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REVIEW

# Long-lasting, biochemically modified mRNA, and its frameshifted recombinant spike proteins in human tissues and circulation after COVID-19 vaccination

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## Abstract

According to the CDC, both Pfizer and Moderna COVID-19 vaccines contain nucleoside-modified messenger RNA (mRNA) encoding the viral spike glycoprotein of severe acute respiratory syndrome caused by corona virus (SARS-CoV-2), administered via intramuscular injections. Despite their worldwide use, very little is known about how nucleoside modifications in mRNA sequences affect their breakdown, transcription and protein synthesis. It was hoped that resident and circulating immune cells attracted to the injection site make copies of the spike protein while the injected mRNA degrades within a few days. It was also originally estimated that recombinant spike proteins generated by mRNA vaccines would persist in the body for a few weeks. In reality, clinical studies now report that modified SARS-CoV-2 mRNA routinely persist up to a month from injection and can be detected in cardiac and skeletal muscle at sites of inflammation and fibrosis, while the recombinant spike protein may persist a little over half a year in blood. Vaccination with 1-methylΨ (pseudouridine enriched) mRNA can elicit cellular immunity to peptide antigens produced by +1 ribosomal frameshifting in major histocompatibility complex-diverse people. The translation of 1-methylΨ mRNA using liquid chromatography tandem mass spectrometry identified nine peptides derived from the mRNA +1 frame. These products impact on off-target host T cell immunity that include increased production of new B cell antigens with far reaching clinical consequences. As an example, a highly significant increase in heart muscle 18-flourodeoxyglucose uptake was detected in vaccinated patients up to half a year (180 days). This review article focuses on medical biochemistry, proteomics and deutenomics principles that explain the persisting spike phenomenon in circulation with organ-related functional damage even in asymptomatic individuals. Proline and hydroxyproline residues emerge as prominent deuterium (heavy hydrogen) binding sites in structural proteins with robust isotopic stability

**Abbreviations:** $^{2}$H-D, deuterium; mRNA, messenger RNA; Polθ, polymerase theta; SARS-CoV, severe acute respiratory syndrome caused by corona virus; WT, natural wild-type.

that resists not only enzymatic breakdown, but virtually all (non)-enzymatic cleavage mechanisms known in chemistry.

**KEYWORDS**
acid hydrolysis, deutenomics, deuterium, DNA polymerase theta, hypoxia, modified mRNA, recombinant spike protein, SARS-CoV-2

## 1 | INTRODUCTION

The Pfizer-BioNTech COVID-19 Vaccine (also known as BNT162b2 and boosters) and Moderna COVID-19 vaccine (messenger RNA [mRNA]-1273 and boosters) work by introducing a modified mRNA molecule that codes for the corona virus spike protein via intramuscular injections.[1] Resident and circulating immune cells attracted to the injection site and regional lymph nodes translate the injected mRNA into copies of the spike protein.[2,3] Shortly after, the injected mRNA degrades due to the inherent intramolecular fragility of RNA, usually within a few days in the injected muscle and regional lymph nodes, as animal studies suggested. The Infectious Disease Society of America estimates that recombinant spike protein fragments, which are generated by host cells from the mRNA in the vaccines, may last up to a few weeks. In fact, clinical studies report that severe acute respiratory syndrome caused by corona virus (SARS-CoV-2) mRNA vaccines commonly persist up to 30 days from injection in humans and can be detected in heart muscle at healing infarcts.[4] Recombinant spike protein may persist in the circulation up to a little over half a year (187 days).[5] In the Pfizer-BioNTech (BNT162b2- Comirnaty) and Moderna (mRNA-1273) injectables, uridine's nitrogen bases were replaced by pseudouridine and methylated, hence they are called $N^1$-methylpseudouridine (m1Ψ). It has been shown that this modification produced more stable nitrogen bases[6] and resulted in less immunogenicity.[7] This is because the substitution of uridines by pseudouridines, or (even better) with methyl-pseudouridine, bypasses surveillance as foreign mRNA from toll-like receptors. Despite the fact that the two mRNA preparations are different (due in part to different codon optimization schemes), they both produce the full-length recombinant spike protein that differs from the wild type spike protein only by two adjacent amino acid substitutions at positions 986 and 987, respectively. In K986P the amino acid lysine and in V987P the amino acid valine is replaced by a proline amino acid, each.[8,9] Proline substitutions readily stabilize the spike protein's conformation for cellular prefusion by abolishing a key tryptic cleavage site. As a result, tryptic digestion[10] opens roads to mass spectrometry analyses that readily differentiate among synthetic (recombinant) spike protein fragments, originated from translations of the injected mRNA vaccines, compared to the natural wild-type (WT) spike of the SARS-COV-2 virus, circulating in biological fluids of humans.[5]

Translational and clinical studies have established that decay modeling of injected mRNA wrapped in liposomes and that of the deriving spike protein is extended beyond what was expected from in vitro culturing and animal studies. Analytical efforts described above indicate significantly longer decay times of both the injected mRNA and its recombinant spike protein in tissues and the circulation. In addition, there are documented pathologies in asymptomatic patients with regard to mRNA immunizations. The distribution and persistence of SARS-CoV-2 mRNA vaccine in human tissues were unclear until specific quantitative reverse transcription polymerase chain reaction-based assays detected each mRNA vaccine in the axillary lymph nodes in the majority of patients as well as in the myocardium in a subset of patients vaccinated within 30 days of death. These results strongly suggest that SARS-CoV-2 mRNA vaccines routinely persist up to 30 days from vaccination in ipsilateral lymphatic organs and can be detected also in the heart.[4]

Mass spectrometry examination of human blood specimens reported the presence of specific fragments of the recombinant spike protein after receiving mRNA-based vaccines in 50% of samples up to 187 days after vaccination.[5] Suggested mechanisms included the integration of the stable injected mRNA into the genome of somatic cells,[11,12] which may lead to the transcription and translation of a constitutively active spike protein pool. The continued persistence and uncertain fate of sustained mRNA and spike formations are of utmost importance due to morphological and functional pathologies increasingly reported in the medical literature, which this viewpoint article addresses.

## 2 | STABLE MESSENGER RNAs AND EUKARYOTE CELL TRANSFORMATION

Stabilized, then injected ribonucleic acids, which remain in various tissues for up to 30 days, can readily serve as templates for the promiscuous reverse transcriptase function of human DNA polymerase theta (Polθ) (EC no. 2.7.7.7) that causes DNA transformation involved in cancer formation.[11] In fact, unlike most Pol I enzymes, Polθ is highly error-prone to carry out translesion synthesis and promotes microhomology-mediated end-joining of double-strand breaks (DSBs) with high RNA fidelity. For the above reasons, Polθ is highly expressed in many cancer cells[13] with significant aneuploidy and a poor clinical outcome.[14] It is also critically important to consider during injectable modified mRNA development efforts that water molecules control the rate constant for nucleotide incorporation in the pre-steady state of RNA and DNA polymerases,[15] including that

of Polθ. This fact is fundamental to better understand how the stable heavy hydrogen isotope called deuterium ($^2$H-D) carried in water molecules of various cell compartments stabilizes mRNA vaccines with undesired DNA transforming effects.

The above discoveries enlighten two basic facts related to nucleic acid chemistry and their use in medicine: (A) RNA and DNA polymerases are inducible by nucleic acid template architectures of various pathogenic, exogenous and/or endogenous origins,[16] and (B) RNA[17] and DNA[18] templates harbor significant $^2$H-D-related conformational stability and resistance against degradation. There is, for example, random integration frequently occurring at DSBs in the genome. During the interphase and facultative heterochromatin formation in the cell cycle, ionizing radiation, viral or modified RNA interference with $^2$H-D-meditated genomic stability increases the frequency of integration.[11] This mechanism supports the previously suggested mRNA integration by the facultative reverse transcriptase function of DNA polymerase enzymes in some cells.[5]

## 2.1 | Half-lives and the biological safety of RNA species in human tissues

Stabilizing RNA by base structural modifications disrupts the inherent instability of messenger ribonucleic acid templates with far reaching biological safety consequences. Transcriptionally produced natural mRNAs including 5′-capped and/or 3′-terminated polyadenylated tail structures show 16.4 h half-lives in human blood, which is the shortest among circular RNAs (circRNAs; 24.56 ± 5.2 h), long non-coding RNAs (lncRNAs; 17.46 ± 3.0 h) and microRNAs (miRNAs; 16.4 ± 4.2 h). Quantitative experiments involving translational events related to mRNAs should be completed within 2 h.[19] For biological safety of preventing uncontrolled multiple translations, mRNA degradation occurs by spontaneous cleavage of the pentose sugar backbone moiety via its phosphodiester linkages and intramolecular transesterification reactions.[20] It is apparent that the protonation or deuteration state of the nucleophilic 2′-hydroxyl group is a critical determinant of the rate of RNA cleavage. The exact geometry of the chemical groups that compose each internucleotide bond has an important influence on cleavage activity as well. Under a broad range of physiological conditions, the rapid and spontaneous breakdown of RNA occurs to preserve genetic integrity by preventing the reverse transcriptase action of polymerase enzymes at its template substrate level, that is, to promptly deplete mRNA templates after translation, in mammalian cells. Additional mechanisms that rapidly dismantle RNA molecules include nucleases to cleave phosphodiester bonds between nucleotides, and ribozymes, involved in splicing. Mass worldwide deliveries of injectable modified mRNA species with unpredictable half-lives, some close to 30 days in human lymphatic tissues and heart muscle,[4] raise serious questions about the biological safety of translatable ribonucleotide therapies. The synthetic mRNAs such as those expressing the SARS-CoV-2 spike protein are equipped with analogue caps that further inhibit physiological mRNA decaying and recycling mechanisms in humans.

## 2.2 | Stable mRNAs, tumor cell metabolism, and deuterium

Nutritional and environmental factors that limit biological plasticity, consistent with slow mRNA turnover and degradation, heavily involve intracellular water chemistry and deutenomics. A recent report corroborates the prime role of intracellular water plasticity in the transformation between a normal and cancerous phenotype of human cells with aneuploidy.[21] While the dynamics of hydration (bulk) water molecules remain virtually unaffected when going from healthy to cancer cells, structured cytoplasmic water (particularly the rotational motions) undergoes significant plastic transformation upon normal-to-cancer transition. Stable ribonucleotide templates are required and available in processed carbohydrate dependent deuterated water loaded cancer cells either by glycolytic activity with semi-deuterated metabolic water formation[22] or by mitochondrial damage that compromises $^2$H-D-depleting proton exchange reactions in the Krebs–Szent–Györgyi cycle during Warburg fermentation.[23–25]

Our review of the deutenomics literature using novel compounds that target RNA virus replication and SARS-COV-2 vaccine development indicates that kinetic isotope effects of $^2$H-D overshadow such efforts via noncovalent interactions between biomolecules, including hydrogen bonding and ionic and van der Waals interactions.[26] For example aurintricarboxylic acid (ATA; Chemical Abstracts Service Registry Number 4431-00-9) strongly inhibits ribonuclease A (RNase; bovine, pancreatic) as a rapid mRNA breakdown inhibitor[27,28] for the development of such vaccines. On the other hand, in its deuterated form aurintricarboxylic acid readily binds to the enzyme's active site just as RNA does, exerting a 3- to 6-fold inhibitory effect on RNA breakdown.[29] This may contribute to the persistence of stable injected RNAs with unpredictable duration, while the potential medium- to long-term complications of such therapies may be devastating in humans.

Vaccines and other therapeutics are now being developed against single-stranded positive-sense enveloped RNA (+ssRNA) viruses such as polio- and yellow fever using $^2$H-D oxide (heavy water) as the stabilizing solvent.[30] Deuterons not only preserve RNA templates to persist in tissues and/or circulation using various mechanisms highlighted above but also trigger conformational changes in polymerase enzyme palm subdomains, catalytic sites and function, which may adversely affect cancer incidence and outcomes, among that of other diseases, after stable mRNA injections.

## 2.3 | Long persistence of injected mRNA with cardiac cell toxicity

Clinical studies report that SARS-CoV-2 mRNA vaccines persist up to 30 days in lymph nodes and the heart muscle at inflammation, fibrosis, and healing infarct sites.[4] Spikevax (mRNA-1273, Moderna) and Comirnaty (BNT162b2, Pfizer/Biontech) vaccines possess cardiac side effects, which for the most part can be classified by their

clinical symptoms as myo- and/or pericarditis. Cardiac complications can be caused by both mRNA-1273 and BNT162b2.[31] More specifically, mRNA-1273 induces arrhythmias as well as irregular contractions associated with localized calcium transients. These indicate significant dysfunction of the cardiac ryanodine receptor (RyR2) after mRNA-1273 injections. In contrast, an increased protein kinase A (PKA) activity was noted in cardiomyocytes using BNT162b2. Both RyR2 impairment and sustained PKA activation may significantly increase the risk of acute cardiac events[31] due to the long-term metabolic and inotropic weakening of cardiomyocytes as detailed below.

## 3 | LATENT SPIKE PROTEIN'S HARM ON THE CARDIAC FUNCTION

Not only may the injected mRNA molecules in vaccines persist for months, but also their recombinant spike protein products can remain in circulation for up to a little over half a year (187 days).[5] This fact deserves a careful look due to potential direct cardiotoxicity. Reports show a comparable rate of mRNA vaccine-related myocarditis after the second dose of the mRNA vaccine in 8 to 27 cases per 100 000 male individuals with that of 59 to 64 cases following SARS-CoV-2 infection (between 12 and 29 years of age).[32] A study including 303 nonvaccinated and 700 vaccinated patients showed that vaccinated patients had overall higher myocardial $^{18}$F-deoxyglucose (FDG) uptake, an indicator of severe cardiac muscle metabolic insufficiency typically as a result of ischemia, regardless of sex or age. Furthermore, severely increased myocardial $^{18}$F-FDG uptake was observed in patients imaged after their second vaccination with increased ipsilateral axillary lymph node uptake when compared with nonvaccinated patients.[33] Important outcomes of these studies are (A) the highly significant increase ($p < 0.001$; one occurrence by chance within a 1000-case cohort) in heart muscle standard deoxyglucose uptake value (SUV) by about 40% on average, and (B) increased glucose uptake was detected in non-symptomatic mRNA injected individuals up to half a year (180 days) after last injection. The latter finding indicates that not only do mRNA vaccinations cause asymptomatic myocardial inflammation, but the effect continues long after vaccination at 6 months later without apparent clinical symptoms.[34] The increasing number of clinically challenging cases of cardiovascular complications with obscure pathologies in modified mRNA injected individuals[35–39] clearly indicates the need to address mechanisms and counter actions to improve outcomes with the imminent suspension of such injections.[40]

### 3.1 | Deuterated proline-related structural rigidity of the pathogenic spike protein

The primary goal of this viewpoint article is to link the medical biochemistry, proteomics and deutenomics-related evidence to the pathogenic role of proline substitutions in recombinant spike protein as potential $^{2}$H-D (heavy hydrogen) binding sites with prominent isotopic stability, persistence and pathogenicity. Injectable mRNA preparations produce the full-length recombinant spike protein that differs from the WT spike protein only by two adjacent amino acid substitutions at positions 986 and 987, respectively. In short, one adjacent lysine and valine amino acids are replaced by a proline amino acid, each.[8,9]

(Hydroxy)Proline substitutions introduced by modified mRNA not only render trypsin digestion ineffective to break down spike proteins but are also targets of $^{2}$H-D accumulation as much as 2.5-fold above natural $^{2}$H-D enrichment under extreme biological conditions.[35] Sustained presence of pathogenic spike proteins after deuterated proline substitutions due to persisting modified mRNA injections with latent local and/or systematic inflammation should be considered a risk factor in vaccine developments for undesirable severe long-lasting complications and morbidities. Although the precise biochemical mechanisms are still in search and most likely involve rapid, compartmentalized and reversible water proton exchange isomerization reactions with significant $^{2}$H-D discrimination properties during aldose-ketose transformations in glycolysis,[23,24] $^{2}$H-D accumulation in (hydroxy)proline readily render such proteins almost impossible to destroy by biological and inorganic chemical reactions.

Carbon-bound $^{2}$H-D of proline is stable under the conditions of acid hydrolysis. When a sample of DL-proline containing 17.0 atom percent D was boiled with 20% HCl for 3 days, the proline isolated from the reaction solution was found to still contain 16.2 atom percent D. Thus, even under these drastic conditions, the exchange of deuterons with protons, if there is any, is very slow in this amino acid.[36] The long-lasting collagen protein preserving effect of (deuterated) proline is well known.[35] It is consistent with a previous model of pseudo-uridines at particular positions to induce the formation of a stable spike protein constitutively and exclusively via introducing double adjacent proline substitutions at critical amino acid positions into this pathogenic protein.[5] The incorporation of two proline residues surpasses resistance to degradation of peptides stabilized by amidation or acetylation, two approaches that are routinely utilized to stabilize drug peptides.[41]

### 3.2 | Cell transforming effect of proline substitutions in spike protein

Proline substitutions in all circulating proteins introduce fundamental biological changes due to altered folding and limited breakdown by exo- and endopeptidase catalyzed enzymatic reactions. In one simplified view, proline disrupts protein secondary structure by inhibiting the backbone to conform to an alpha-helix or beta-sheet conformation.[37] In turn, proline possesses multifaceted roles in cell behavior and contributes to the progression of devastating pathologies such as fibrosis and metastatic cancer.[38] Proline diminishes the ability of virtually all endopeptidases including trypsin, chymotrypsin, elastase, thermolysin, and pepsin to cleave adjacent peptide bonds. As a result, biological properties of the recombinant spike protein with vicinal proline substitutions can act as small proline-rich proteins with structural properties shifted towards that of the cornified envelope, which

provides structural stability to exert biological barrier functions and is recognized as a marker for terminal squamous cell differentiation.[39] Proline-rich proteins, especially adjoining proline structures, are rarely found in normal non-squamous tissues, and their increased expression has been reported in some types of non-squamous cell carcinoma, such as colorectal, breast and pancreatic cancer.[40,42] Proline-rich peptides and proteins are involved in the pathogenesis of non-squamous cellular transformation that may lead to unpredictable biological behaviors by worsening the prognosis of cancer. Aggressive malignancies take up proline rich proteins, such as collagen fragments, under nutrient limited conditions, whereby mostly protein-derived proline contributes to cancer cell metabolism.[43]

The administration of proline-stabilized immunoglobulin products in order to boost immune functions in patients with defects in proline metabolism are contraindicated.[44] This further highlights the close relationship between proline substitutions and potential deuteration with a crucial role in the long persistence of recombinant spike proteins that may collectively act in favor of proline rich extracellular matrix contamination. The cell transforming effect of recombinant spike proteins represents a nutrient reservoir for tumor cells highlighting the need to reevaluate the substitutions of this amino acid in mRNA-based vaccine and drug development efforts in the future. This is more so as clinical and translational observations in mRNA vaccinated individuals reveal numerous pathologies involving multifocal necrotizing encephalitis,[45] acute autoimmune myocarditis,[46] acute (epi-)myocarditis with patchy interstitial myocardial T-lymphocytic infiltration, mild myocyte damage,[47] myocarditis with regional dysfunction[48] and unbound spike antibodies of postvaccine myocarditis[49]; which are all very severe conditions.

### 3.3 | Frameshifting during mRNA transcription triggers off-target immunity

Surprisingly little is known about how ribonucleotide modifications affect protein synthesis despite their widespread use in mRNA vaccines. A very recent study found that there is a large increase in ribosomal +1 frameshifting during translation of 1-methylΨ (pseudouridine) mRNA, which produced both the expected in-frame product and two additional bands at higher molecular weight.[50] After vaccination of mice with BNT162b2 there was an apparent T cell response to both the in-frame SARS-CoV-2 spike protein and that of the +1 frameshifted products. The clinically approved SARS-CoV-2 mRNA vaccines also produced +1 ribosome frameshifting during recombinant antigen mRNA translation that readily elicited off-target cellular immune responses. The study found that responses to +1 frameshifted spike peptides were significantly increased in vaccinated mice compared to untreated mice, which suggested that +1 frameshifted products encoded in BNT162b2 spike mRNA are T cell antigens for inbred mice.

In a subsequent investigation of 21 individuals vaccinated with BNT162b2 and 20 controls there was a significantly higher interferon (IFNγ) response to +1 frameshifted antigen in the BNT162b2 vaccine group while there was no association between T cell responses to +1 frameshifted antigen and age, sex or HLA subtype.[50] Cellular immune responses to +1 frameshifted products were observed only in individuals vaccinated with BNT162b2. These data suggest that vaccination with 1-methylΨ mRNA can elicit cellular immunity to peptide antigens produced by +1 ribosomal frameshifting in major histocompatibility complex (MHC)-diverse people and MHC-uniform mice. Further studies to gain insight into +1 ribosome frameshifting during translation of 1-methylΨ mRNA using liquid chromatography tandem mass spectrometry (LC–MS/MS) identified a library of six in-frame peptides and nine peptides derived from the mRNA +1 frame. All in-frame peptides were mapped to the N-terminal region of the spike protein, whereas +1 frameshifted peptides were mapped downstream. These data demonstrated that the elongated polypeptide was indeed a chimeric product consisting of in-frame N-terminal residues and +1 frameshifted C-terminal residues. Alongside the above impact on host T cell immunity, the off-target effects of ribosomal frameshifting could include increased production of new B cell antigens with far reaching medical consequences that explain clinically observed increases in muscle inflammation, or the highly significant increase in glucose uptake of heart muscle cells as described above.

## 4 | SUMMARY AND CONCLUSIONS

Trends in mRNA vaccine and medicine development efforts ignore many basic principles of medical biochemistry, physiology, proteomics and deutenomics. Although in vitro/vivo–transcribed mRNAs encoding clinically important proteins have broad potentials for therapeutic applications,[51–53] mRNA modified by pseudouridination and other changes, including methylation, is infeasible for clinical use because of its long-lasting and potentially permanent and immunostimulatory nature. Nucleoside modification is believed to be an effective approach to enhance stability by making the product resistant to ribonucleases. As a result, there is increased translational capacity of mRNA while potentially diminishing its immunogenicity in vivo. The persistent nature of mRNA coding for SARS-CoV-2 spike protein provides a dangerously long exposure to an unlimited dose of this pathogenic protein, and thus, it needs re-evaluation for continued human use. We have provided the molecular basis for a wide distribution of injuries, disabilities, and deaths resulting from spike protein-related diseases, which derive from ill-advised continued use of these products. Understanding the above overarching proteomics and deutenomics mechanisms, especially in cell growth and transformation,[25] in modified mRNA vaccines-related severe adverse events is necessary for a scientifically informed benefit/risk evaluation of such vaccinations.

### AUTHOR CONTRIBUTIONS

All authors were involved in the conception, drafting, and execution of the manuscript, as well as its critical editing and revisions. AMK performed the literature review of vaccination-related aseptic inflammatory reactions and provided discussions. CB and MP performed in-frame and reviewed frameshifted protein analyses with discussions. PAM and SS prepared final discussions and conclusions regarding

cardiology outcomes as well as environment-/nutrition-related sciences. LGB designed the flow of reviewed material for this review and provided all deutenomics discussions. All authors critically reviewed the final version of this manuscript and approved its submission.

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## DATA AVAILABILITY STATEMENT

All data used in this article were retrieved from peer reviewed items in the literature, as cited. The data and compilation strategy that support the findings of this study are available on request from the corresponding author.

## ETHICS STATEMENT

Ethical approval was not sought for this study because the data to be collected are not linked to individuals. Citing peer reviewed literature is also exempt from the Institutional Review Board (IRB) surveillance.

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