

IMMUNOINFORMATICS-BASED DESIGN AND IN SILICO EVALUATION OF HPV-16 L1 MULTI-EPITOPE VACCINE AGAINST CERVICAL CANCER

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Abstract

HPV-16 is a high-risk subtype of human papillomaviruses associated with cervical cancer cases worldwide. Vaccine research for this virus is currently an area of interest both for preventive and therapeutic purposes. This study used an immunoinformatics approach to design a multi-epitope vaccine (MEV) from highly conserved regions of the major capsid protein L1 of HPV-16. The amino acid sequence of L1 protein was analyzed for conservation and homology. Potential B-cell, CTLs and HTLs epitopes were predicted and selected from the conserved regions. The selected epitopes were then fused together to construct a single MEV in linear form. MEV was analyzed for several physico-chemical, immunological and structural parameters. The MEV consisted of 505 amino acids with predicted molecular mass of 56.28 kDa, instability index of 36.57%, theoretical isoelectric point of 8.55, aliphatic index of 74.51 and GRAVY value of -0.347. MEV exhibited good antigenicity and non-allergenic nature. The MEV possessed organized secondary and 3D structures. The plot of Ramachandran showed that majority of the MEV residues were residing in the favored and allowed regions which are indicative of good stereochemical quality. MEV was further docked with TLR3 receptor and the results showed that MEV can fit well into the TLR3 receptor interaction site with a docking score of -11.35. Optimization of MEV for expression in *Escherichia coli* and in silico cloning into pET-28a(+) expression vector was also performed.

INTRODUCTION

Human papillomavirus type 16 (HPV-16) is known as high-risk, non-enveloped, double-stranded DNA virus belongs to Papillomaviridae family. HPV-16 genome is 8 kb in size consist of genes that are essential for replication, for control of host cell programs, and for virus assembly. HPV-16 is among the most prevalent oncogenic HPV

genotypes and is strongly associated with cervical cancer as well as other anogenital and oropharyngeal malignancies (Ramakrishnan, Partricia, and Mathan 2015). Persistent infection is particularly important in cervical carcinogenesis because prolonged viral persistence can promote the progression of cervical epithelial abnormalities toward intense lesions and invasive cancer.

HPV-16 is carcinogenic human papillomavirus, encodes for viral oncoproteins E6 and E7. The E6 protein causes degradation of the tumor suppressor p53. In addition, E6 may stimulate the expression of telomerase, the enzyme that extends telomeres. E7 interferes with the function of retinoblastoma protein (Rb) to control cell cycle progression. Cells with extended life span, causing uncontrolled growth, are produced by E6 and E7 proteins. The mechanisms by which HPV-16 prevents the immune system from recognizing and eliminating infected epithelial cells also contribute to HPV-associated carcinogenesis. Persistent infection and the disrupted cellular control mechanisms lead to malignant transformation of infected cells (Tomać 2016; Schaal 2024). Amador-Molina et al. (2013) and Stanley (2021) described several mechanisms of HPV-infected cells to avoid recognition by the immune system. Vaccination is one of the most effective approaches for preventing HPV infection and related cervical disease. Current prophylactic vaccines are predominantly based on virus-like particles formed by major capsid protein L1 and provide strong protection against vaccine-targeted HPV types. However, these vaccines are intended primarily to prevent infection and are not designed to eliminate established HPV infection or treat existing HPV-associated lesions. Moreover, vaccine accessibility and delivery remain challenging in some resource-limited settings (Dadari and Zgibor 2021). These considerations continue to motivate investigation of complementary vaccine strategies that may be economical, precisely targeted and capable of engaging multiple components of the immune system.

HPV-16 L1 major capsid protein represents an attractive antigen for computational vaccine design because of its structural importance and immunological relevance. L1 constitutes the major component of the viral capsid, participates in virion assembly and host-cell interactions, and can self-assemble into virus-like particles that induce neutralizing antibody responses. Its antigenic properties have therefore made it an established

target in HPV vaccine research. Conserved immunogenic regions within L1 may also provide suitable sources of B-cell, T-cell epitopes for incorporation into epitope-based vaccine constructs (Sanami et al. 2022).

The current advancement in the field of bioinformatics and immunoinformatics have allowed identification of potential antigenic sites and the design of multi-epitope vaccines (MEVs) by using computational approaches. Epitopes for B-cell epitopes, cytotoxic T-lymphocytes cells and helper T-lymphocytes cells can be evaluated by taking into account several parameters such as antigenicity, allergenicity, toxicity, binding to Major Histocompatibility Complex (MHC) molecules (Chakraborty et al. 2021; Bukhari et al. 2022). Selected epitopes can subsequently be assembled using suitable linkers to generate a single vaccine construct. Additional computational assessment of physicochemical characteristics, structural properties, receptor interactions and recombinant expression feasibility can further support the prioritization of promising constructs before experimental investigation.

In the present study, an immunoinformatics-based strategy was used to develop multi-epitope vaccine targeting L1 protein of HPV-16. The L1 sequence was initially examined for sequence similarity and conservation. B-cell, MHC class I-II epitopes further subsequently predicted, screened for incorporation into final vaccine construct. The resulting multi-epitope vaccine was evaluated for its physicochemical characteristics, immunological properties, secondary and tertiary structural features and stereochemical quality. The molecular docking, further done to examine binding interaction between vaccine construct and human Toll-like receptor 3 (TLR3). For potential recombinant production, the vaccine coding sequence was codon optimized and computationally inserted into pET-28a(+) expression vector for *Escherichia coli*. Overall, the proposed HPV-16 multi-epitope vaccine represents promising in silico construct, although

experimental validation is required to confirm biological activity and effectiveness.

Materials and Methods

Target Protein Selection and Sequence Analysis

The amino acid sequence of Human Papillomavirus type 16 (HPV-16) major capsid protein L1 was obtained from UniProt. This sequence was used for all subsequent immunoinformatic studies. The sequence was also used to assess the degree of conservation for HPV-16 isolates using BLASTp homology searches. In addition, sequence homology to proteins from the human proteome as well as to several commensal species including *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus johnsonii* was assessed using NCBI BLASTp searches against the complete proteomes of these organisms.

Epitope Prediction and Screening

Cytotoxic T-lymphocyte (CTL) epitopes derived from HPV-16 L1 protein were identified using Immune Epitope Database (IEDB), with analysis focused on human MHC class I alleles. The predicted peptides were prioritized according to their estimated binding performance, and most suitable candidates were retained for subsequent assessment. The population coverage analysis performed for shortlisted T-cell epitopes. Helper T-lymphocyte (HTL) epitopes were evaluated for their predicted ability stimulate cytokine responses, including IFN- γ using IFNepitope, IL-4 using IL4pred, and IL-10 using IL10pred.

Multi-Epitope Vaccine Construction

Selected CTL, HTL and B-cell epitopes were assembled into linear multi-epitope vaccine construct. The suitable peptide linkers were incorporated between epitope regions to maintain their separation and structural organization. A C-terminal polyhistidine tag was included to facilitate subsequent purification. The resulting amino acid sequence was used for further physicochemical, immunological and structural analyses.

Physicochemical and Immunological Characterization

The final vaccine construct was analyzed with ExPASy ProtParam server to determine its physicochemical characteristics. All essential parameters were computed. Immunological profile of construct was also examined by assessing its predicted antigenicity and allergenic potential. Allergenicity was evaluated using the Allergen Online server.

Secondary and Tertiary Structure Prediction

A secondary structure prediction for final vaccine generated by PSIPRED, showing percentage of alpha-helices, beta-strands and coils. A tertiary structure prediction for vaccine was generated using Phyre2 after submission of the vaccine amino acid sequence as a FASTA formatted file. The resulting model was then subjected to structural refinement and validation

Structural Refinement and Validation

The amino acid sequence of vaccine construct was converted into corresponding nucleotide sequence, optimized for efficient expression in *Escherichia coli* using GenSmart. Optimized gene sequence was subsequently inserted computationally into pET-28a(+) expression vector with SnapGene to generate recombinant bacterial expression construct.

Molecular Docking

The molecular docking was performed to evaluate predicted interaction between refined multi-epitope vaccine construct and Toll-like receptor 3 (TLR3). AutoDock Vina was used for docking, and resulting complex was evaluated according to its docking score and predicted binding orientation. The docked complex was subsequently visualized to examine the vaccine-TLR3 interaction interface.

Codon Optimization and In Silico Cloning

The vaccine peptide amino acid sequence was reverse translated and codon optimized for expression in *E. coli* by GenSmart, resulting

nucleotide sequence was then in silico cloned into pET-28a(+) expression vector using SnapGene to create a potential expression construct in bacteria.

Results

Retrieval and Selection of the Target Protein

L1 protein was chosen as the target for a vaccine designed using immunoinformatics methods. The amino acid sequence of L1 protein of HPV-16 was obtained from the UniProt database, accession number P03101. The retrieved protein consists of 505 amino acids and, according to UniProt, was annotated at the protein level with evidence. The sequence was then used for homology, conservation and epitope prediction.

Homology and Conservation Analysis

Homology analysis of the HPV-16 L1 protein showed no significant sequence similarity with selected proteins of *Homo sapiens*, *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus johnsonii*. This finding supported the selection of L1 for vaccine design by reducing the likelihood of selecting epitopes with similarity to host or selected commensal proteins and, consequently, minimizing the potential for unintended cross-reactivity. Conservation analysis further indicated that the L1 protein was conserved among the HPV-16 sequences included in the analysis, supporting its selection for subsequent epitope prediction and multi-epitope vaccine construction. The characteristics and screening results of selected protein are summarized in Table 1.

Table 1. Characteristics and screening results of selected HPV-16 L1 protein

Protein	UniProt ID	Gene	Length (aa)	Protein existence	Homology with selected host/commensal proteins	Conservation
Major capsid protein L1	P03101	L1	505	Evidence at protein level	No significant homology detected	Conserved

Epitope Prediction and Screening

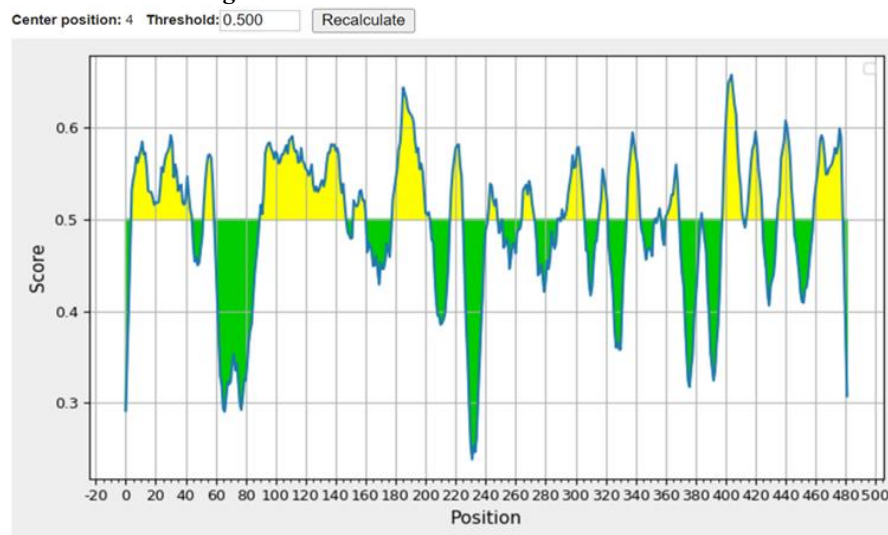


Figure 1. Prediction of linear B-cell epitopes in the HPV-16 L1 capsid protein

Table 2. Predicted physicochemical and immunological properties of final multi-epitope vaccine construct.

Parameter	Predicted value
Number of amino acids	505
Molecular weight	56,278.19 Da
Instability index	36.57
Theoretical pI	8.55
GRAVY	−0.347
Aliphatic index	74.51
Allergenicity	Non-allergen
Antigenicity	Antigenic

GAMDFTTLQANKSEVP**GCGCG**GRKFLQAGLKAKPK**GCGCG**NASAYAANAGVD
 NREG**GCGCG**LGRKFLQAGLKAKPK**GCGCG**DDTENASAYAANAGV**GCGCG**LDDTE
 NASAYAANAG**GCGCG**GENASAYAANAGVDNR**GCGCG**ASAYAANAGVDNREGC
GCGLQPPPGTLEDTYRF**GCGCG**LKAKPKFTLGKRKAG**GCGCG**GKSPCTNVAV
 NPGD**GCGCG**GDMVDTGFGAMDFTT**GCGCG**YLPPVPVSKVVSTDE**GCGCG**TTSST
 STTAKRKKR**GCGCG**LRHGEEYDLQFIFQL**GCGCG**TVIQDGMVDTGFGAG**GCG**
GHLDPNKF**G**FPDTS**GCGCG**TVGENVPDDLYIK**GCGCG**VIQDGMVDTGFGA
 M**GCGCG**AIA**C**QKHTPPAPK**EDGCGCG**TSTSTTAKRKKRKL**GCGCG**GISGHPLLN
 KLDDTE**GCGCG**DTTRSTNMSLCA**ISGCGCG**IHLDPNKF**G**FPDTS**GCGCG**RIHLP
 DPNKF**G**FPDTS**GCGCG**DYKQ**T**QLCLIGCKPP**KKEK**KTVYLPVPVSKVVSTDEYV**K**
KNKK**F**GFDPDTSFYNPDT**K**KLNLDDTENASAYAANAGVDN**K**KIGEHWGKGSPCTN
KKIQ**K**KDM**K**KTLQANKSEV**K**KYIKMVSEPY**K**KAGTVGENVPDDLYIKGSGSTANL
 KKQIFN**K**KTSETTYKNTNFKEYLRHGEE**K**KSTILEDWNFGLQPPPGTLEDTYRFV**K**
 KIACQKHTPPAPKEDPL**K**KY**K**K**K**EF**S**ADLDQ**F**K**K**GLKAKPKFTLGKRKATPTSS
 TSTTAKRKKHHHHHHH

The HPV-16 L1 capsid protein sequence was analyzed to identify suitable B-cell and T-cell epitopes for inclusion in multi-epitope vaccine construct. Linear B-cell epitopes were predicted using a threshold value of 0.500. Several regions of the L1 protein produced scores above this cutoff and were therefore considered as potential B-cell epitope candidates (Figure 1). T-cell epitope prediction was also carried out to identify peptides with the ability to bind human MHC class I and MHC class II molecules. Predicted epitopes were ranked according to their respective scores and further examined for MHC-binding potential and other important immunological features, including antigenicity, allergenicity, and toxicity. Based on these screening criteria, the most suitable B-cell, MHC class I-II epitopes were selected. These shortlisted epitopes were then assembled to develop final multi-epitope vaccine construct, as described in following section.

Construction of the Multi-Epitope Vaccine

Shortlisted epitopes were assembled into linear multi-epitope vaccine (MEV) construct. Final design included selected MHC class I-II, and B-cell epitopes, which joined using appropriate linker sequences to maintain separation between the individual epitope regions. GCGCG and KK linkers were incorporated at suitable positions within the construct. A six-histidine tag (HHHHHH) was also added at C-terminal end to facilitate purification of recombinant vaccine protein. The final MEV contained 505 amino acid residues. The complete amino acid sequence of designed construct is provided below

Physicochemical and Immunological Properties of the Vaccine Construct

The physicochemical characteristics of final MEV were evaluated to determine its predicted stability and hydrophobic properties. The construct had a

molecular weight of 56,278.19 Da (56.28 kDa) and consisted of 505 amino acid residues. Its instability index was 36.57, which was below the commonly used threshold of 40 and therefore classified the construct as stable. The theoretical isoelectric point (pI) was 8.55, indicating a basic protein.

The aliphatic index of the construct was 74.51, while the grand average of hydropathicity (GRAVY) was -0.347 . The negative GRAVY value indicated an overall hydrophilic nature of the vaccine construct. Immunological assessment further classified the final construct as antigenic and non-allergenic. The predicted physicochemical and immunological characteristics are summarized in Table 2.

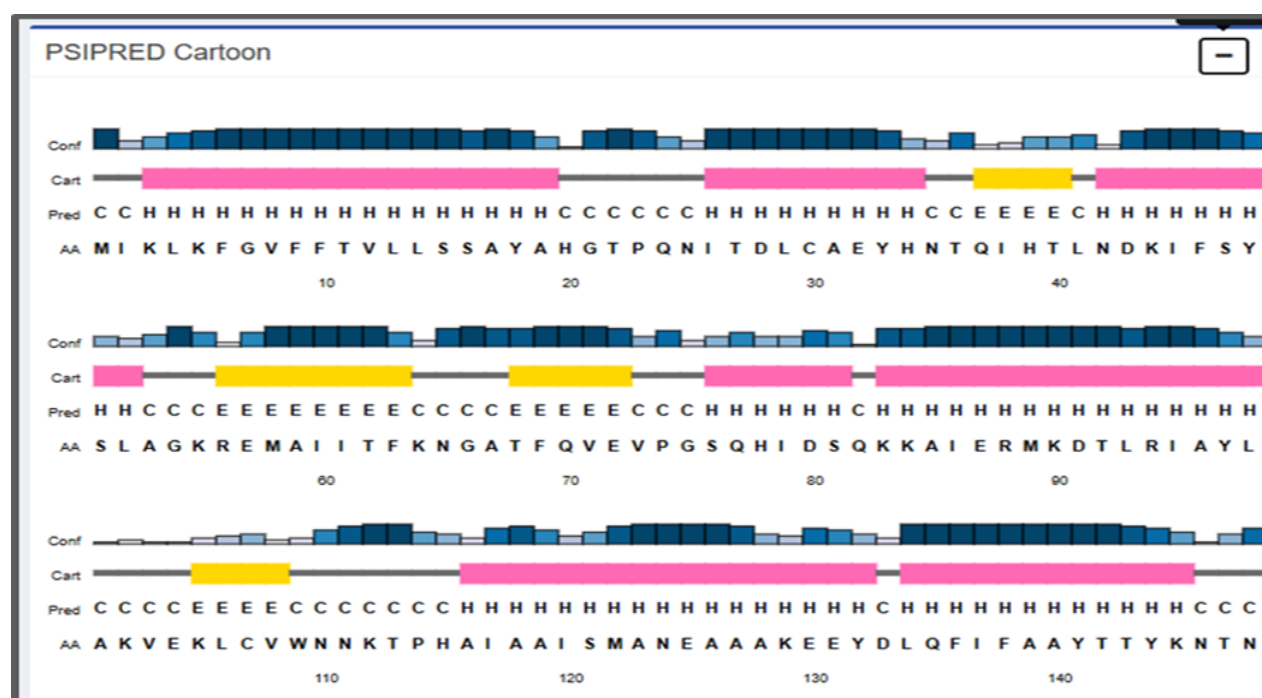


Figure 2. Predicted secondary structure of multi-epitope vaccine construc

Tertiary Structure Prediction

A three-dimensional model of final multi-epitope vaccine construct was generated to visualize its predicted tertiary conformation. The model showed folding of vaccine sequence into compact three-dimensional structure composed of interconnected secondary structural elements and

loop regions (Figure 3). The predicted tertiary model was subsequently used for structural evaluation and molecular docking analyses. The structure is displayed using a color gradient from N-terminal to C-terminal region of the protein chain.

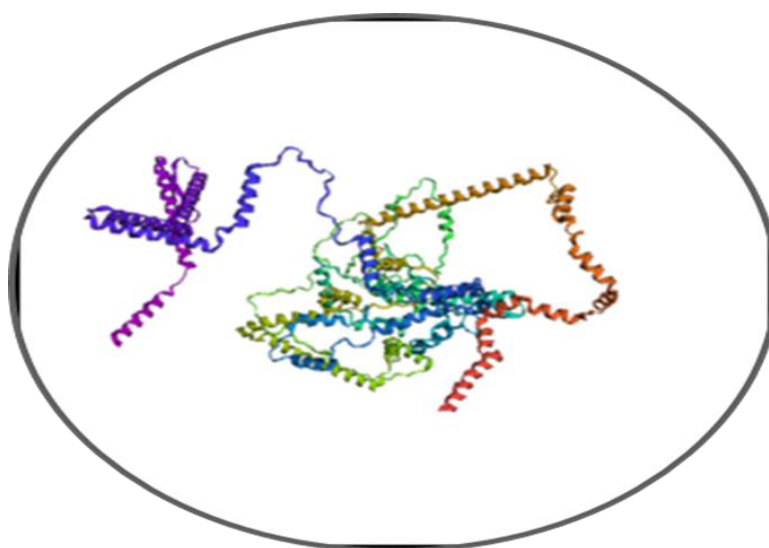


Figure 3. Predicted three-dimensional structure of final multi-epitope vaccine construct

Structural Validation of the Vaccine Model

Following energy minimization, refined three-dimensional structure of multi-epitope vaccine was evaluated using Ramachandran plot analysis. The plot examined phi (ϕ) and psi (ψ) backbone dihedral angles of the amino acid residues. Most residues were located within favored and additionally allowed regions, whereas only a small proportion appeared in disallowed regions (Figure

4). This distribution indicated that the refined vaccine model had acceptable stereochemical quality and was therefore considered suitable for subsequent molecular docking analysis.

Most amino acid residues were distributed within the favored and allowed conformational regions, while comparatively few residues were observed in disallowed regions, indicating acceptable stereochemical quality of predicted structure.

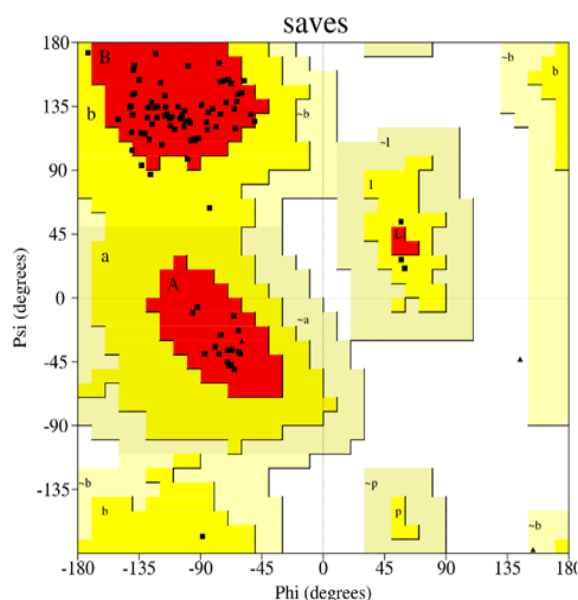


Figure 4. Ramachandran plot analysis of refined multi-epitope vaccine structure

Molecular Docking

The molecular docking analysis between final multi-epitope vaccine construct and Toll-like receptor 3 (TLR3) of innate immune system which is crucial for antiviral immunity by AutoDock Vina showed a good interaction with a docking score of -11.35 for the TLR3-vaccine complex.

The representative docked complex indicated good contact of vaccine construct with putative binding site of TLR3. In addition, a good structural complementarity between vaccine construct and TLR3 molecules was observed (Figure 5).

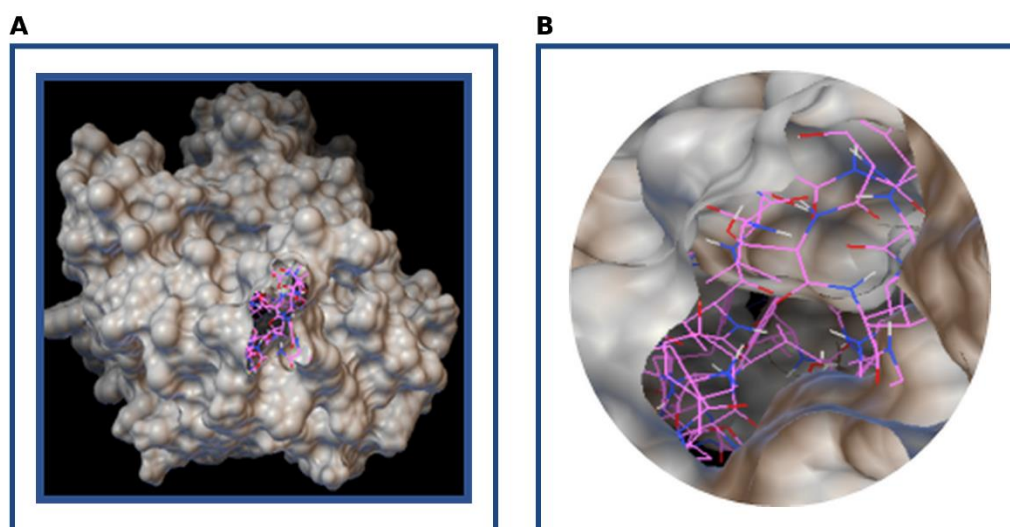


Figure 5. Predicted interaction of the multi-epitope vaccine construct with TLR3

Codon Optimization and In Silico Cloning

The final multi-epitope vaccine construct was reverse-translated and codon optimized for expression in *Escherichia coli* by GenSmart codon optimization software. The resulting optimized nucleotide sequence was then cloned in silico into the pET-28a(+) expression vector by SnapGene. The resulting vaccine construct was then made

sure to include all of the necessary vector parts to allow expression in bacterial cells, such as a gene that confers resistance to the antibiotic kanamycin (KanR), the origin of replication for *E. coli* (ori), an f1 origin for single-stranded DNA production, a T7 terminator, and a 6×His tag for protein purification (Figure 6).

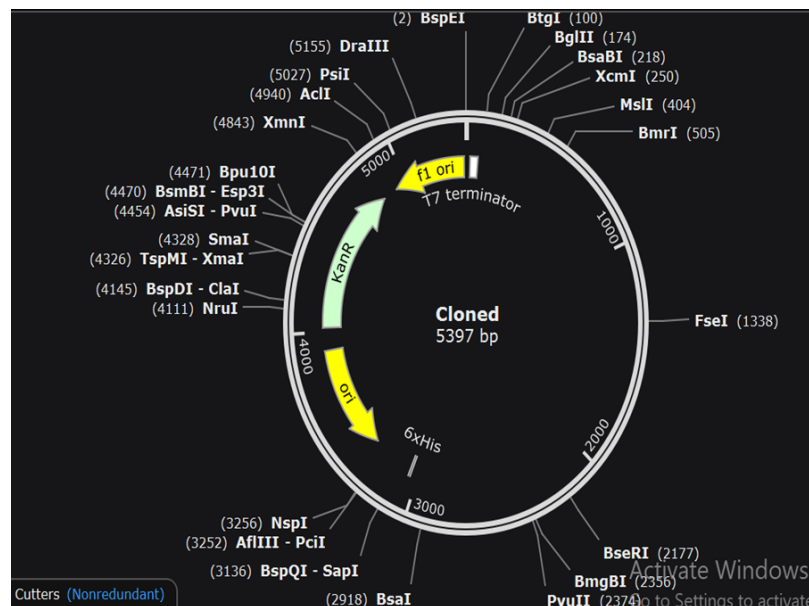


Figure 6. In silico cloning of the codon-optimized multi-epitope vaccine construct into the pET-28a(+) expression vector.

Discussion

HPV-16 is major high-risk HPV genotypes associated with cervical carcinogenesis. Persistent infection can promote development of precancerous cervical lesions and eventually invasive cervical cancer through prolonged viral persistence and disruption of normal host-cell regulatory mechanisms (Ojha et al. 2022; Ullah et al. 2023). Current prophylactic human papillomavirus (HPV) vaccines are primarily designed to prevent infections with HPV types covered by the vaccine. As the vaccines are not intended to cure existing HPV infections or to treat already established lesions caused by HPV, there is a need for alternative approaches to prevent and/or treat HPV-related lesions. Therefore, the search for new vaccine types has led to the design of so-called multi-epitope vaccines which are even compiled by means of computers. These vaccines consist of several so-called epitopes, i.e. parts of viruses that are recognized by immune system.

In the present study, major capsid protein L1 of HPV-16 was selected as target antigen for immunoinformatics-based vaccine development. L1 represents the principal structural protein of

the viral capsid and contains immunologically accessible regions capable of recognition by the host immune system. Sequence analysis showed no significant homology between the selected HPV-16 L1 protein and the examined proteins of *Homo sapiens* and selected *Lactobacillus* species. Conservation analysis further supported the use of L1 as the source protein for subsequent epitope prediction. These characteristics provided a suitable basis for screening potentially immunogenic regions while reducing the likelihood of selecting sequences with similarity to the examined host and commensal proteins.

To identify immunologically relevant regions within the HPV-16 L1 protein, both humoral and cellular immune responses were considered. Several linear B-cell epitopes exceeding selected prediction threshold were identified. In parallel, peptides with predicted affinity for MHC class I- II molecules were screened for potential CTL and HTL epitopes, respectively. The shortlisted candidates were further evaluated for important immunological features, including antigenicity, allergenicity and toxicity. Such multi-parameter screening helps select epitopes capable of contributing to both antibody-mediated and T-

cell-mediated immune responses (Parvizpour et al. 2020). The most suitable epitopes were subsequently combined using appropriate linker sequences to generate final multi-epitope vaccine construct.

The resulting vaccine consisted of 505 amino acid residues and had estimated molecular weight of 56.28 kDa. Physicochemical analysis further characterized the construct. Its instability index of 36.57 was below reference value of 40, suggesting that protein is predicted to be stable. An aliphatic index of 74.51 indicated a considerable proportion of aliphatic side chains within the sequence. The GRAVY value of -0.347 reflected an overall hydrophilic nature of the vaccine construct. In addition to that predicted theoretical isoelectric point (pI) of 8.55 indicated that protein has a basic character. These properties supported the use of the designed construct for subsequent structural evaluation and other *in silico* analyses.

Moreover, the sequence predicted to be used for structural studies and to interact with receptor was both antigenic and non-allergenic. Although these *in silico* analyses did not provide evidence of the proposed vaccine's safety and immunogenicity, they did provide useful criteria for ranking putative vaccines for further studies.

Structural characterization further supported the results obtained from MEV design by computer. Secondary-structure prediction of designed MEV showed the presence of alpha-helices, beta-strands as well as coils spread all over the sequence. Thus, parts of the sequence are ordered whereas others are flexible. The three-dimensional model was subsequently refined and evaluated using Ramachandran plot analysis. Most amino acid residues occupied the most favored and additionally allowed conformational regions, while comparatively few residues were present in generously allowed or disallowed regions. The predominance of residues within acceptable ϕ and ψ conformational space indicated satisfactory stereochemical quality of the refined model. Ramachandran analysis is widely used for evaluating predicted protein structures because residues located within favored and allowed

regions are generally associated with more acceptable backbone geometry (Nagarajan et al. 2013; ul Qamar et al. 2020).

Interaction of the designed vaccine with an innate immune receptor was further explored through molecular docking with Toll-like receptor 3 (TLR3). TLR3, important pattern-recognition receptor involved in antiviral innate immune signaling and contributes to initiation of downstream inflammatory and adaptive immune responses. The TLR3-vaccine complex produced a docking score of -1135.7 in the applied docking workflow. Structural visualization showed close accommodation of vaccine construct within the predicted TLR3 interaction region and demonstrated structural complementarity between the two molecules. These findings suggest that the designed MEV is computationally capable of interacting with TLR3. However, docking represents a predicted molecular interaction and does not by itself demonstrate receptor activation; therefore, this interaction should be confirmed experimentally.

Finally, codon optimization and *in silico* cloning were performed to examine feasibility of subsequent recombinant production of designed construct. The vaccine sequence was reverse-translated and optimized according to the codon usage pattern of *Escherichia coli*, followed by virtual insertion into pET-28a(+) expression vector. The recombinant plasmid retained important expression-vector components, including the kanamycin resistance marker, origin of replication, T7 terminator and 6×His tag. The pET-28a(+)/*E. coli* system is widely employed for recombinant protein production, and the successful *in silico* construction provides a practical framework for future synthesis and expression studies. Nevertheless, actual expression level, protein solubility and biological activity will require experimental confirmation.

Overall, the present immunoinformatics workflow integrated L1 protein selection, epitope prediction and screening, multi-epitope vaccine construction, physicochemical characterization, structural modelling and validation, TLR3 docking, codon

optimization and in silico cloning. Combined computational findings support the HPV-16 L1-based MEV as a candidate for further investigation. Future in vitro and in vivo studies will be performed further to determine its expression, immunogenicity, safety and ability to generate protective or therapeutic immune responses against HPV-16-associated disease.

Conclusion

In the present study, a multi-epitope vaccine based on HPV-16 L1 has been designed by using immunoinformatics approach. The designed vaccine construct of HPV-16 L1 showed predicted favorable physicochemical properties, structural stability, antigenicity and non-allergenicity. The modeled structure of vaccine construct was checked on Ramachandran plot and showed acceptable stereochemical quality after refinement. Also, predicted molecular interaction of vaccine with TLR3 using docking study indicated favorable interaction. Moreover, in silico codon optimization and cloning into pET-28a(+) vector followed by in silico expression study in *E. coli* showed potential of designed multi-epitope vaccine to be expressed as recombinant in *E. coli* and could be explored further as potential vaccine candidate to be tested for immunogenicity and protective efficacy against diseases caused by HPV-16 in in vitro and in vivo studies and its therapeutic application.

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