

Mimicking Biochemical Traits with a Synthetic Lipid Nanoparticles SARS-CoV-2 Model

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The continuous evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) during the global pandemic challenges diagnostic technologies, underscoring the urgent need for quick adaptation and innovation to improve accuracy and efficiency against new variants. The study introduces a synthetic Lipid Nanoparticles (LNPs) model of SARS-CoV-2, utilizing advancements in LNPs vaccine technology to mimic the virus's key biochemical and genetic traits. This liposomal model encapsulates the characteristic SARS-CoV-2 mRNA and nucleocapsid (N) proteins within LNPs and further conjugates with spike (S) protein derivatives on its outer membrane, closely replicating the virus's structure and inducing accurate antigen-antibody responses in diagnostic tests. Furthermore, validation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with commercial SARS-CoV-2 reagents confirms its effectiveness in simulating viral RNA amplification. This establishes it as an efficient tool for assessing the diagnostic efficacy of newly marketed diagnostic products. This LNPs model represents a significant advancement in diagnostic development, offering potential for therapeutic and vaccine research while ensuring safety and scalability.

1. Introduction

As of February 2024, according to the World Health Organization (WHO), the global Coronavirus Disease 2019 (COVID-19) pandemic has recorded over 774 million confirmed cases and more than seven million deaths.^[1] The virus identified as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) continues to pose a significant threat to global health and is not expected to be easily mitigated in the short term. In response, like most pandemics, the healthcare sector has been heavily engaged in efforts toward prevention, diagnosis, and treatment. Despite the development of relatively rapid diagnostic devices^[2] and new vaccines,^[3,4] the fundamental threat that the virus continues to evolve into variants over time and place remains.^[5,6] Indeed, polymerase chain reaction (PCR) and strip-based antigen devices optimized for the virus's genetic material or proteins from the early stages of the

pandemic are now failing to detect or are significantly reducing the accuracy of diagnosis for variants that have emerged mere months into the pandemic.^[7–9] For example, the SPERA COVID-19 antigen test manufactured by Xtrava Health was revoked by the Food and Drug Association (FDA) for use in the United States of America (USA) on August 3, 2023. This decision was based on its ineffectiveness in diagnosing the BA.1 and BA.2 omicron sub-variants of SARS-CoV-2, characterized by mutations in the nucleocapsid protein.^[7]

Addressing the emergence and continuous evolution of virus variants necessitates the development of rapid response technologies. Research on SARS-CoV-2 is escalating to identify optimal countermeasures, which typically require facilities with a Biosafety Level (BSL) of 3 or higher. Maintaining such high biosafety standards is expensive, and as of now, there are only ~50 BSL-4 facilities worldwide.^[10,11] Both in-development and existing diagnostic devices must undergo validation in these facilities using actual highly pathogenic viruses. The limited accessibility to highly pathogenic samples significantly impedes the swift transition of innovative technologies from the laboratory to practical field applications. Furthermore, simplifying the process to evaluate the efficacy of existing diagnostic devices against new variants would facilitate strategic responses, such as repurposing effective devices in regions affected by those variants, rather than mass disposal.^[12]

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To this end, if there were standard samples that mimic the features of pathogenic viruses without the risk of infection, it would enable more rapid evaluation of newly developed diagnostic devices. There has been, indeed considerable research on model systems that resemble viruses, such as virus-like particles (VLPs),^[13] pseudoviruses,^[14] and bacteriophages.^[15] Currently, commercially available systems for SARS-CoV-2 related studies are primarily limited to pseudotyped virus.^[16,17] These are recombinant viral particles with a core derived from one virus such as lentiviruses or rhabdoviruses, particularly vesicular stomatitis virus (VSV) and surface proteins derived from SARS-CoV-2, such as the spike protein.^[18] These particles allow research to be conducted in BSL-2 laboratories, making them accessible to a broader range of researchers.^[19] However, for evaluating analytical devices, pseudotyped virus present several limitations, including higher costs, unnecessary biological complexity, and the requirement for specific storage and handling conditions to maintain stability, which restricts their suitability for routine diagnostic test development.

As an alternative to the pseudoviruses, synthetic virus models have been proposed,^[20] such as polymeric nanoparticles,^[21,22] mimetic nanoparticles,^[23,24] and lipid-based nanoparticles.^[25,26] These methods offer a safer option and allow for broader customization according to the purpose of the research. During the COVID-19 pandemic, lipid nanoparticles (LNPs) emerged as a critical component in the development of effective and safe mRNA vaccines.^[27] Their popularity arises from the ability to engineer LNPs with tunable properties, such as size, surface charge, and lipid composition, to optimize delivery efficiency and immune response.^[28] Beyond their role in vaccine delivery, recent studies have highlighted the potential of LNPs as SARS-CoV-2 virus-mimicking particles through surface functionalization with viral proteins (Table S1, Supporting Information), offering innovative approaches for understanding viral behavior and developing preventative strategies.^[17,29,30] In the context of diagnostic systems, effective evaluation models are essential for improving both antigen-based self-diagnostic kits and RNA-targeting PCR assays, often applied simultaneously to the same clinical samples. The unique capability of LNPs to encapsulate RNA and conjugate surface proteins positions them as promising synthetic virus models for evaluating diagnostic devices. Therefore, developing robust virus-like models that facilitate cross-platform testing and validation represents a crucial step toward advancing diagnostic technologies and enhancing pandemic preparedness.

This study develops a synthetic Lipid Nanoparticles (LNPs) SARS-CoV-2 model that mimics the typical biochemical properties and genetic material of the SARS-CoV-2 virus in a similar structure. Initially, we encapsulated mRNA encoding the spike (S) protein within LNPs, minimizing damage to mRNA molecules while enhancing encapsulation efficiency. Nucleocapsid (N) protein, responsible for mRNA synthesis, was co-encapsulated into LNPs with mRNA using a modified ethanol injection method. Moreover, the S protein, utilized by most rapid diagnostic kits, was conjugated onto the lipid surface encapsulating the mRNA-N protein, mirroring the coronavirus's distinctive form. When applied to the majority of market-released rapid diagnostic kits, the externally exposed S protein and the internal N protein induced antigen-antibody reactions similar to the actual virus. Additionally, the inserted mRNA was amplified in reverse

transcription-quantitative polymerase chain reaction (RT-qPCR) identically to the virus. This multifunctional LNPs SARS-CoV-2 model, structurally very similar to the COVID virus and possessing most of its specific biochemical fingerprints, can be easily utilized for evaluating the efficacy of diagnostic devices. It holds promise for application in various research areas, not only in validating new diagnostic methods for the virus but also in developing therapeutics, vaccines, and studying infection mechanisms.

2. Results and Discussion

2.1. LNPs SARS-CoV-2 Model

SARS-CoV-2, a member of the coronavirus family, comprises four essential structural proteins: spike (S) protein, envelope (E) protein, membrane (M) protein, nucleocapsid (N) protein, and carries a single-stranded positive RNA (Figure 1a). The N protein binds to RNA to form a capsid, which is then enclosed by the envelope structure composed of the S, E, and M proteins.^[31,32] The transmission electron micrograph (TEM) reveals SARS-CoV-2's structure, displaying the S protein (colored red) on the surface of the virus particle (colored orange) (Figure 1b). Following SARS-CoV-2 exposure, the human immune response identifies S and N proteins as the primary immunogens.^[33,34] The N protein plays a crucial role in viral replication and transcription, is abundantly expressed during infection, and possesses high immunogenicity.^[35,36] Conversely, S protein is vital for viral infection and pathogenesis and is highly immunogenic.^[37,38] Therefore, S and N proteins are selected for incorporation into the LNPs, along with mRNA encoding the S protein, as a model RNA in the proposed LNPs SARS-CoV-2 model (Figure 1c). The TEM image of the LNPs SARS-CoV-2 model in Figure 1d successfully captures the conjugated S protein on the surface of the LNPs, providing a structure that mimics the actual virus.

In this model, mRNA is co-encapsulated with N protein inside the LNPs, representing the inner component of SARS-CoV-2, while the S protein is conjugated on the LNPs' surface. A modified ethanol injection method is employed for LNPs formation, simultaneously encapsulating mRNA and N protein. The LNP's lipid component includes an ionizable lipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), stabilizing and facilitating the interaction between the anionic charge of mRNA and the lipid's cationic charge, thus enhancing mRNA encapsulation efficiency. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol contribute to the LNPs' structural stability and integrity, while 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000 PE) prevents LNPs aggregation. Additionally, 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)) is incorporated into the lipid composition as Ni-NTA LNP for subsequent S protein conjugation on the surface (Figure 1e). Overall, the LNPs SARS-CoV-2 model closely replicates the essential biochemical contents, structural architecture, and sizes similar to the actual virus. It was investigated whether this model could serve as a substitute and simulant for the deadly virus, potentially paving the way for safer and more accessible research and diagnostic development.

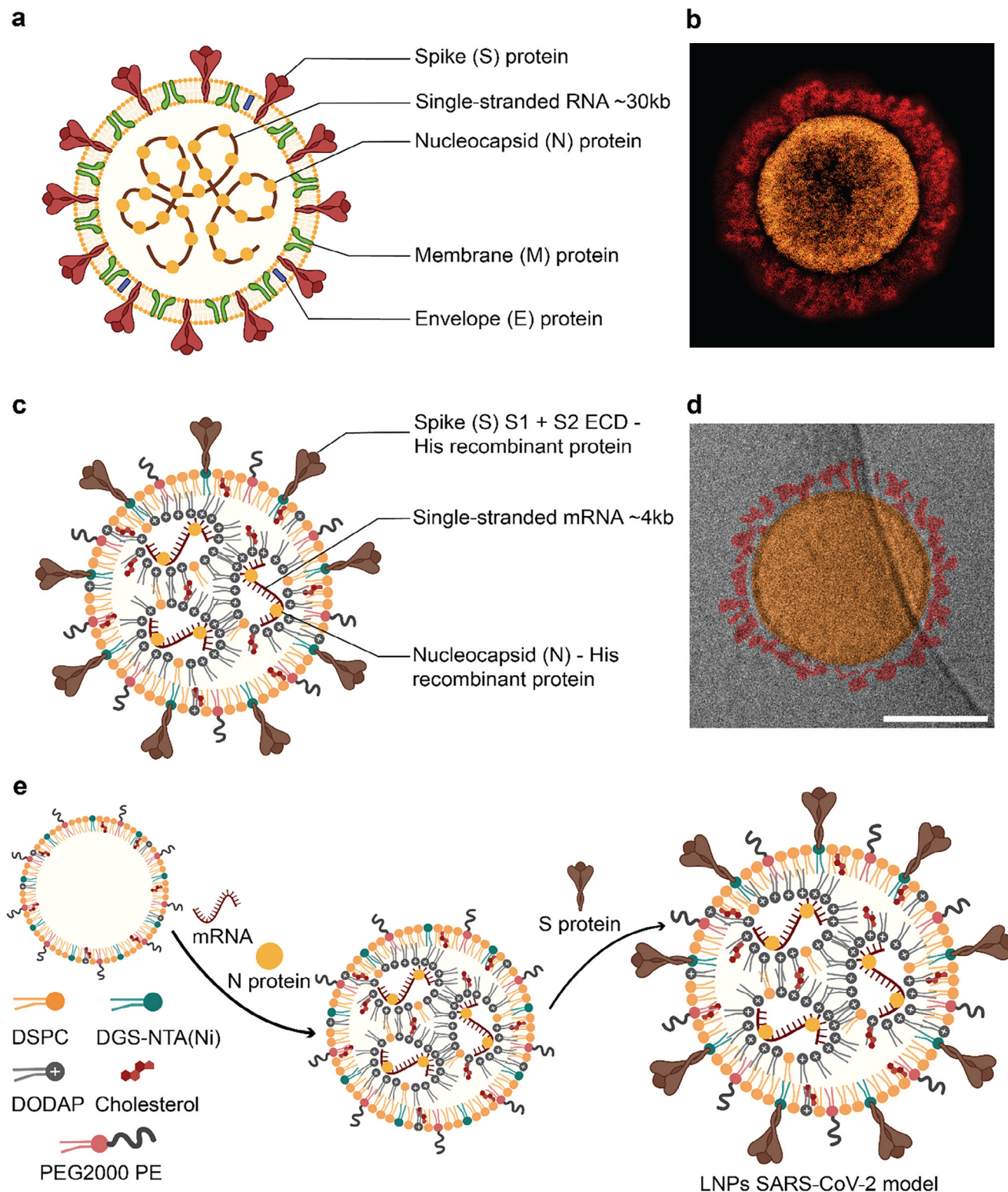


Figure 1. Comparative visualization of SARS-CoV-2 and its LNPs model. a) Representative image of SARS-CoV-2 virus with its component. b) Transmission electron micrograph (TEM) image of SARS-CoV-2 virus particle (UK B.1.1.7 variant), isolated from a patient sample and cultivated in cell culture. Image captured at the National Institute of Allergy and Infectious Diseases (NIAID) Integrated Research Facility (IRF) in Fort Detrick, Maryland. Credit: NIAID. c) LNPs SARS-CoV-2 model with its component. d) Cryogenic transmission electron microscopy (Cryo-TEM) image of fabricated LNPs SARS-CoV-2 model. Scale bar = 100 nm. e) Fabrication step of LNPs SARS-CoV-2 model. Figure a,c,e are created by Biorender.

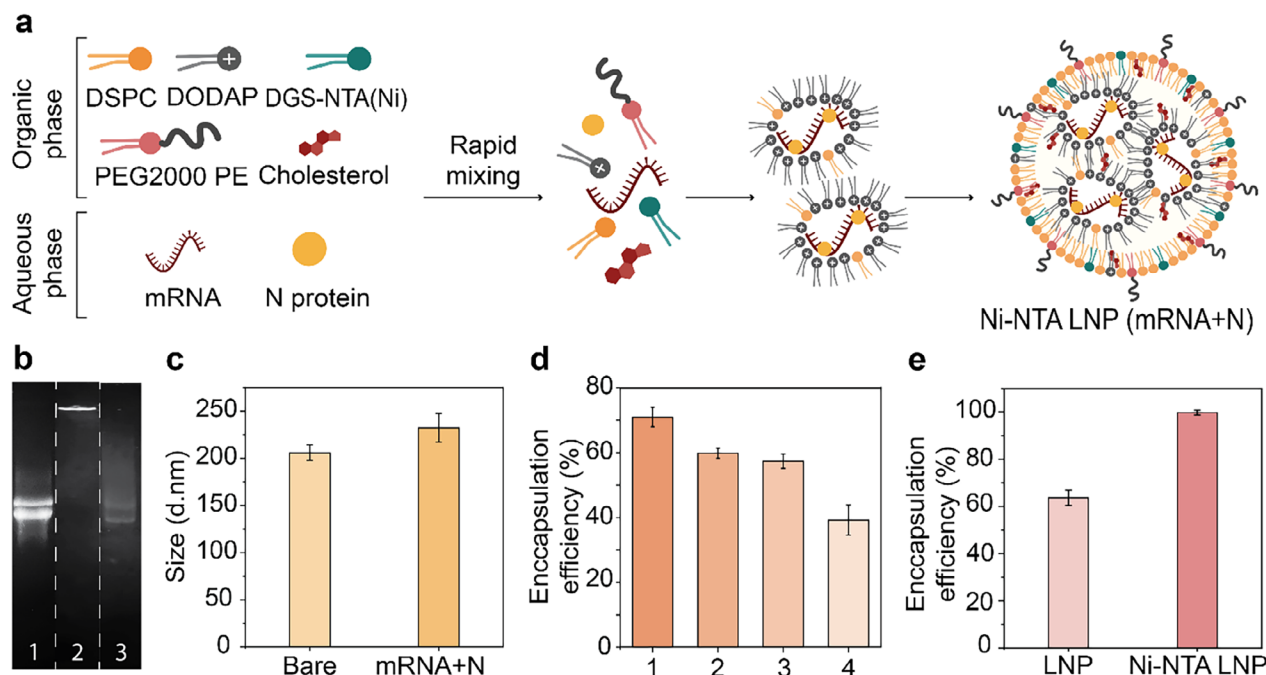


Figure 2. mRNA and N protein co-encapsulation in LNP SARS-CoV-2 model. a) mRNA and N protein encapsulation in Ni-NTA LNP by modified ethanol injection method. Created by Biorender. b) Qualitative characterization of mRNA encapsulation in LNP by gel electrophoresis. Lane 1: free mRNA, lane 2: LNP (mRNA) before lysis, and lane 3: mRNA encapsulated in LNP after lysis with Triton X-100. The gel image was spliced from different experiments for comparison. Dotted lines indicate where the gels were merged for clarity. c) The size average of bare Ni-NTA LNP and Ni-NTA LNP (mRNA+N), obtained with DLS. d) Encapsulation efficiency of mRNA in 1: LNP (mRNA), 2: LNP (mRNA+N), 3: Ni-NTA LNP (mRNA), and 4: Ni-NTA LNP (mRNA+N), determined by RiboGreen assay. e) Encapsulation efficiency of N protein in LNP and Ni-NTA LNP, determined by ELISA.

2.2. mRNA and N Protein Encapsulation in Ni-NTA LNP

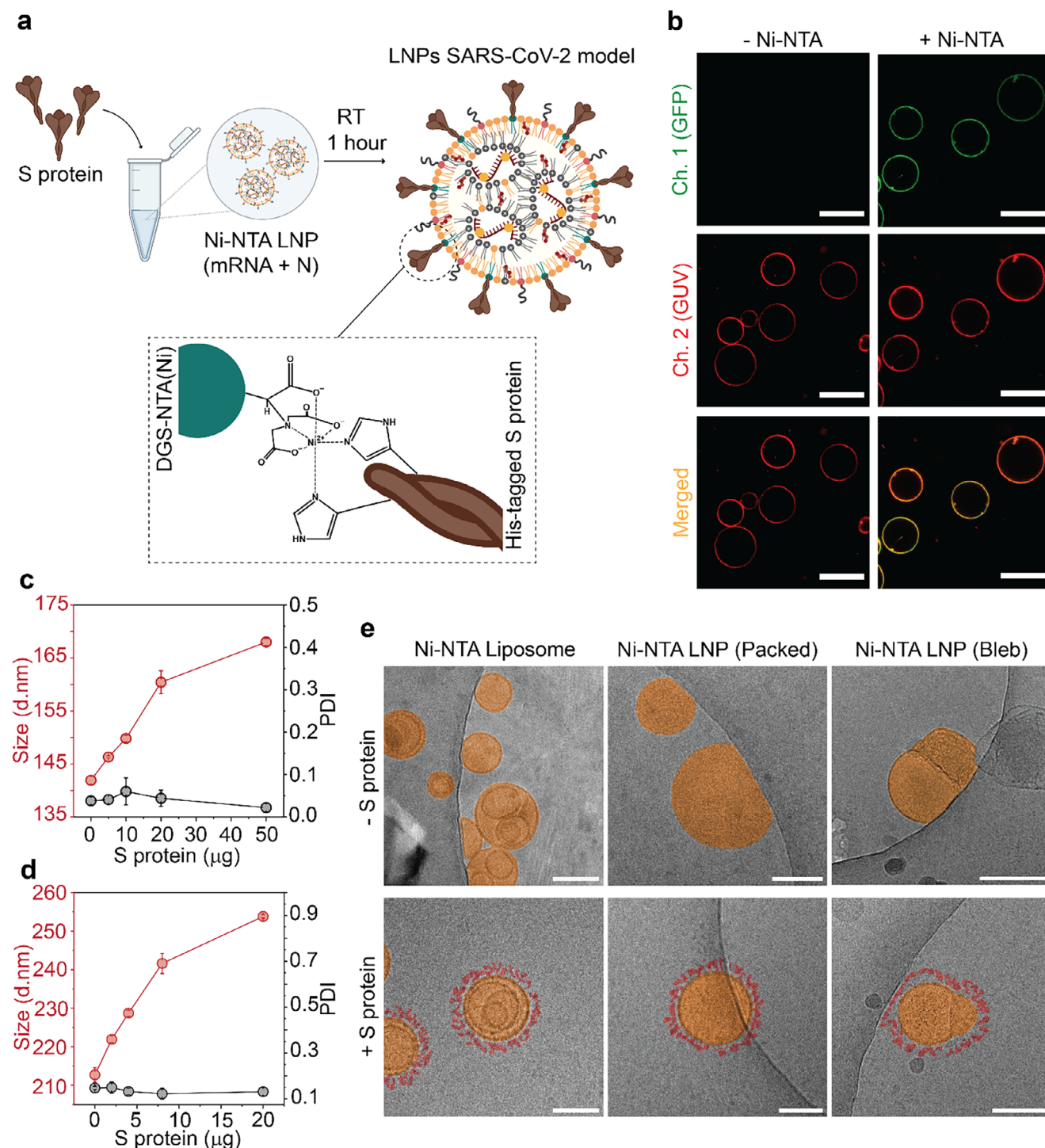
RNA is fundamental to viruses as genetic material, making its encapsulation a critical aspect of virus model exploration. In this model, 30 μg of mRNA was encapsulated at an ionizable lipid nitrogen (DODAP) to oligonucleotide phosphate (mRNA) ratio of 6 (N:P = 6:1), mirroring the ratio used in Pfizer and Moderna vaccine formulation^[27] and co-encapsulated with N protein inside the LNPs (Figure 2a). To verify mRNA encapsulation within the LNPs, gel electrophoresis compared LNP samples before and after lysis to 30 μg of mRNA. As shown in Figure 2b, a strong band appears for mRNA in line 1. For the non-lysed LNP sample (line 2), the mRNA remains protected within the LNP, inhibiting its migration under the electrical field. However, lysis releases the mRNA, allowing it to migrate through the gel, producing a band similar to the standalone mRNA sample (line 3). This confirms successful mRNA encapsulation inside the LNP using the modified ethanol injection method. Additionally, 10 μg of N protein was co-encapsulated with 30 μg of mRNA in the Ni-NTA LNP, denoted as Ni-NTA LNP (mRNA+N). Dynamic light scattering (DLS) analysis indicated the Ni-NTA LNP size to be ≈ 205 nm, with an insignificant increase to 232 nm after mRNA and N protein encapsulation (Figure 2c; Figure S1, Supporting Information).

Subsequently, encapsulation efficiency was quantified using RiboGreen assay for mRNA and enzyme-linked immunosorbent assay (ELISA) for N protein. Efficiency was calculated by dividing the concentration of encapsulated mRNA/N protein by the total concentration in the sample. Figure 2d demonstrates that

mRNA encapsulation was influenced by co-encapsulation with N protein and the presence of Ni-NTA in the lipid composition. The highest efficiency observed was 71.0% in the LNP sample with only mRNA encapsulation. Co-encapsulation of N protein with mRNA resulted in a decreased mRNA encapsulation efficiency to 59.9%. The presence of Ni-NTA further reduced mRNA encapsulation efficiency to 57.3% and 39.2%, respectively, when co-encapsulated with N protein. This suggests co-encapsulation with N protein occupies space inside the LNPs, reducing mRNA encapsulation. Additionally, Ni-NTA's anionic charge interference may alter the LNPs' electrostatic properties, affecting negatively charged mRNA encapsulation. Conversely, the N protein encapsulation efficiency in Ni-NTA LNP significantly increased from 63.6% to 99.8% compared to only LNP (Figure 2e). This higher efficiency is attributed to increased interaction between the his-tagged N protein used in the experiment and the DGS-NTA(Ni) lipid in the Ni-NTA LNP.

2.3. Ni-NTA LNP Surface Conjugating with S Proteins

The S protein, a critical structural component of SARS-CoV-2, offers significant benefits for vaccine development and viral studies when integrated into a virus model. Utilizing DGS-NTA(Ni) lipid at a 4% molar ratio^[39] facilitates the conjugation of the S protein onto the Ni-NTA LNP surface through a specific affinity-based chelating process involving the polyhistidine residue of his-tagged S protein and nickel ions (Figure 3a). A preliminary study using liposomes explored Ni-NTA's interaction with his-



tagged green fluorescence protein (GFP), showing clear GFP attachment on the Ni-NTA liposome surface under confocal microscopy (Figure 3b). This confirms the feasibility of applying Ni-NTA and his-tagged protein interaction to anchor the S protein on Ni-NTA LNP surfaces.

DLS measurements indicated an increase in Ni-NTA liposome size with rising S protein concentration, without particle aggregation, as suggested by polydispersity index (PDI) values below 0.1 (Figure 3c). The notable size expansion in samples with a Ni-NTA to protein ratio 20:1 underscores a saturation point in Ni-NTA chelation (Table S2, Supporting Information), essential for controlling protein conjugation. This mechanism was also applied to Ni-NTA LNP (mRNA+N) (Figure 3d), demonstrating successful S protein conjugation as evidenced by increased particle size proportional to the S protein amount, similar to earlier Ni-NTA liposome results (Table S3 and Figure S2, Supporting Information). The LNPs, encapsulating both mRNA and N protein, show the viability of the final SARS-CoV-2 model.

The final LNPs SARS-CoV-2 model, consisting of 10 µg S protein conjugated to Ni-NTA LNP (mRNA+N), was chosen for further investigation, as it demonstrated a similar trend of diameter increase after the S protein conjugation. Additionally, the zeta potential was analysed, showing a shift toward a more positive value after conjugation. Specifically, the zeta potential of bare Ni-NTA LNP was −8.024 mV, while the LNPs SARS-CoV-2 model was −5.256 mV. The shift value is likely due to the presence of His-tagged N and S proteins in the model, as the imidazole group in histidine residues can be slightly protonated at physiological pH (≈7.4), contributing positive charges (Figure S3, Supporting Information).

Cryo-TEM images further validate the LNPs SARS-CoV-2 model, showcasing liposomes and LNPs with and without S protein conjugation (Figure 3e). Bare liposomes lack corona features, while S protein-conjugated liposomes exhibit SARS-CoV-2-like corona structures (Figure S4, Supporting Information). Ni-NTA LNP samples, particularly those with encapsulated mRNA and N protein, reveal a denser internal structure due to the encapsulated components. In Figure 3e, the Ni-NTA LNP samples display packed (Figure S5, Supporting Information) and bleb (Figure S6, Supporting Information) structures. This can be explained by the condensation of mRNA and lipids, which is often attributed to the clear presence of the encapsulated mRNA.^[40] Nevertheless, both structures in Ni-NTA LNP (mRNA+N) show a corona feature in the sample with S protein conjugation. The cryo-TEM images also reveal a range of size distributions in the LNPs SARS-CoV-2 model, showing a similar trend to the DLS measurement results (Figure S7, Supporting Information). In summary, cryo-TEM imaging confirms the successful conjugation of S protein on both liposome and LNPs surfaces through Ni-NTA chelation, effectively mimicking the corona and envelope features of the virus model.

2.4. Qualitative and Quantitative Detection of LNPs SARS-CoV-2 Model

The LNPs SARS-CoV-2 model, comprising 30 µg mRNA, 10 µg N protein, and 10 µg S protein conjugated to Ni-NTA LNP, underwent both qualitative and quantitative detection. This step is

critical as the model aims to replicate not just the physical attributes but also the detection capabilities of real viruses. Initially, qualitative detection through rapid antigen tests and quantitative analysis via RT-qPCR were performed, reflecting the model's accuracy in simulating real SARS-CoV-2 virus detection. RT-qPCR was first employed to detect the encapsulated mRNA by converting it to cDNA for PCR quantification. To ensure accuracy, the LNPs were treated with RNase A (RN) before lysis to remove external, non-encapsulated mRNA, followed by RNase inhibitor (RI) addition to halt RNA degradation post-lysis (Figure 4a). The optimization of RN and RI amounts is shown in Figure S8 (Supporting Information). The sample with RN and RI treatment gave a lower quantification cycle (Cq) value, indicating higher cDNA concentration, compared to the sample with RN treatment only (Table S4, Supporting Information). The Cq values were used to calculate the concentration of cDNA (Figure S9, Supporting Information), and a significant increase in the amount of cDNA upon the RI treatment was observed. These results confirm the robust presence of the encapsulated mRNA and the improved integrity of encapsulated mRNA in this virus model (Figure 4b).

The LNPs SARS-CoV-2 model then underwent qualitative detection using rapid antigen tests to identify the S and N proteins. Utilizing the Humasis rapid antigen kit, which targets both S and N proteins, we established a baseline with Ni-NTA LNP alone (negative control or NC), showing no detection, indicating the absence of these proteins (Figure 4c). Testing the LNPs SARS-CoV-2 model revealed successful detection of the conjugated S protein and enhanced detection of N protein post-lysis, confirming the model's ability to qualitatively identify both antigens. Given the commercial availability of N protein rapid antigen tests, the LNPs SARS-CoV-2 model was further evaluated with various kit brands targeting N protein (Figure 4d). Bare Ni-NTA LNP as NC and LNPs SARS-CoV-2 were tested to verify that detection results stemmed from the encapsulated N protein. Only LNPs SARS-CoV-2 samples showed positive results across different brands, indicating that N protein, encapsulated within the LNPs SARS-CoV-2 model, is released upon lysis and detectable by rapid antigen tests. These qualitative analyses based on conventional immunoassays and the previously mentioned quantitative evaluations via molecular assays demonstrate that the LNPs SARS-CoV-2 model accurately replicates most of the detection characteristics of the actual virus. These capabilities show that our LNP models have the potential to serve as benchmarks for evaluating devices designed to detect new variants of the virus, even without the actual virus present.

3. Conclusion

We successfully developed the LNPs SARS-CoV-2 model by encapsulating mRNA and N protein, and by conjugating the S protein onto the surface of LNPs. This innovative approach not only replicates the physical and biochemical features of the virus but also demonstrates the capability for both qualitative and quantitative detection, mirroring real-world testing scenarios for SARS-CoV-2. The encapsulation efficiency for mRNA and N protein reached as high as 71.0% and 99.8%, respectively, highlighting the model's effectiveness in simulating the internal composition of the virus. Additionally, the conjugation of S proteins on the surface provides structural similarity to the actual virus, further

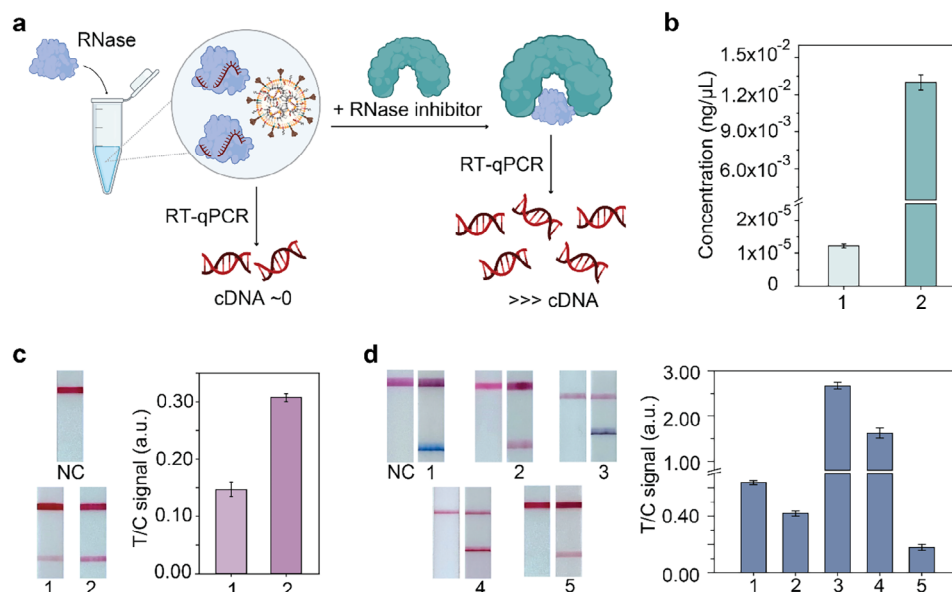


Figure 4. Qualitative and quantitative detection of LNPs SARS-CoV-2 model. a) Pre-treatment of LNPs SARS-CoV-2 model with RNase A (RN) and RNase inhibitor (RI) for quantification by RT-qPCR. Created by Biorender. b) cDNA concentration obtained by RT-qPCR, 1: without and 2: with RI treatment. c) Rapid antigen test kit results and quantitative T/C signal for SARS-CoV-2 S and N protein detection of 1: Ni-NTA LNP with S protein conjugation and 2: LNPs SARS-CoV-2 model. Humasis rapid kit was used for the detection. d) Various results from different brands of rapid antigen test and quantitative T/C signal for N protein detection of LNPs SARS-CoV-2 model (1: Wells Bio, 2: Abbott, 3: Rapigen, 4: SD Biosensor, 5: Humasis).

underscoring the high potential of our LNPs SARS-CoV-2 model as a valuable tool for evaluating and refining diagnostic devices. It offers a safer and more accessible option for research and development, facilitating crucial insights into viral behavior and the efficacy of detection technologies without the associated risks of handling live pathogens.

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Supporting Information

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

artificial virus, diagnostics, lipid nanoparticles, SARS-CoV-2

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