

IN SILICO DESIGN OF A MULTI-EPITOPE VACCINE AGAINST THE INFLUENZA B VIRUS MATRIX 1 (M1) PROTEIN

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DOI: (<https://doi.org/10.71146/kjmr1012>)

Article Info



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Abstract

Influenza B virus (IBV) is a major cause of seasonal influenza epidemics, contributing substantially to global morbidity and mortality, particularly among children and the elderly. Current seasonal vaccines primarily target the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), which are highly variable and subject to antigenic drift, limiting long-term vaccine effectiveness. The Matrix 1 (M1) protein, an internal structural protein essential for viral assembly, budding and replication, is far more conserved across IBV strains and lineages, making it an attractive target for a broadly protective vaccine. This study used an immunoinformatics approach to design and characterise a multi-epitope vaccine against the M1 protein of IBV. The M1 protein sequence was retrieved from NCBI and validated for antigenicity, allergenicity and toxicity before being screened for linear B-cell and T-cell (MHC class I and II) epitopes using IEDB prediction tools. Epitopes that were antigenic, non-allergenic and non-toxic were combined with the adjuvant 50S ribosomal protein L7/L12 and appropriate linkers (EAAAK, GPGPG and AYY) into a single multi-epitope construct terminating in a 6xHis purification tag. The physicochemical properties, secondary and tertiary structure, population coverage, molecular docking against immune receptors, codon optimisation, in silico cloning, immune simulation and molecular dynamics behaviour of the construct were then evaluated. The final vaccine construct comprised 895 amino acids incorporating 32 MHC class I, 30 MHC class II and 3 B-cell epitopes, and was predicted to cover 97.82% of the world population when class I and II responses were combined. The construct was stable, thermostable and basic in nature, formed stable docking interactions with HLA-A*02:01 and TLR4, expressed favourable codon adaptation for *E. coli*, and elicited a simulated antibody response (IgM and IgG1/IgG2) consistent with an effective humoral immune response. These findings indicate that a multi-epitope vaccine directed against the conserved M1 protein of IBV is a computationally promising candidate for broader, more durable protection against influenza B, warranting further in vitro and in vivo validation.

Keywords: *Influenza B virus; Matrix 1 protein; multi-epitope vaccine; immunoinformatics; epitope prediction; molecular docking; in silico vaccine design.*

1. Introduction

Influenza viruses remain a major global health burden, causing seasonal epidemics and carrying pandemic potential. Belonging to the family Orthomyxoviridae, influenza viruses are classified into four types – A, B, C and D (Cottey et al., 2001) – of which types A and B are of greatest clinical importance in humans. Influenza B virus (IBV), first isolated in 1940, is serologically distinct from influenza A virus (Koutsakos et al., 2016) and, unlike IAV, is essentially restricted to human hosts. IBV possesses a segmented, negative-sense, single-stranded RNA genome of eight segments that together encode eleven proteins, including three polymerase subunits (PA, PB1, PB2), the nucleoprotein (NP), the nonstructural protein (NS1), the nuclear export protein (NEP/NS2), the matrix protein (M1), the M2 ion channel, and the surface glycoproteins haemagglutinin (HA), neuraminidase (NA) and NB (Chen and Holmes, 2008). Two antigenically and genetically distinct lineages of IBV currently co-circulate: B/Victoria/2/1987-like and B/Yamagata/16/1988-like viruses, which, together with the seasonal H1N1 and H3N2 subtypes of influenza A, are responsible for recurring seasonal epidemics roughly every two to four years.

Current influenza vaccines are directed mainly against HA and NA, the two surface glycoproteins responsible for viral attachment, entry and release. Because these proteins are highly variable and subject to antigenic drift, they necessitate frequent updates to vaccine composition (Yuan et al., 2024), and effectiveness can be further reduced when there is a mismatch between vaccine strains and circulating viruses (Cao et al., 2021). The M1 protein offers a promising alternative target. As an internal structural protein that organises the viral envelope and mediates interactions with the viral ribonucleoprotein complexes during assembly, budding and replication, M1 is far more conserved across IBV strains and lineages than HA or NA, making it less prone to mutation and a more stable target for immune responses (Ruigrok et al., 2000). A vaccine built around conserved M1 epitopes could in principle provide broader and more durable protection across both circulating IBV lineages.

Multi-epitope, or epitope-based, vaccines have emerged as an attractive alternative to traditional whole-protein or live-attenuated vaccines. In silico vaccine design can be viewed as a process of selecting good fragments of viral proteins and then assembling them into a final construct (Yang et al., 2021). By selecting immunogenic linear B-cell epitopes together with MHC class I-restricted (cytotoxic T lymphocyte, CTL) and MHC class II-restricted (helper T lymphocyte, HTL) epitopes from a target antigen – a strategy attracting increasing attention for its safety, stability and reduced risk of allergic or autoimmune reactions relative to traditional vaccines (Koutsakos et al., 2016) – and joining them with functional linkers and an immunostimulatory adjuvant, it is possible to design a single recombinant construct that engages both the humoral and cell-mediated arms of the adaptive immune response. Incorporating the adjuvant directly into the construct, rather than mixing it separately, may enhance immunogenicity by ensuring simultaneous delivery of adjuvant and antigen to the same antigen-presenting cells (Maleki et al., 2021), and this combined approach can further stimulate both humoral (antibody-mediated) and cellular (T-cell-mediated) immunity (Behbahani et al., 2021).

This study applies an immunoinformatics pipeline to the M1 protein of influenza B virus with the aim of designing a multi-epitope vaccine capable of eliciting a broad and robust immune response. We hypothesised that a construct built from conserved, antigenic, non-allergenic and non-toxic M1 epitopes, combined with a suitable adjuvant and linkers, would form a stable, immunogenic protein with the potential to confer cross-protective immunity against diverse circulating strains of influenza B virus.

2. Materials and Methods

2.1 Protein sequence retrieval

The amino acid sequence of the Matrix 1 (M1) protein of influenza B virus was retrieved in FASTA format from the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>) database and used as the basis for all subsequent computational analyses (Maleki et al., 2021).

2.2 Protein sequence validation

Before epitope prediction, the retrieved M1 sequence was evaluated for antigenicity, allergenicity and toxicity. Antigenicity was assessed using VaxiJen (<https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>); allergenicity was assessed using AllerTOP (<https://www.ddg-pharmfac.net/AllerTOP/>); and toxicity was assessed using ToxinPred (<https://webs.iitd.edu.in/raghava/toxinpred/algo.php>). Physicochemical properties of the native M1 protein were additionally computed using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>).

2.3 B-cell epitope prediction

Linear B-cell epitopes, intended to elicit neutralising antibodies capable of blocking infectivity of the pathogen harbouring the protein antigen (Yuan et al., 2024), were predicted from the validated M1 sequence using the IEDB B-cell epitope prediction server (<http://tools.iedb.org/bcell/>). Predicted epitopes were subsequently screened for antigenicity, allergenicity and toxicity using the same tools described above.

2.4 T-cell epitope prediction

MHC class I epitopes, recognised by cytotoxic T lymphocytes (CTLs), and MHC class II epitopes, recognised by helper T lymphocytes (HTLs), were predicted using the IEDB T-cell epitope prediction server (<http://tools.iedb.org/main/tcell/>). As with the B-cell epitopes, all predicted T-cell epitopes were validated for antigenicity, allergenicity and toxicity (Behbahani et al., 2021), and only epitopes that were antigenic, non-allergenic and non-toxic were retained for vaccine construction.

2.5 Population coverage analysis

Because human leukocyte antigen (HLA) allele frequencies vary substantially between populations, the worldwide population coverage of the selected T-cell epitopes was estimated using the IEDB population coverage tool (<http://tools.iedb.org/population/>), evaluating 16 HLA class I and class II alleles with the area set to “World” and the calculation option set to “Class I and II combined” (Sanami et al., 2022).

2.6 Design of the recombinant multi-epitope vaccine

Antigenic, non-allergenic and non-toxic epitopes with prediction scores above 0.5 were selected from the high-scoring CTL, high-affinity HTL and B-cell epitope sets to construct the multi-epitope vaccine. The 50S ribosomal protein L7/L12 was selected, after comparison of three candidate peptide adjuvants, as the adjuvant for the final construct on the basis of its favourable properties. The adjuvant was fused to the N-terminus of the construct using the rigid, α -helical EAAAK linker, which promotes an extended, stable domain-spacer conformation. Individual CTL, HTL and B-cell epitopes were joined using GPGPG and AYY linkers respectively, which minimise the formation of junctional neo-epitopes and promote correct antigen processing. A 6xHis tag was appended to the C-terminus of the construct to facilitate downstream purification and identification.

2.7 Evaluation of physicochemical properties

The physicochemical properties of the assembled vaccine construct – including molecular weight, theoretical isoelectric point (pI), amino acid and atomic composition, chemical formula, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) – were computed using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>) (Rostaminia et al., 2021).

2.8 Secondary and tertiary structure prediction

The secondary structure of the vaccine construct (helices, strands and coils) was predicted using the PSIPRED 4.0 web server (<http://bioinf.cs.ucl.ac.uk/psipred/>), using the vaccine amino acid sequence as input. The tertiary (3D) structure was predicted using I-TASSER (<https://zhanggroup.org/I-TASSER/>) and the resulting PDB model was visualised using Chimera software.

2.9 Molecular docking

To evaluate the potential of the vaccine construct to engage the host immune system, the modelled vaccine structure was docked, as ligand, against two immunologically relevant receptors – HLA-A*02:01 and Toll-like receptor 4 (TLR4) – using AutoDock Vina.

2.10 Codon optimisation and in silico cloning

The Java Codon Adaptation Tool (JCat; <http://www.jcat.de/Start.jsp>) was used to reverse-translate and optimise the codon usage of the final vaccine construct for expression in *Escherichia coli* strain K12 (Grote et al., 2005), with options set to avoid rho-independent transcription terminators, prokaryotic ribosome-binding sites and restriction-enzyme cleavage sites. The resulting codon adaptation index (CAI) and GC content were used to gauge the likely expression efficiency of the construct. SnapGene software was then used to clone the optimised sequence, via the Eco53KI and BstZ17I restriction sites, into the pET-32a+ expression vector (Rcheulishvili et al., 2023).

2.11 Immune response simulation

The immune response elicited by the vaccine construct was simulated using the C-ImmSim server over a 35-day period, generating predicted antigen, IgM and IgG (IgG1 and IgG2) titre profiles over time.

2.12 Molecular dynamics simulation

The structural stability of the vaccine construct and of the vaccine–TLR4 docked complex was assessed over a 50 ns molecular dynamics simulation, with root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) calculated for the C-alpha backbone to evaluate conformational stability and residue-level flexibility.

3. Results

3.1 Retrieval and validation of the M1 protein sequence

The M1 protein sequence of influenza B virus was successfully retrieved from NCBI in FASTA format. On validation, the protein returned a VaxiJen antigenicity score of 0.5583, above the 0.5 threshold used to classify a protein as antigenic. AllerTOP predicted the protein to be non-allergenic, and ToxinPred predicted it to be non-toxic, confirming its suitability as a vaccine target. The physicochemical properties of the native M1 protein, as computed by ExPASy ProtParam, are summarised in Table 1. The 248-residue protein has a molecular weight of 27,386.83 Da, a theoretical pI of 9.09 (basic), an instability index of 39.95 (classifying it as marginally

stable), a negative GRAVY value of -0.291 (indicating a hydrophilic character), and an aliphatic index of 81.49 , consistent with reasonable thermostability.

Table 1. Physicochemical properties of the influenza B virus Matrix 1 (M1) protein.

Physicochemical property	Matrix Protein 1 (M1)
Number of amino acids	248
Molecular weight (Da)	27,386.83
Chemical formula	C1191H1949N339O358S20
Theoretical pI	9.09
Instability index	39.95 (stable)
GRAVY	-0.291
Aliphatic index	81.49
Estimated half-life	30 h (mammalian reticulocytes, in vitro); >20 h (yeast, in vivo); >10 h (E. coli, in vivo)

3.2 B-cell and T-cell epitope prediction

IEDB analysis of the validated M1 sequence yielded eight candidate linear B-cell epitopes, of which three were antigenic, non-allergenic and non-toxic and were carried forward for vaccine construction. For the T-cell response, IEDB prediction and subsequent filtering for antigenicity, allergenicity and toxicity yielded 32 MHC class I (CTL) epitopes and 30 MHC class II (HTL) epitopes. The full set of 65 selected B-cell and T-cell epitopes is listed in Table 2.

Table 2. B-cell, MHC class I and MHC class II epitopes selected from the M1 protein for inclusion in the multi-epitope vaccine construct.

B-cell epitopes (n=3)	MHC class I epitopes (n=32)	MHC class II epitopes (n=30)
RKMRKCVSFHEAFEIAEGHEPGNYSM SHSHRAHSRAARSSVPGVRREM ALEWIKNKR	ALIGASICF	AERKMRKCVSFHEAF
	ALIGASICFL	ALLYCLMVMYLNPGN
	ALLYCLMVMY	ATKKKGLILAERKMR
	ASHSHRAHS	ERKMRKCVSFHEAFE
	ASICFLKPK	FEIAEGHESSALLYC
	ATKKKGLIL	FGGKEFDLDSALEWI
	ERKMRKCVSF	GGKEFDLDSALEWIK
	GASICFLKPK	GKEFDLDSALEWIKN
	GKEFDLDSAL	GVRREMOMVSAMNTA
	GMGTTATKK	KEFDLDSALEWIKNK
	GVRREMOMV	KKKGLILAERKMRKC
	IGASICFLK	LILAERKMRKCVSFH
	ILAERKMRK	LLYCLMVMYLNPGNY
	LGTLCALCEK	LYCLMVMYLNPGNYS
	LILAERKMRK	MRKCVSFHEAFEIAE
	LLYCLMVMYL	PGVRREMOMVSAMNT
	MVMYLNPGNY	REMOMVSAMNTAKTM
	MYLNPGNYSM	RKMRKCVSFHEAFEI
	QMVAMNTA	RREMOMVSAMNTAKT

	QVKLGTLCAL	SALLYCLMVMYLNPG
	REMQMVSAM	SFHEAFEIAEGHESS
	REMQMVSAMN	TDIQKALIGASICFL
	RKMRKCVSF	TEPLSGMGTTATKKK
	RKMRKCVSFH	TKKKGLILAERKMRK
	RREMQMVSAM	VLRLGASQKNGEGI
	SGMGTTATK	VPGVRRREMQMVSAMN
	SSMGNSALV	VRREMQMVSAMNTAK
	VLRLGASQK	VSFHEAFEIAEGHES
	VMYLNPGNY	WFGGKEFDLDSALEW
	VMYLNPGNYS	YCLMVMYLNPGNYSM
	VSFHEAFEI	
	YLNPGNYSM	

3.3 Population coverage

Combined analysis of the selected MHC class I and class II epitopes against 16 HLA alleles predicted a worldwide population coverage of 97.82%, with an average of 10.04 epitope–HLA hits per individual and a PC90 value of 3.8, indicating that the vast majority of the global population is expected to present at least one of the selected epitopes.

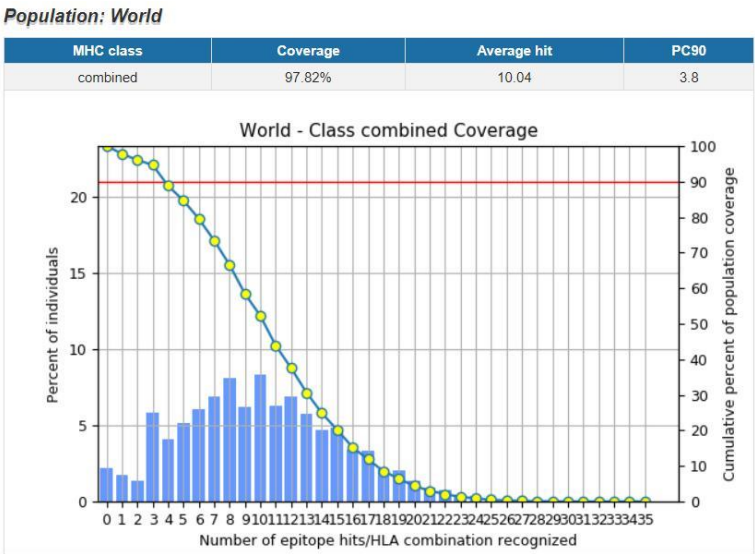


Figure 1.. Predicted worldwide population coverage (combined MHC class I and class II) of the selected M1 epitope set, generated using the IEDB population coverage tool.

3.4 Design and composition of the vaccine construct

The final multi-epitope vaccine construct comprises 895 amino acids and incorporates a total of 65 epitopes – 32 MHC class I, 30 MHC class II and 3 B-cell epitopes. The 50S ribosomal protein L7/L12 adjuvant is fused to the N-terminus via the EAAAK linker; the CTL, HTL and B-cell epitopes are joined using GPGPG and AYY linkers respectively; and a 6xHis tag is appended at the C-terminus to aid purification and detection. The linkers are intended to provide conformational flexibility, support correct folding, keep functional domains appropriately separated, and increase the overall stability of the fusion protein.

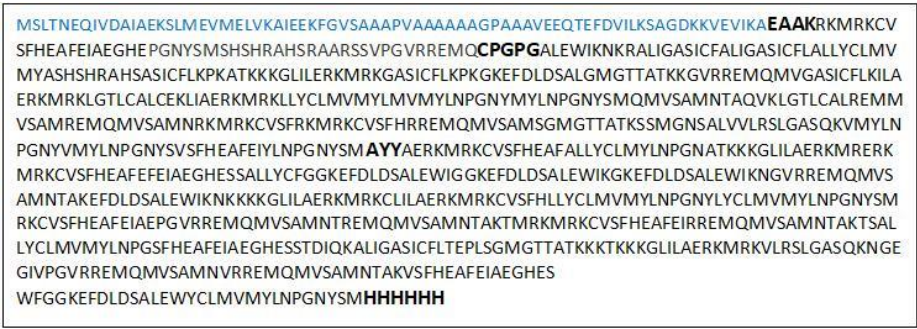


Figure 2. Final multi-epitope vaccine construct. The adjuvant (50S ribosomal protein L7/L12, blue) is fused via the EAAAK linker (bold); CTL, HTL and B-cell epitopes are joined by GPGPG and AYY linkers (bold); the construct terminates in a 6xHis tag.

3.5 Physicochemical properties of the vaccine construct

ProtParam analysis of the assembled 895-residue construct predicted a molecular weight of 101,232.47 kDa and a theoretical pI of 9.42, indicating an overall basic protein, consistent with the 130 positively charged residues against 87 negatively charged residues in the sequence. The estimated half-life was 30 h in mammalian reticulocytes in vitro, more than 20 h in yeast in vivo, and more than 10 h in E. coli in vivo. The molecular formula was calculated as C4449H7151N1241O1244S103 (14,188 atoms in total). The instability index was 31.19, classifying the construct as a stable protein (an index above 40 would indicate instability), and the aliphatic index was 76.69, indicating good thermostability. The GRAVY value of -0.143 confirms that, like the native M1 protein, the vaccine construct is hydrophilic in character, a property generally favourable for solubility and downstream expression.

3.6 Secondary and tertiary structure

PSIPRED secondary structure prediction identified a mixture of helical, strand and coil regions distributed across the length of the construct, consistent with the expected architecture of a linear, linker-joined multi-epitope fusion protein. The corresponding I-TASSER-predicted three-dimensional model, visualised in Chimera, showed the adjuvant and linked epitope segments arranged as an elongated, multi-domain chain, in agreement with the secondary structure assignment.

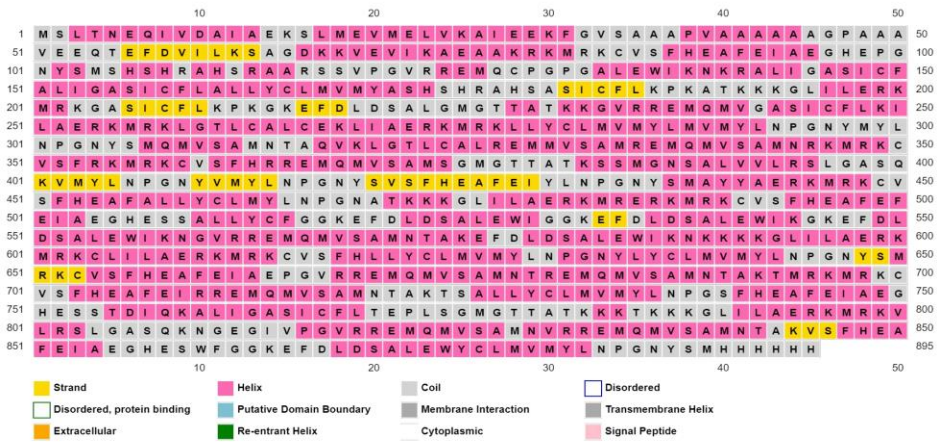


Figure 3A. Predicted secondary structure of the vaccine construct (PSIPRED), showing helix, strand and coil regions across the 895-residue sequence.

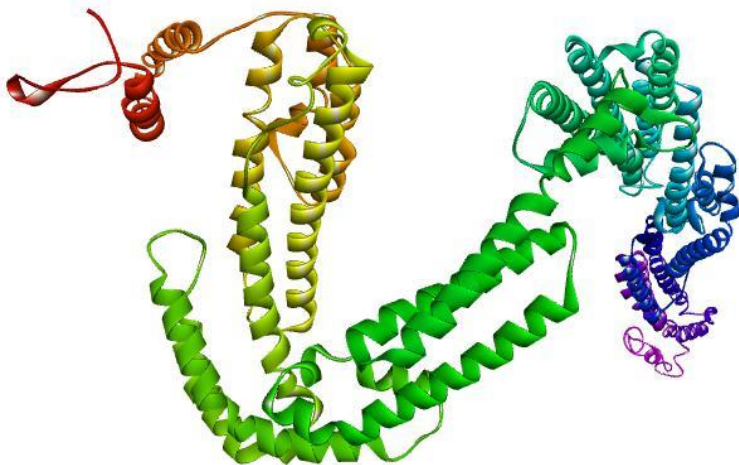


Figure 4B. Predicted three-dimensional structure of the vaccine construct (I-TASSER, visualised in Chimera; rainbow colouring from N-terminus, blue, to C-terminus, red).

3.7 Molecular docking

Docking of the vaccine construct against HLA-A*02:01 and TLR4 using AutoDock Vina indicated that the construct forms stable interactions with both immune receptors, supporting its potential capacity to be presented to, and to activate, the host immune system through both MHC-restricted antigen presentation and innate TLR4-mediated signalling.

3.8 Codon optimisation and in silico cloning

JCat-based codon optimisation of the 895-residue construct for expression in E. coli strain K12 produced an optimised nucleotide sequence with a codon adaptation index (CAI) of 0.98 (an ideal score is 1.0, with values above 0.8 considered excellent) and a GC content of 46.51%, within the favourable 30–70% range for efficient bacterial expression. In silico cloning into the pET-32a+ vector, using the Eco53KI and BstZ17I restriction sites at the N- and C-termini of the insert respectively, produced a final construct of 5,380 bp.

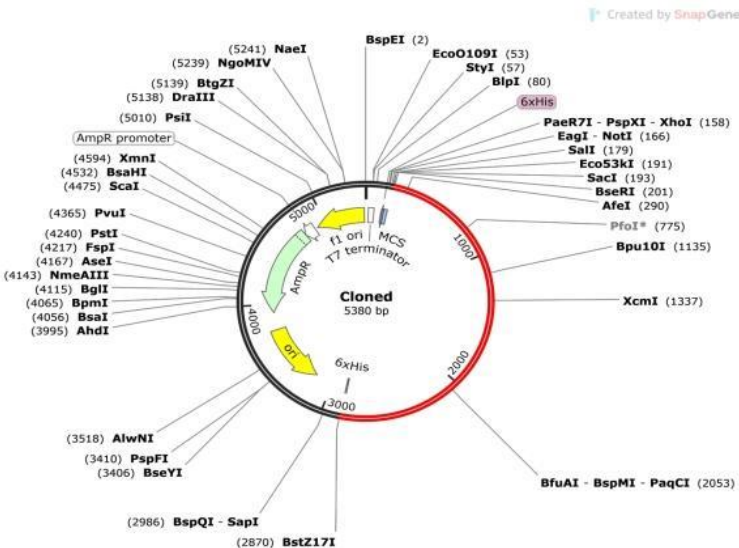


Figure 5.. In silico cloning of the optimised vaccine construct (red segment) into the pET-32a+ expression vector via the Eco53KI and BstZ17I restriction sites (5,380 bp total).

3.9 Immune response simulation

C-ImmSim simulation of the immune response over 35 days showed a rapid rise in antigen concentration that peaked on day 1, followed by a sharp decline, indicating effective antigen clearance facilitated by the immune system (An et al., 2017). IgM titres rose early, peaking around day 10, consistent with a typical primary humoral response. This was followed by a gradual rise in IgG1 and IgG2 titres, both peaking around day 15, with the combined IgM+IgG response remaining elevated and sustained through the simulated period. The balanced IgG1/IgG2 response suggests broad neutralisation capability against the virus, underscoring the potential efficacy of the multi-epitope vaccine in inducing a strong and durable immune response (Pappalardo et al., 2016).

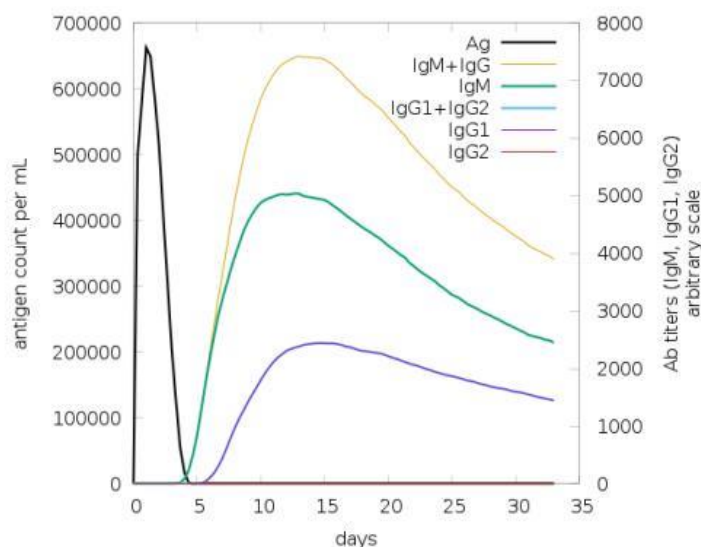


Figure 6.. C-ImmSim-simulated antigen and antibody (IgM, IgG1, IgG2) kinetics over 35 days following administration of the vaccine construct.

3.10 Molecular dynamics simulation

Over a 50 ns simulation, the average RMSD of the vaccine model alone was 3.0 Å, while the vaccine–TLR4 complex showed an RMSD of 1.4 Å with minimal further drift, both within the 1–3 Å range generally considered acceptable for a stable complex. The RMSD trajectory showed an initial adaptation phase over roughly the first 20 ns, during which the complex explored conformational space before settling into a stable state that was maintained for the remainder of the simulation. The corresponding RMSF profile showed fluctuations concentrated in specific residue ranges, consistent with localised flexibility required for the complex to reach and maintain a stable bound conformation, while the overall backbone remained structurally coherent.

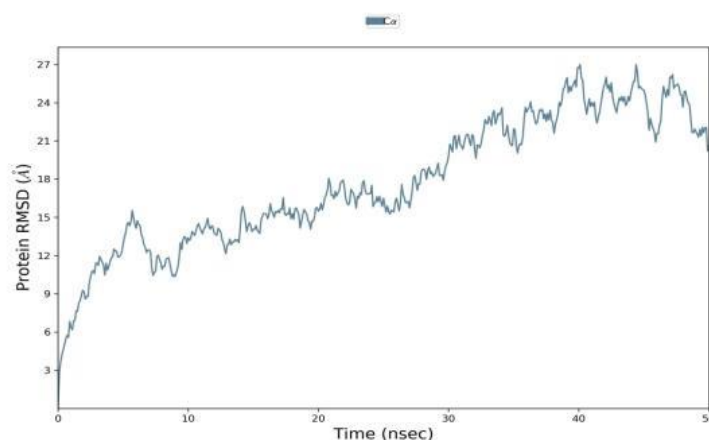


Figure 7A. Protein backbone (C α) RMSD of the vaccine–TLR4 docked complex over a 50 ns molecular dynamics simulation.

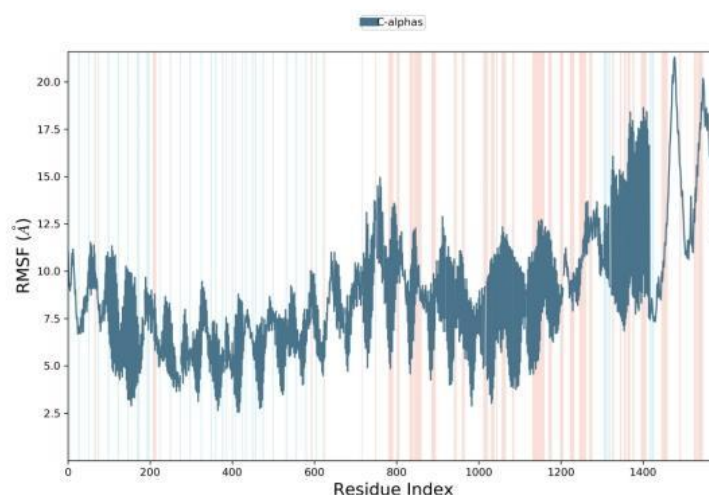


Figure 8B. Per-residue RMSF of the vaccine–TLR4 docked complex over the 50 ns simulation, showing regions of localised flexibility.

4. Discussion

The results of this study support the feasibility of a multi-epitope vaccine strategy directed against the M1 protein of influenza B virus. Because M1 is a highly conserved internal structural protein, epitopes drawn from it are less likely to be lost through the antigenic drift that regularly erodes the effectiveness of HA- and NA-based seasonal vaccines. By combining validated B-cell and T-cell epitopes with a well-characterised adjuvant and functional linkers, the resulting 895-residue construct was predicted to be antigenic, non-allergenic, non-toxic, thermostable and capable of covering nearly 98% of the world population through combined MHC class I and class II presentation – a coverage level that compares favourably with other reported in silico multi-epitope vaccine designs against influenza A and B.

The favourable docking behaviour against HLA-A*02:01 and TLR4, together with the stable RMSD/RMSF profile obtained from molecular dynamics simulation, suggest that the construct is likely to be correctly presented to T cells while also engaging innate immune signalling through TLR4, which could enhance adjuvanticity. The simulated C-ImmSim immune response, characterised by an early IgM peak followed by a

sustained IgG1/IgG2 response, is consistent with the pattern expected of an effective prophylactic vaccine capable of supporting both a rapid primary response and durable humoral memory. The favourable codon adaptation index (0.98) and GC content (46.51%) obtained for the *E. coli*-optimised construct further indicate that recombinant expression of the vaccine in a standard bacterial host, such as via the pET-32a+ system used for in silico cloning here, should be technically feasible.

These findings should nonetheless be interpreted within the limits of an entirely computational study. All predictions of antigenicity, allergenicity, toxicity, structure, docking and immune response are model-based and require confirmation through wet-laboratory expression, purification and immunogenicity testing, followed by appropriate animal and, ultimately, clinical studies before any conclusions can be drawn about real-world safety or efficacy. In addition, the pET-32a+ expression system used for in silico cloning is known to exhibit occasional low-level promoter leakage and is best avoided for proteins that are toxic in small amounts or highly hydrophobic; the GRAVY value obtained here (−0.143) indicates that the hydrophilic construct designed in this study should not pose this particular risk. Future work should focus on recombinant expression and purification of the construct, in vitro immunogenicity assays, and, where warranted, animal challenge studies against representative B/Victoria and B/Yamagata strains to confirm the cross-lineage protection suggested by these in silico results.

5. Conclusion

This study presents a novel, entirely computational approach to influenza B vaccine design by targeting the highly conserved Matrix 1 (M1) protein rather than the variable surface glycoproteins used by current seasonal vaccines. The resulting multi-epitope construct – incorporating 32 MHC class I, 30 MHC class II and 3 B-cell epitopes fused to the 50S ribosomal protein L7/L12 adjuvant – was predicted to be stable, non-allergenic, non-toxic and capable of eliciting a broad, sustained immune response, with an estimated global population coverage of 97.82%. Molecular docking and dynamics simulations further support the construct's ability to engage immune receptors in a stable manner, and codon optimisation confirms its suitability for recombinant expression in *E. coli*. By focusing on a conserved internal antigen, this approach offers the potential for broader and more durable protection against circulating influenza B lineages than current HA/NA-based vaccines, while reducing the need for frequent reformulation. Further in vitro and in vivo studies are required to validate the immunogenicity, safety and protective efficacy of this candidate, but the present findings provide a promising computational foundation for the development of a more universal influenza B vaccine.

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