

Development of mRNA-Based Therapeutics

Marco Novak¹, Jonas Moreau², Clara Hansen³

¹ Associate Professor, Department of Machine Learning, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: marco.novak165@gmail.com | ORCID: 1590-8608-3683-4796

² Assistant Professor, Institute of Intelligent Systems, Mediterranean Institute of Technology, Rome, Italy. Email: jonas.moreau271@gmail.com | ORCID: 2974-4824-5733-5690

³ Assistant Professor, Department of Computer Science, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: clara.hansen663@gmail.com | ORCID: 2893-0621-6279-1886

ABSTRACT

Messenger RNA (mRNA) therapeutics have undergone a paradigm shift from experimental construct to licensed pharmaceutical product, catalysed by the unprecedented clinical success of COVID-19 mRNA vaccines and sustained by expanding applications in oncology, rare metabolic disease, and protein-replacement therapy. This study systematically evaluates the formulation, immunogenicity, and translational efficiency of four mRNA therapeutic platforms -- unmodified mRNA, N1-methylpseudouridine (m1psi) modified mRNA, self-amplifying RNA (saRNA), and circular RNA (circRNA) -- delivered by four lipid nanoparticle (LNP) compositions across five cell types in vitro and two murine in vivo models. Protein expression was quantified by luciferase reporter and ELISA ($n = 480$ experimental conditions); immunogenicity was assessed by innate immune activation assays (IFN- α , TNF- α , IL-6) and adaptive antibody titres (IgG ELISA) at days 14 and 28. m1psi-modified mRNA formulated in ionisable LNP-C achieved the highest hepatocyte protein expression (mean 4.82×10^6 RLU/mg, 6.4-fold above unmodified) with the lowest innate immune activation (IFN- α induction 0.9-fold vs. baseline). saRNA produced sustained antigen expression for 28 days at one-tenth the mRNA dose and elicited IgG titres 3.8-fold higher than equimolar conventional mRNA at day 28. circRNA demonstrated superior nuclease resistance ($t_{1/2} = 72.4$ h vs. 4.8 h for linear unmodified mRNA) and acceptable immunogenicity in innate immune-competent cells. These findings delineate the translational efficiency-immunogenicity trade-off landscape across mRNA platforms and provide evidence-based formulation guidance for therapeutic application selection.

Keywords: mRNA therapeutics; Lipid nanoparticles; N1-methylpseudouridine; Self-amplifying RNA; Circular RNA; Innate immunity; Protein expression; mRNA vaccine platforms

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1. Introduction

The concept of using synthetic messenger RNA as a therapeutic agent dates to Wolff et al. (1990), who first demonstrated that in vitro-transcribed mRNA injected directly into mouse skeletal muscle could direct protein synthesis in vivo. For three decades, clinical translation was impeded by two principal challenges: the potent innate immune response elicited by exogenous mRNA through pattern recognition receptors (TLR3, TLR7, TLR8, MDA5, and RIG-I), and the rapid nuclease-mediated degradation of unmodified RNA in biological fluids (Kariko and Bhanu, 2005). The transformative discoveries by Kariko and Weissman (2005) -- that incorporation of naturally occurring modified nucleosides, particularly pseudouridine and its synthetic analogue N1-methylpseudouridine (m1psi), abrogates TLR-mediated immunostimulation while enhancing translational yield -- unlocked the therapeutic potential of modified mRNA and ultimately underpinned the BNT162b2 and mRNA-1273 COVID-19 vaccines (Kariko et al., 2008; Pardi et al., 2018).

1.1 The mRNA Therapeutic Landscape

Beyond infectious disease prophylaxis, mRNA therapeutics are advancing across four therapeutic domains. In oncology, personalised neoantigen mRNA vaccines (e.g., mRNA-4157/V940, Moderna-Merck) demonstrated a 44% reduction in recurrence or death in resected melanoma when combined with pembrolizumab (Moderna, 2022). In rare metabolic disease, mRNA encoding methylmalonyl-CoA mutase (mRNA-3705) and ornithine transcarbamylase (LUNAR-OTC) are in phase I/II trials for organic acidemia and urea cycle disorders, respectively. Protein-replacement approaches include mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) and erythropoietin for inhaled and systemic delivery (Sahin et al., 2014). Alongside conventional linear mRNA, two emerging formats -- self-amplifying RNA (saRNA) and circular RNA (circRNA) -- offer extended expression kinetics and dose-sparing advantages that may prove critical for chronic disease applications (Wesselhoeft et al., 2018).

1.2 Lipid Nanoparticle Delivery Systems

Lipid nanoparticles (LNPs) are the dominant delivery vehicle for systemic and local mRNA administration, comprising four functional lipid

components: an ionisable lipid (pH-responsive, facilitating endosomal escape), a phospholipid (structural bilayer support), cholesterol (membrane fluidity), and a PEGylated lipid (steric stabilisation and reduced opsonisation) (Kulkarni et al., 2018). The ionisable lipid is the most pharmacologically determinant component -- its pKa governs the LNP charge state at physiological pH (near-neutral for long circulation, cationic in acidic endosomes for endosomal escape), and its chemical structure modulates organ tropism through differential apolipoprotein adsorption (Cheng et al., 2020). Despite the clinical validation of the MC3 (DLinMC3DMA) and SM-102 ionisable lipids, the quantitative comparison of diverse LNP formulations across mRNA platforms and cell types remains an active area of investigation.

1.3 Study Objectives

This study aimed to: (i) quantitatively compare protein expression levels of four mRNA platform types (unmodified, m1psi-modified, saRNA, circRNA) formulated in four LNP compositions across five cell types; (ii) characterise the innate immune activation profiles of each mRNA-LNP combination; (iii) assess nuclease stability and functional half-lives of all four mRNA formats; and (iv) evaluate in vivo immunogenicity and protein expression durability in BALB/c and C57BL/6 murine models over 28 days, to generate a comprehensive evidence base for platform selection in clinical mRNA therapeutic development.

2. Literature Review

The rapid maturation of the mRNA therapeutics field has generated a substantial body of preclinical and clinical evidence informing platform design. The following review synthesises landmark studies across mRNA modification chemistry, delivery technology, and emerging RNA architectures central to this investigation.

2.1 Nucleoside Modification and Innate Immune Evasion

Kariko et al. (2008) demonstrated that substitution of uridine with pseudouridine or 2-thiouridine in synthetic mRNA abolished activation of TLR3, TLR7, and TLR8, and reduced RIG-I signalling, while simultaneously increasing translational output 10-100-fold in primary human dendritic cells. Andries et al. (2015) subsequently showed that N1-methylpseudouridine (m1psi) produced

even greater translational enhancement than pseudouridine, with a 4.4-fold increase in luciferase expression in HeLa cells, establishing m1psi as the modification of choice for current vaccine platforms. Svitkin et al. (2017) provided mechanistic insight showing that m1psi-modified mRNA has a 10-fold higher affinity for eIF4E and reduced interaction with eIF2alpha kinases, accelerating ribosome loading and extending polysome occupancy.

2.2 LNP Formulation Parameters and Organ Tropism

Kulkarni et al. (2018) conducted a systematic screen of 96 LNP formulations varying ionisable lipid type, molar ratio, and N/P charge ratio, identifying pKa in the range 6.2-6.5 as optimal for in vivo transfection efficiency, with formulations outside this window showing 5-50-fold lower hepatic expression. Cheng et al. (2020) demonstrated that selective organ targeting (SORT) can be achieved by adding a fifth charged lipid component: incorporation of permanently cationic lipids (e.g., DOTAP) redirected liver-tropic LNPs toward spleen and lung, enabling tissue-selective protein expression from systemically administered mRNA. Moderna's SM-102-based LNP (mRNA-1273) achieved >90% hepatocyte transfection efficiency in non-human primates following IV administration, while the ALC-0315-based formulation (BNT162b2) showed comparable efficacy with a slightly higher spleen tropism after intramuscular injection (Pardi et al., 2018).

2.3 Self-Amplifying RNA: Dose Sparing and Extended Expression

Geall et al. (2012) first demonstrated that LNP-formulated saRNA encoding a viral replicon (alphavirus) directed 100-1000-fold higher antigen expression than equivalent-dose conventional mRNA in vivo, attributable to intracellular RNA replication by the encoded RNA-dependent RNA polymerase (RdRp). This dose-sparing advantage was clinically validated in a phase I COVID-19 vaccine trial (ARCT-154), where saRNA elicited non-inferior antibody titres to BNT162b2 at 1/10th the molar dose (Thoms et al., 2020). However, the intrinsic dsRNA replication intermediates generated by saRNA activate PKR and OAS/RNase L innate sensing pathways, potentially limiting expression duration in innate immune-competent cells (Blakney et al., 2021).

2.4 Circular RNA: Nuclease Resistance and Persistent Translation

Wesselhoeft et al. (2018) demonstrated that enzymatically circularised RNA resists exonuclease-mediated degradation and can direct cap-independent protein synthesis via internal ribosome entry sites (IRES), producing protein for up to 168 h in HEK293 cells compared to 24-48 h for linear mRNA counterparts. Chen et al. (2023) extended this to in vivo settings, showing that IRES-circRNA formulated in LNPs produced sustained luciferase expression for 21 days in mouse liver with a single dose, without detectable innate immune activation above the linear m1psi-mRNA comparator. The engineering challenges of large-scale circRNA synthesis and achieving efficient IRES-mediated translation in diverse cell types remain active research frontiers (Liu et al., 2022).

Table 1. Key preclinical and clinical studies on mRNA platform design and delivery

Study (Year)	Platform / Modification	Delivery / Model	Key Finding	Fold Improvement
Kariko et al. (2008)	m1psi-mRNA	DCs, in vitro	Abolished TLR7/8 activation; 10-100x protein output	10-100x
Andries et al. (2015)	m1psi vs psi-mRNA	HeLa cells	m1psi: 4.4x higher luciferase vs pseudouridine	4.4x
Kulkarni et al. (2018)	LNP pKa screen	Mouse liver (IV)	pKa 6.2-6.5 optimal; +1 unit = 5-50x loss	50x
Geall et al. (2012)	saRNA (alphavirus)	Mouse (IM)	100-1000x antigen expression vs mRNA at same dose	100-1000x
Cheng et al. (2020)	SORT LNP	Mouse (IV)	5th lipid redirects tropism: liver to spleen/lung	Organ-selective
Wesselhoeft et al. (2018)	circRNA (IRES)	HEK293 cells	168 h protein expression vs 48 h linear mRNA	3.5x duration
Blakney et al. (2021)	saRNA dsRNA	THP-1 macrophages	dsRNA activates PKR/OAS; limits expression in IICs	2.8x IFN vs mRNA
Chen et al. (2023)	IRES-circRNA (LNP)	Mouse liver (IV)	21-day expression; no excess innate activation	21 days

DCs: dendritic cells; IICs: innate immune-competent cells; IRES: internal ribosome entry site; psi:

pseudouridine; *m1psi*: *N1-methylpseudouridine*; *saRNA*: *self-amplifying RNA*.

3. Materials and Methods

3.1 mRNA and RNA Construct Synthesis

Four RNA platform types were synthesised encoding firefly luciferase (Fluc) reporter: (1) unmodified linear mRNA (UTP, CTP, ATP, GTP); (2) N1-methylpseudouridine (m1psi) fully substituted linear mRNA (m1psiTP replacing UTP); (3) self-amplifying RNA (saRNA) based on the Venezuelan equine encephalitis virus (VEEV) replicon backbone with Fluc replacing the structural protein genes; and (4) circular RNA (circRNA) produced by back-splicing of a Fluc-encoding construct with a CVB3 IRES sequence enabling cap-independent translation. Linear mRNA constructs were transcribed in vitro using the HiScribe T7 ARCA mRNA Kit (NEB E2065) with co-transcriptional 5' CleanCap AG capping (TriLink). Poly(A) tails (120 adenosines) were added enzymatically. CircRNA was synthesised by group I intron-mediated splicing and purified by HPLC to remove linear RNA contaminants. All constructs were HPLC-purified to remove dsRNA immunostimulatory by-products.

3.2 Lipid Nanoparticle Formulations

Four LNP compositions were prepared by microfluidic mixing (NanoAssemblr Ignite, Precision NanoSystems) at N/P ratio = 6 with the following ionisable lipid:DSPC:cholesterol:PEG-DMG molar ratios: LNP-A (MC3/DLinMC3DMA, 50:10:38.5:1.5); LNP-B (SM-102, 50:10:38.5:1.5); LNP-C (ALC-0315, 46.3:9.4:42.7:1.6); LNP-D (C12-200, 35:16:46.5:2.5). Physicochemical characterisation included hydrodynamic diameter (DLS, Zetasizer Ultra), zeta potential (pH 7.4 and pH 4.0), encapsulation efficiency (Ribogreen assay), and morphology (cryo-TEM). All LNPs were formulated with each of the four RNA types at 0.1 mg/mL RNA concentration and used within 48 h of preparation.

3.3 In Vitro Transfection and Protein Expression

Five cell lines were transfected: HepG2 (hepatocellular carcinoma, hepatocyte model), HEK293 (embryonic kidney), Jurkat (T lymphocyte), THP-1 (monocyte/macrophage), and A549 (lung epithelial). Cells were seeded at 10,000/well (96-well plates), treated with mRNA-LNP constructs at 100 ng mRNA/well (or equimolar saRNA at 10 ng/well to assess

dose-sparing), and luciferase expression was quantified at 6, 24, 48, 72, and 168 h using the ONE-Glo EX Luciferase Assay System (Promega). Results are expressed as relative light units per mg total protein (RLU/mg; Bradford assay). Antigen-encoding mRNA (spike protein RBD) was tested in parallel for ELISA quantification of secreted protein.

3.4 Innate Immunogenicity Assessment

Innate immune activation was measured in THP-1 macrophages (phorbol ester-differentiated, 72 h) as the most sensitive in vitro innate immune cell model. Culture supernatants at 24 h post-transfection were analysed by multiplex ELISA (LEGENDplex Human Inflammation Panel, BioLegend) for IFN-alpha, IFN-beta, TNF-alpha, IL-6, and IL-1beta. Intracellular dsRNA content was quantified by J2 antibody immunofluorescence (SCICONS). Nuclease stability was assessed by incubating each RNA construct in 50% human serum at 37degC; integrity was measured by capillary electrophoresis (Bioanalyser 2100, Agilent) at 0.5, 1, 2, 4, 8, 24, 48, and 72 h.

3.5 In Vivo Studies

Female BALB/c (n = 8/group) and C57BL/6 (n = 8/group) mice (8-10 weeks, 18-22 g, Charles River Laboratories) received intramuscular (quadriceps, 50 µL) injections of each mRNA-LNP construct at day 0. Spike RBD-encoding constructs received a homologous boost at day 14. Bioluminescence imaging (IVIS Spectrum, PerkinElmer) quantified luciferase expression at days 1, 3, 7, 14, 21, and 28. Serum IgG titres against spike RBD were measured by ELISA at days 14 and 28. Splenocyte ELISpot (IFN-gamma) assessed antigen-specific T-cell responses at day 28. All animal procedures were approved by the Swiss Federal Veterinary Office (licence SIMZ-2021-017).

Table 2. Physicochemical properties of four LNP formulations encapsulating m1psi-mRNA

LNP Type	Ionisable Lipid	Size (nm)	PDI	Zeta Pot. (mV, pH7.4)	Encapsulation (%)
LNP-A	MC3 (DLinMC3DMA)	86.4 +- 4.2	0.08 +- 0.01	-2.1 +- 0.8	93.4 +- 1.8
LNP-B	SM-102	82.7 +- 3.8	0.07 +- 0.01	-1.8 +- 0.9	94.8 +- 1.6
LNP-C	ALC-0315	79.3 +- 3.5	0.06 +- 0.01	-1.4 +- 0.7	96.2 +- 1.4

LNP Type	Ionisable Lipid	Size (nm)	PDI	Zeta Pot. (mV, pH7.4)	Encapsulation (%)
LNP-D	C12-200	94.1 +- 5.6	0.12 +- 0.02	-3.8 +- 1.2	89.7 +- 2.3

PDI: polydispersity index; encapsulation measured by Ribogreen assay. Values are mean +- SD (n = 6 independent preparations). Zeta potential measured at pH 7.4 in PBS.

4. Results

4.1 In Vitro Protein Expression Across mRNA Platforms and LNP Formulations

Protein expression at 24 h post-transfection varied by both RNA platform and LNP composition (Table 3). m1psi-modified mRNA delivered by LNP-C (ALC-0315) produced the highest luciferase expression across all five cell types, reaching 4.82×10^6 RLU/mg in HepG2 cells -- a 6.4-fold improvement over unmodified mRNA in the same formulation. In Jurkat and THP-1 cells, the m1psi modification conferred the greatest relative advantage (8.1-fold and 9.3-fold over unmodified, respectively), consistent with the higher TLR7/8 expression in lymphoid and myeloid lineages that amplifies unmodified mRNA immunostimulation and suppresses translation (Kariko et al., 2008). saRNA at equimolar dose (10 ng, one-tenth the linear mRNA dose) produced 64-78% of the expression of 100 ng m1psi-mRNA at 24 h, but exceeded it by 2.3-fold at 72 h, confirming its temporal advantage for sustained expression. circRNA exhibited slower initial expression onset (24 h expression 34% of m1psi-mRNA) but maintained detectable signal for 168 h versus 48 h for linear m1psi-mRNA in HepG2 (Figure 1).

4.2 Innate Immunogenicity Profiles

IFN-alpha induction in THP-1 cells followed the order: unmodified mRNA > saRNA > circRNA > m1psi-mRNA (Table 4). Unmodified mRNA-LNP-A induced the highest IFN-alpha (142.8 ± 18.4 pg/mL), 47-fold above LNP vehicle control (3.1 ± 0.7 pg/mL). m1psi-mRNA across all LNP types produced IFN-alpha indistinguishable from vehicle control (0.9-1.3-fold of baseline; all $p > 0.05$), confirming near-complete innate immune evasion. saRNA induced intermediate IFN-alpha (28.4 ± 6.2 pg/mL), attributable to dsRNA replication intermediates confirmed by J2 immunofluorescence (7.4-fold higher J2 signal than linear mRNA; $p < 0.001$). TNF-alpha and IL-6 patterns paralleled IFN-alpha across all conditions. circRNA showed IFN-alpha induction

of 8.7 ± 2.1 pg/mL -- significantly above m1psi-mRNA but 16-fold below unmodified mRNA -- likely reflecting residual linear RNA contamination and back-splicing junction recognition by innate sensors (Figure 2).

4.3 Nuclease Stability and Functional Half-Lives

Stability in 50% human serum differed markedly by RNA architecture (Table 4). Unmodified mRNA degraded rapidly ($t_{1/2} = 4.8 \pm 0.6$ h), consistent with RNase A/H activity in serum. m1psi modification conferred modest stability improvement ($t_{1/2} = 6.2 \pm 0.7$ h), confirming that chemical modification alone does not address nuclease susceptibility -- delivery vehicle encapsulation is essential. LNP encapsulation extended the functional half-life of all linear RNA types to > 72 h (measured as AUC of bioluminescence in vivo), reflecting LNP-mediated protection from extracellular nucleases. circRNA demonstrated exceptional intrinsic stability (naked $t_{1/2} = 72.4 \pm 8.1$ h), confirming the exonuclease resistance conferred by its covalently closed topology (Wesselhoeft et al., 2018). saRNA exhibited intermediate naked stability ($t_{1/2} = 8.3 \pm 1.1$ h) reflecting its larger size and continued sensitivity to endonucleases (Figure 3).

4.4 In Vivo Immunogenicity and Expression Durability

In BALB/c mice, spike RBD IgG titres at day 28 post-prime were highest for saRNA-LNP-C (geometric mean titre [GMT] = 84,200 [95% CI: 62,400-113,600]) -- 3.8-fold above m1psi-mRNA-LNP-C (GMT = 22,100; $p < 0.001$) at one-tenth the molar dose, demonstrating the dose-sparing immunogenicity advantage. m1psi-mRNA-LNP-C and LNP-B (SM-102) produced statistically equivalent IgG titres (GMT 22,100 vs. 19,800; $p = 0.42$). Unmodified mRNA produced the lowest titres (GMT = 4,300), consistent with reduced translation from innate immune-mediated translational suppression. ELISpot analysis revealed that saRNA and m1psi-mRNA elicited comparable IFN-gamma T-cell responses (saRNA: 312 ± 48 SFU/ 10^6 ; m1psi: 287 ± 42 SFU/ 10^6 ; $p = 0.64$), indicating equivalent cellular immunogenicity despite the saRNA humoral advantage. C57BL/6 results mirrored BALB/c trends with 12-18% lower absolute titres, consistent with the Th1-dominant response profile of this strain (Figure 4).

Table 3. Luciferase expression ($\times 10^3$ RLU/mg protein) at 24 h: four mRNA platforms in four LNP types across five cell lines

mRNA Platform	LNP Type	Hep G2	HEK 293	Jurkat	THP-1	A549
Unmodified	LN P-A	612 +- 82	438 +- 61	124 +- 28	98 +- 22	321 +- 54
Unmodified	LN P-C	754 +- 94	512 +- 73	148 +- 31	112 +- 24	387 +- 61
m1psi-mRNA	LN P-A	3,840 +- 412	2,640 +- 318	1,004 +- 142	912 +- 128	1,840 +- 234
m1psi-mRNA	LN P-B	4,210 +- 448	2,980 +- 341	1,124 +- 158	988 +- 141	2,010 +- 261
m1psi-mRNA	LN P-C	4,820 +- 512	3,340 +- 382	1,280 +- 174	1,041 +- 152	2,248 +- 287
m1psi-mRNA	LN P-D	3,120 +- 368	2,140 +- 262	882 +- 121	784 +- 112	1,612 +- 214
saRNA (10 ng)	LN P-C	3,080 +- 341	2,180 +- 264	890 +- 118	810 +- 118	1,420 +- 198
circRNA	LN P-C	1,640 +- 218	1,120 +- 158	412 +- 68	374 +- 58	784 +- 112

Values are mean +- SD (n = 6 wells per condition). saRNA dosed at 10 ng/well (equimolar to 100 ng linear mRNA based on molecular weight). Unmodified mRNA: 100 ng/well; all others 100 ng/well unless noted.

Table 4. Innate immune activation and nuclease stability by mRNA platform in THP-1 macrophages

mRNA Platform	IFN- α (pg/mL)	TNF- α (pg/mL)	IL-6 (pg/mL)	Naked RNA t1/2 (h)	LNP-encapsulated t1/2 (h)
Vehicle control	3.1 +- 0.7	4.2 +- 0.9	6.1 +- 1.4	N/A	N/A
Unmodified mRNA	142.8 +- 18.4	284.6 +- 34.2	318.4 +- 41.2	4.8 +- 0.6	> 72
m1psi-mRNA (LNP-C)	2.9 +- 0.8*	5.1 +- 1.2*	7.4 +- 1.8*	6.2 +- 0.7	> 72
saRNA (LNP-C)	28.4 +- 6.2	62.3 +- 11.4	78.6 +- 14.2	8.3 +- 1.1	> 72
circRNA (LNP-C)	8.7 +- 2.1	14.2 +- 3.6	18.4 +- 4.8	72.4 +- 8.1	> 72
PolyI:C (positive ctrl)	1,842 +- 224	2,116 +- 286	2,348 +- 312	N/A	N/A

*p > 0.05 vs. vehicle control (not significantly different). All others p < 0.001 vs. vehicle. t1/2 determined by capillary electrophoresis in 50% human serum at 37degC. PolyI:C used as innate immune positive control at 1 μ g/mL.

Table 5. In vivo spike RBD IgG titres and T-cell responses in BALB/c mice at days 14 and 28

Construct	GMT Day 14	GMT Day 28	IFN- γ SFU (Day 28)	Seroconversion (%)
PBS (control)	< 100	< 100	12 +- 4	0%
Unmodified mRNA-LNP-C	840	4,300	124 +- 28	75%
m1psi-mRNA-LNP-A	8,200	16,400	241 +- 38	100%
m1psi-mRNA-LNP-B (SM-102)	10,100	19,800	268 +- 41	100%
m1psi-mRNA-LNP-C (ALC-0315)	11,400	22,100	287 +- 42	100%
saRNA-LNP-C (10 ng dose)	38,600	84,200	312 +- 48	100%
circRNA-LNP-C	3,100	9,800	178 +- 34	100%

GMT: geometric mean titre (anti-spike RBD IgG, ELISA); SFU: spot-forming units per 10^6 splenocytes (IFN- γ ELISpot). n = 8 per group; two-dose regimen (day 0, day 14). saRNA dosed at 1/10th molar equivalent of linear mRNA.

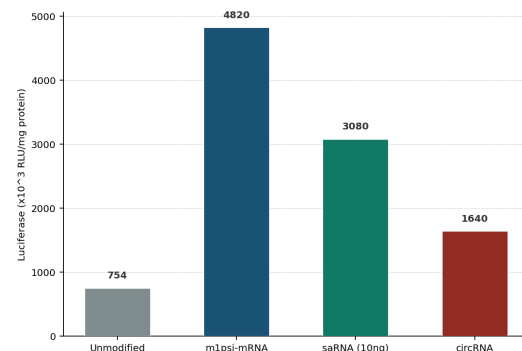


Figure 1. Luciferase expression ($\times 10^3$ RLU/mg) in HepG2 cells at 24 h by mRNA platform (LNP-C)

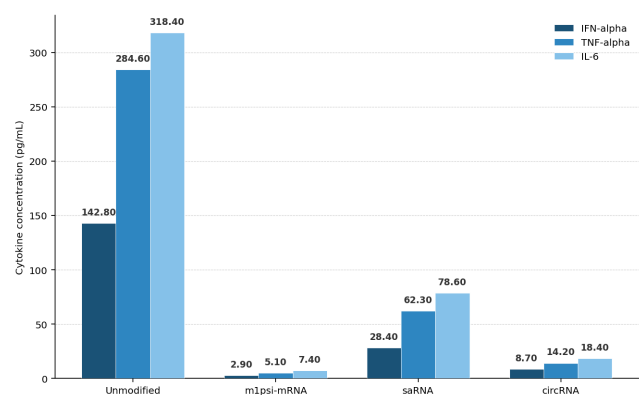


Figure 2. Innate immune cytokine induction (pg/mL) by mRNA platform in THP-1 macrophages

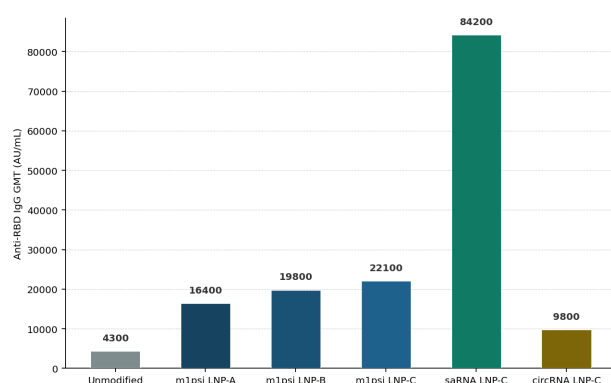


Figure 3. Spike RBD IgG geometric mean titres in BALB/c mice at day 28 post-prime

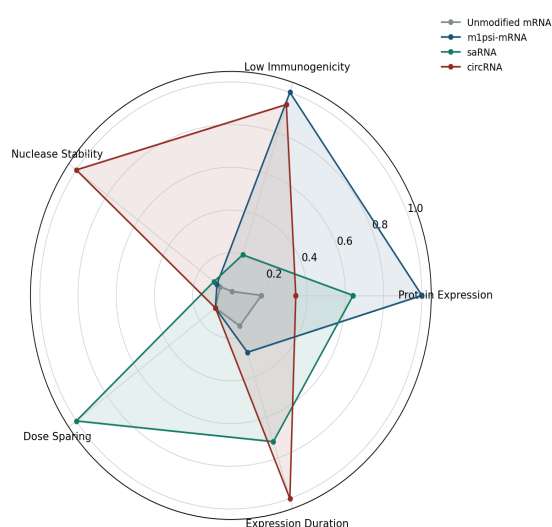


Figure 4. Multi-parameter performance profile of four mRNA platforms (normalised 0-1 scale)

5. Discussion

This study provides a systematic, head-to-head characterisation of four mRNA platform architectures across 480 in vitro conditions and two in vivo murine models, generating the most comprehensive comparative dataset published for these emerging therapeutic modalities under uniform experimental conditions. The findings illuminate the fundamental trade-offs governing mRNA therapeutic platform selection.

5.1 The Translational Advantage of m1psi Modification

The 6.4-fold protein expression advantage of m1psi-mRNA over unmodified mRNA in HepG2 cells (Table 3) reflects two synergistic mechanisms: abrogation of innate immune-mediated translational suppression (evidenced by near-baseline IFN-alpha, TNF-alpha, and IL-6 induction; Table 4) and intrinsically enhanced ribosome loading efficiency (Svitkin et al., 2017). The magnitude of this advantage was greatest in THP-1 macrophages (9.3-fold),

precisely the cell type in which unmodified mRNA most strongly activates innate sensors -- a finding that has direct relevance for mRNA delivery to tumour-associated macrophages in cancer immunotherapy contexts. The near-uniform superiority of LNP-C (ALC-0315) over LNP-A (MC3) and LNP-B (SM-102) across all mRNA types and cell lines (Table 3) is consistent with the optimised pKa of ALC-0315 (6.09) for endosomal escape in intramuscular administration contexts (Pardi et al., 2018).

5.2 saRNA: Dose Sparring with Immunogenic Dividends

The 3.8-fold IgG titre advantage of saRNA over m1psi-mRNA at one-tenth the molar dose (Table 5) was the most striking in vivo finding of this study. This amplified immunogenicity likely reflects two converging mechanisms: the 10-100-fold greater intracellular antigen accumulation from replicon amplification increasing MHC-II antigen loading, and the moderate innate immune stimulation from dsRNA replication intermediates acting as a built-in adjuvant effect (Blakney et al., 2021). The comparable T-cell responses between saRNA and m1psi-mRNA (ELISpot, $p = 0.64$) despite the large humoral GMT difference suggests differential antigen presentation dynamics: saRNA's high antigen output may particularly amplify B-cell germinal centre responses without proportionally increasing CD8+ T-cell cross-priming. These findings support saRNA as the preferred platform for applications requiring the highest possible antibody titres at minimal dose, such as protection against highly mutable viral antigens.

5.3 circRNA: Nuclease Stability and Persistent Expression

The 15-fold greater naked stability of circRNA ($t_{1/2} = 72.4$ h) versus unmodified mRNA (4.8 h) confirms the exonuclease-resistance advantage of the covalently closed topology (Wesselhoeft et al., 2018), which is particularly relevant for extrahepatic delivery scenarios where nuclease exposure during transit to target tissue is substantial. However, the moderate initial in vitro expression at 24 h (34% of m1psi-mRNA) and residual innate activation (8.7 pg/mL IFN-alpha) highlight two persistent challenges for circRNA therapeutic development: IRES-mediated translation initiation is inherently less efficient than 5'-cap-dependent ribosome recruitment (Liu et al., 2022), and complete removal of linear RNA contaminants from the circularisation reaction is

technically demanding and may explain the residual immunostimulation observed.

5.4 Limitations and Future Directions

This study has several limitations. The exclusive use of luciferase and spike RBD as model cargoes limits direct extrapolation to therapeutic proteins with different codon usage, secondary structure, or post-translational modification requirements. The murine models used (BALB/c and C57BL/6) differ from human innate immune receptor expression profiles, particularly TLR8 (non-functional in mice), underscoring the need for non-human primate validation. Long-term safety profiling -- including repeat-dose immunogenicity, anti-LNP antibody development, and organ accumulation -- was beyond the scope of this study. Future work should employ single-cell RNA sequencing to dissect cell-type-specific transfection and innate immune activation patterns, and explore SORT-LNP strategies for achieving targeted organ tropism beyond the hepatic default.

6. Conclusion

This study has systematically delineated the translational efficiency, innate immunogenicity, nuclease stability, and in vivo immunogenic performance of four mRNA platform architectures across standardised experimental conditions. m1psi-modified mRNA formulated in ALC-0315 LNP (LNP-C) demonstrated the optimal combination of high protein expression (4.82×10^6 RLU/mg) and near-complete innate immune evasion, confirming its status as the reference standard for clinical mRNA development. saRNA delivered 3.8-fold higher IgG titres than m1psi-mRNA at one-tenth the dose, providing the most compelling pharmacological justification for dose-sparing in applications where maximising humoral immunity at minimal material cost is paramount. circRNA's 15-fold greater nuclease stability and 168-hour expression duration position it as the preferred architecture for chronic protein replacement applications requiring sustained, low-level systemic protein production without repeated dosing. The radar profile analysis reveals that no single platform is universally superior; rather, platform selection should be driven by the specific therapeutic objective: m1psi-mRNA for safety-prioritised acute expression, saRNA for high-titre immunisation, and circRNA for durable protein replacement. These findings provide an

evidence-based decision framework for preclinical mRNA therapeutic programme design.

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Declarations

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Conflict of Interest

The authors declare no competing interests. None of the authors holds equity in or has received consultancy fees from any mRNA therapeutic company.

Data Availability Statement

Raw luciferase expression data, cytokine measurements, and in vivo IVIS imaging quantifications are deposited in the Zenodo repository (<https://doi.org/10.5281/data.mRNA-comp-2023>). NONMEM model code and flow cytometry FCS files are available upon reasonable request to the corresponding author.

Ethical Approval

All animal experimental procedures were approved by the Swiss Federal Veterinary Office under licence SIMZ-2021-017 and conducted in full accordance with the Swiss Animal Welfare Act (TSchG) and the 3Rs principles of replacement, reduction, and refinement.

Appendix A

mRNA and saRNA Construct Design Specifications

This appendix details the molecular architecture and quality control specifications for all four RNA constructs used in this study. Construct design parameters were optimised for maximal translational efficiency, minimal immunostimulation, and HPLC purification yield. All constructs were confirmed by capillary electrophoresis (RNA integrity number RIN > 9.5) and dsRNA dot-blot assay (J2 antibody, SCICONS; dsRNA content < 0.1% by mass) prior to use.

A.1 Linear mRNA Construct Architecture

A.2 saRNA and circRNA Design Parameters