

A Matter of State: Destabilizing Postfusion Spike to Preserve the Prefusion Form

By Carson Gray* and JF Trembl†

Nucleic acid vaccines possess several advantages over previous approaches to vaccination and have been an object of study for several decades; only recently, however, has a nucleic acid vaccine had large scale, international use, with the development of a vaccine against SARS-CoV-2 in 2020. mRNA vaccines in particular have garnered significant attention due to their rapid antigen production, generally improved immune response, modularity, and potentially improved safety.¹ In contrast to vaccination with purified protein antigens, mRNA vaccines enable endogenous antigen production, more closely recapitulating viral antigen presentation pathways.² The SARS-CoV-2 mRNA vaccine has been demonstrated to produce a more robust cellular immune response compared to the recombinant protein vaccine in murine models, consistent with enhanced MHC class I presentation.³ mRNA vaccines may also be an improvement over viral platforms in certain contexts. Inactivated viruses have similar issues to purified protein, in that there is no endogenous expression of antigenic material; live attenuated vaccines are a more complete system, however they are prone to inflammation and can in rare cases cause an infection. An advantage of mRNA above almost every other system is that it does not require cell-based pipelines or protein purification during production, making it highly scalable via in vitro transcription-based manufacturing.⁴

A major focus in the current mRNA research landscape is optimization, both in delivery systems and the chemical structure of the RNA itself. Lipid nanoparticles are the only clinically validated delivery mechanism for mRNA vaccines, and work has been done to optimize immunogenicity while making the nanoparticles more tolerable.⁵ Additionally, mRNA base modifications have historically been observed to be critical for mRNA stability and expression, especially the presence of pseudouridine which reduces innate immune activation and enhances translational efficiency.⁶ Synthesizing these findings is important for

generating more effective vaccines, and the continued maturation of mRNA vaccination strategies.⁷ An interesting manifestation of this is in rational structure optimization, which has the objective of modifying antigen structure to more effectively display relevant epitopes. The spike proteins of many viruses are metastable, undergoing large structural changes to facilitate membrane fusion.⁸ The elicitation of neutralizing antibodies often depends on recognition of the pre-fusion conformation as many neutralizing epitopes become distorted or hidden after the spike transitions to its post-fusion conformation, making stabilization of the pre-fusion structure important for vaccine efficacy. To generate a potent neutralizing response, mRNA vaccines therefore do not necessarily code for the native sequence, but rather a modified form that stabilizes the pre-fusion state. The SARS-CoV-2 spike is a trimeric class I fusion glycoprotein that changes shape

during membrane fusion. The Heptad Repeat 2 (HR2) region plays an important role in this process and has therefore become a focus for engineering mutations that stabilize the spike in its pre-fusion conformation. A landmark publication by Pallesen et al. in 2017 involved a rational double mutation, K986P/V987P, situated at the boundary of HR2 and the central helix hinge in the MERS-CoV spike protein. The simple addition of these two prolines stabilizes pre-fusion conformation by restricting conformational rearrangement of the central helix / hinge region.⁹ These mutations are included in the SARS-CoV-2 mRNA vaccine, testifying to the clinical relevance of rational structure optimization of antigens.

Recently, Chakraborty *et al.* investigated a new approach to stabilizing the pre-fusion conformation: rather than constricting the helix extension that occurs during the transition process, they destabilized the post-fusion conformation by substituting charged residues in positions that are exposed and buried in the pre-fusion and post-fusion conformations, respectively.¹⁰ Specifically, N969 and I973 were replaced with aspartic acid. This represents a conceptual shift from trapping the pre-fusion state with rigidifying mutations to thermodynamically disfavoring the post-fusion

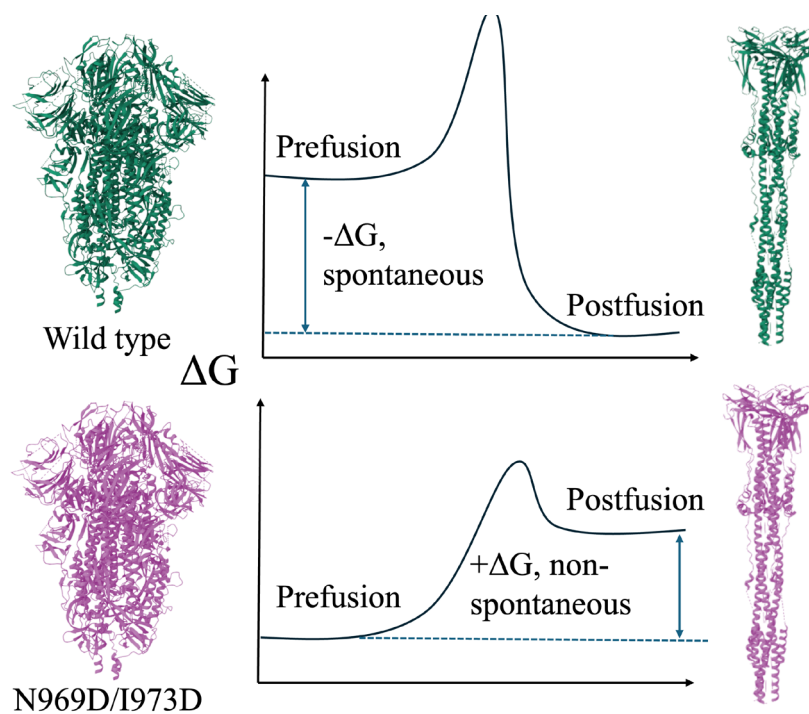


Figure 1 | Stabilizing optimal spike conformation. The introduction of charged residues at positions 969 and 973 destabilize the post-fusion conformation of the SARS-CoV-2 Spike, resulting in a thermodynamically favored pre-fusion conformation.

conformation through targeted electrostatic destabilization (See Fig 1).

Hereafter, constructs are named based on their mutations (Spike-#P = number of proline substitutions; Spike-#D = number of aspartic acid substitutions). When expressed in mammalian cells, the Spike-2D ectodomain exhibited significantly higher expression than WT and yields comparable to those of Spike-2P and Spike-6P. A combined construct (Spike-4P-2D), incorporating both proline and aspartate substitutions while reverting the original K986P/V987P mutations, showed similar expression levels. Both Spike-2D and Spike-4P-2D were consistent with pre-fusion stabilization, as assessed by binding to the CR3022 antibody.

Hamsters were vaccinated with all constructs to assess their efficacy in eliciting neutralizing antibodies. All constructs exhibited significant antibody titers upon prime vaccination, and Spike-4P-2D performed similarly to Spike-2P and Spike-6P after a boost vaccination. All were able to effectively neutralize B.1.351 pseudovirus. When animals were challenged with SARS-CoV-2 virus, all vaccinations conferred similar protection in terms of weight and lung histopathology. Taken together, the results of this research suggest that the proposed aspartic acid mutations are an effective means of stabilizing the pre-fusion Spike conformation and can elicit neutralizing antibodies.

Development of new approaches to stabilize the pre-fusion conformation is an important aspect of mRNA vaccine optimization. Although two different constructs may successfully stabilize the pre-fusion conformation, subtle differences in epitope exposure, expression efficiency, and glycosylation patterns may alter the performance of the vaccine. More broadly, this raises the possibility that charge-based destabilization strategies may be applied to other class I fusion proteins, offering an alternative route to pre-fusion stabilization beyond proline substitution. However, while antibody binding and pseudovirus neutralization support pre-fusion stabilization, high-resolution structural and biophysical measurements would be required to fully establish equivalence between the stabilization strategies.

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

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