). PQQ, VC, and Glu were added to the virus solution, and incubated at 37 °C for 1 h. The virus solutions were deactivated by adding 1% glutaraldehyde and 1% formaldehyde into the solution. Then, buffer exchange to PBS buffer was performed using

the vivaspin column (MWCO 3K, Cytiva). A small sample volume was used to determine particle size distribution as shown in Table S4.

Table S4: Average particle size and distribution of size particles for each sample mixture.

Sample	Average of size particle (nm)	Distribution of size particle
Virus control	498.8	
Virus + optimized PQQ	1839.3	

TEM observation and imaging

Additional TEM images of the virus control sample and the virus + optimized PQQ sample are shown in Figure S6 and Figure S7.

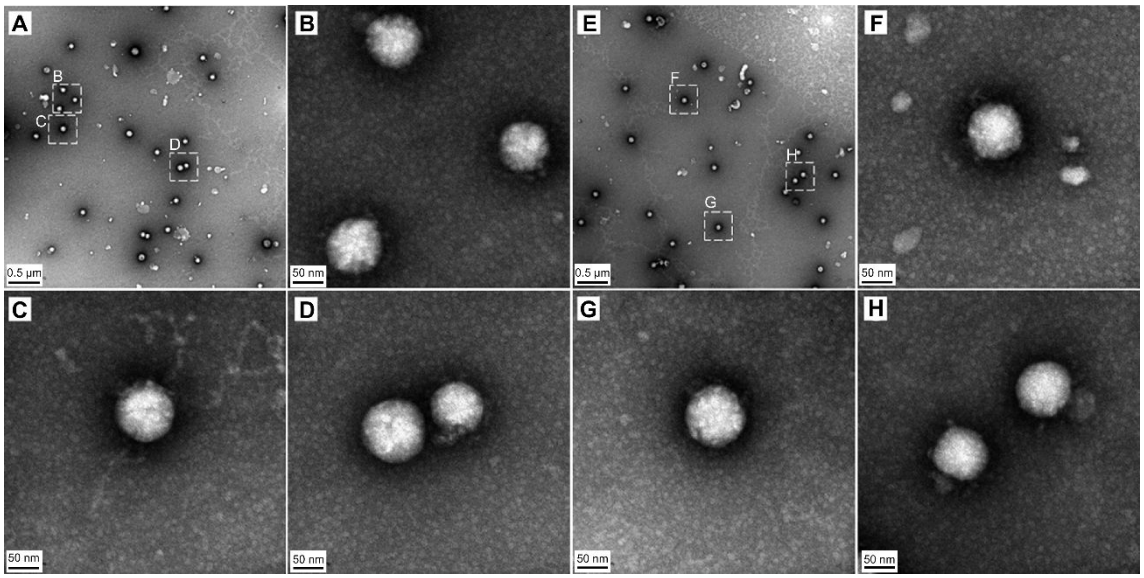


Figure S6: In addition to the images presented in Figure 4d-g, TEM images of the viral control sample from two different fields (a and e) were captured, and zoomed-in regions of each field

were boxed and labeled with b-d and f-h, respectively.

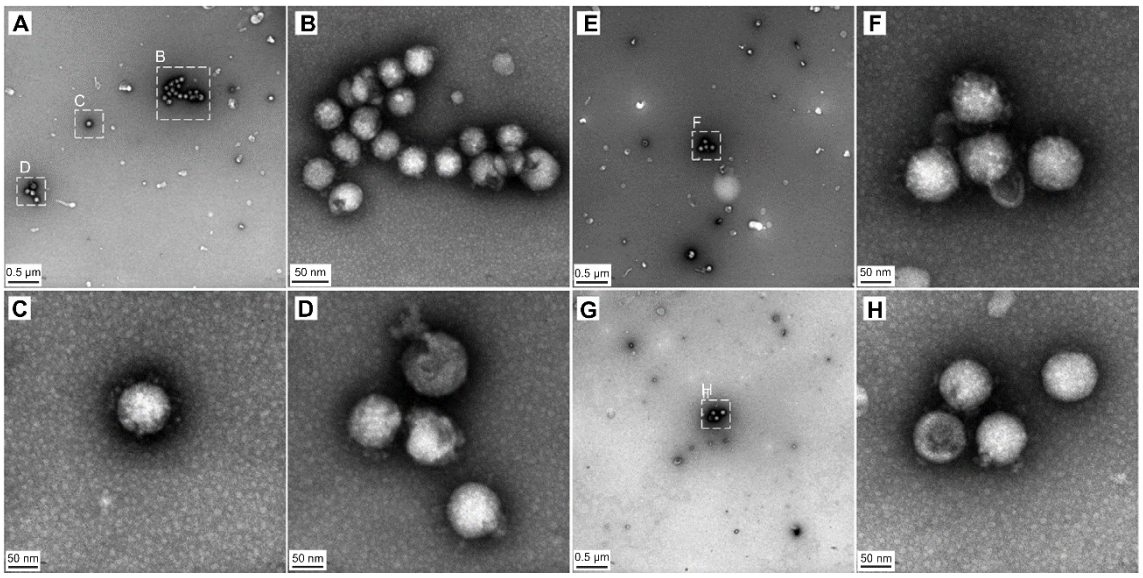


Figure S7: In addition to the images presented in Figure 4h-k, TEM images of the virus + optimized PQQ sample from two different fields (a and e) were captured, and zoomed-in regions of each field were boxed and labeled with b-d and f-h. Viral aggregation (b and f) and damaged viral capsids (d and h) were observed in the optimized PQQ-treated samples.

STEM observation and imaging

For STEM observation, we used the same samples mentioned in TEM experiment. For negative staining, a cooper grid with a weakly hydrophilic plastic support film attached was used and 5 µL of the purified virus sample was deposited onto the grid for 5 mins, washed with distilled water, and negatively stained with 2% phosphotungstic acid solution (pH7.0) for 5 mins. After drying, the samples were observed under a scanning transmission electron microscope (HD-2300; Hitachi, Ltd., Tokyo, Japan) at an accelerating voltage of 200 kV, 120µA with Z-contrast and transmission electron modes. STEM images of the virus control sample and the virus + optimized PQQ sample are shown in Figure S8a-c and Figure S8d-f, respectively.

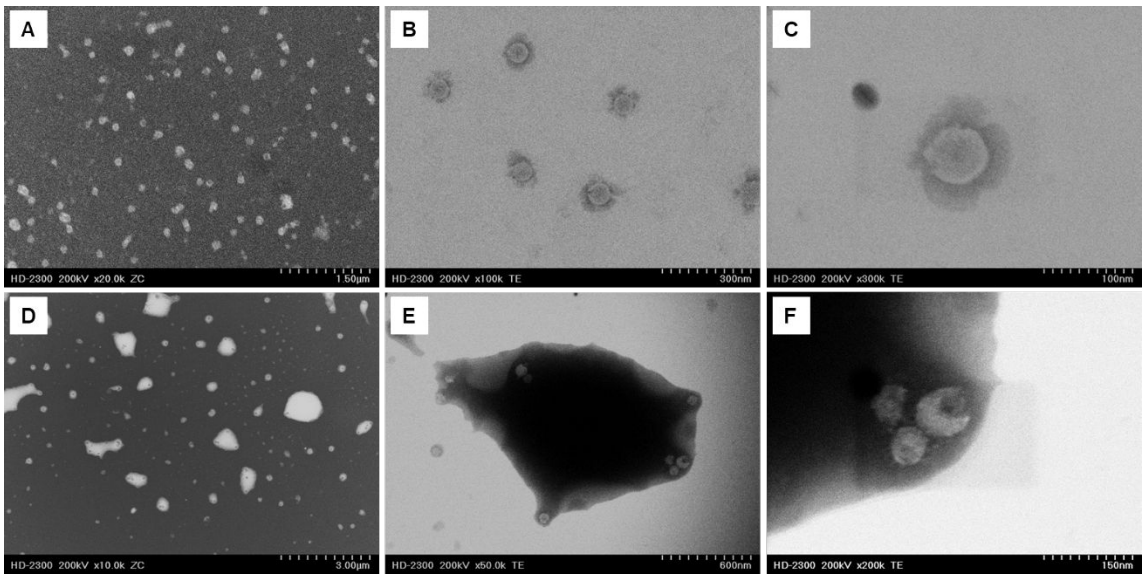


Figure S8: STEM images of the viral control sample (a-c) and virus + optimized PQQ (d-f). Images of a and d were captured in Z-contrast mode whereas b, c, e and f were captured in transmission electron mode. Coincidence with TEM result, damaged viral capsid was observed in the optimized PQQ-treated samples (f) but not in control sample (c).

SDS-PAGE experiment

We examined viral aggregation using SDS-PAGE since aggregation could slow down the diffusion of viral particles[7]. After incubated for 1h at 37 ˚C, virus control, virus treated with PQQ and optimized-PQQ samples were stained with EzLabel FluroNeo (WSE-701, Atto, Tokyo, Japan) heated at 95 ˚C for 3 min. Then, samples were treated with DTT reducing agent and heated at 95 ˚C for another 3 mins. All samples were loaded onto 12.5% ePAGEL-HR (Atto) in SDS-PAGE running buffer (Nippo Gene, Tokyo, Japan) and run for 30 mins at 21 mA. The result indicates that influence of viral aggregation was not readily evident in this experiment, as there was little difference in bands distance between samples (Figure S9).

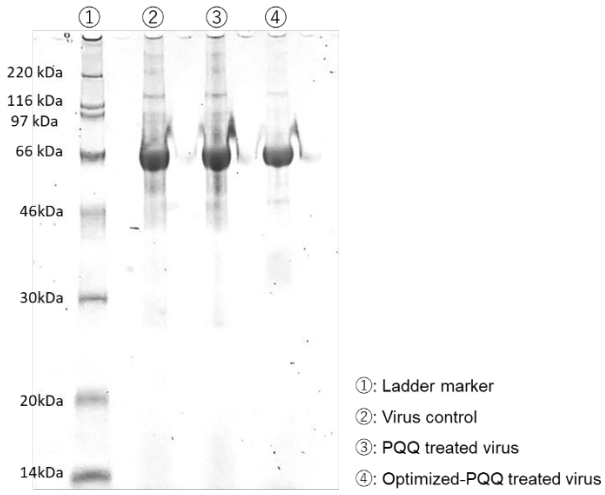


Figure S9: SDS-PAGE analysis of viral particles.

In-vitro SARS-CoV-2 M^{pro} inhibition assay

Inhibition effect of PQQ was evaluated by the FRET assay using the SARS-CoV-2 MBP-tagged 3CL Protease Assay Kit (BPS Bioscience; San Diego, USA). Table S5 shows Mpro activity (%) and inhibition rate (%) of PQQ in different concentrations.

Table S5: SARS-CoV-2 Mpro inhibition assay.

PQQ μM	**Mpro activity (%)**					**Inhibition (%)**
	1	**2**	**3**	**average**	**std**	
20	21.2	8.2	5.4	11.6	8.4	88.4
10	18.5	18.0	14.9	17.1	2.0	82.9
5	27.6	29.7	25.5	27.6	2.1	72.4
2.5	48.4	47.2	42.6	46.1	3.1	53.9
1.25	91.3	96.1	84.3	90.6	5.9	9.4
0.625	93.8	97.7	89.9	93.8	3.9	6.2
0	102.3	104.7	92.9	100.0	6.3	0.0

FIPV spike1 polyclonal antibody immune test

Rabbit serum containing polyclonal antibody targeting FIPV spike1 was purchased from Eurofins Japan (Kanagawa, Japan). A synthetic peptide was used as the Spike1-FCOV antigen to generate a rabbit polyclonal antibody. The acid sequence (N→C) of the synthetic peptide is NH2-C+MEAMENSTGNARGK-COOH. Polyclonal antibodies were used as whole

blood antibody sera, supplemented with 0.09% sodium azide.
The reduction plaque assay was performed. In this experiment, 20× and 200× diluted antibody serum was added to the virus solution and cell culture to evaluate the infection inhibition. The results confirmed that the antibody serum had a strong inhibitory effect on FIPV (Table S6). In addition, the inhibition effect was enhanced by the addition of 100 μg/μl PQQ.

Table S6: Viral inhibition rate of the plaque assay.

Test solution	Average of plaque number	Inhibition (%)
Antibody serum（20×dilution）	3	97
Antibody serum（200×dilution）	24	71
Antibody serum（20×dilution）, 100 μg/μl PQQ	0	100
Antibody serum（200×dilution）, 100 μg/μl PQQ	2	98
Control	83	0

The polyclonal antibodies were also tested for viral aggregation using the filtration method. Antibody serum, 20x and 200x diluted, were added to the viral solution and viral aggregation was evaluated. PQQ was added to the antibody serum at 100 μg/μl. The results revealed that the polyclonal antibody had no aggregation effect (Table S7).

Table S7: Viral aggregation test.

Test solution	Viral reduction (%) after filtration
Antibody serum（20×dilution）	5
Antibody serum（200×dilution）	8
Antibody serum（20×dilution）, 100 μg/μl PQQ	27
Antibody serum（200×dilution）, 100 μg/μl PQQ	22
Control	0

References

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