Design of a candidate multi-epitope vaccine against SARS-CoV-2 using reverse vaccinology approach

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Research Article

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Abstract

Coronavirus 2019 (COVID-19) infection as a global epidemic that is spreading dramatically day to day. Currently, many efforts have been made against COVID-19 through the designing or developing of specific vaccine or drug, worldwide. In this study, we used the bioinformatics approach to design an effective multi-epitope vaccine against COVID-19 based on Spike (S) protein. Here, we employed in silico tools to identify potential T and B cell epitopes from S protein that have the ability to induce cellular and humoral immunity. Then, the peptide sequence of potential T, B cell epitopes and flagellin (as adjuvant molecule) were joined together by suitable linkers to construct of candidate multi-epitope vaccine (MEV). Subsequently, immunological and structural evaluations such as antigenicity, allergenicity, 3D modeling, molecular docking, fast flexibility simulations as well as in silico cloning were performed. Immunological and structural computational data showed that designed MEV potentially has proper capacity for inducing of cellular and humoral immune responses against COVID-19. Based on the preliminary results, in vitro and in vivo experiments are required for validation in the future.

1. Introduction

A novel strain of coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), in December 2019, appeared in Wuhan City, Hubei province, China. It was first seen at the seafood Huanan store, which might be due to contact with the animal and then transmitted very quickly from human to human throughout China. The new strain was named by the World Health Organization (WHO) as 2019 novel coronavirus (2019-nCoV) or coronavirus disease 2019 (COVID-19) and announced. On January 30th, 2020, due to rapid human-to-human transmission of the 2019-nCoV disease and reporting of laboratory-confirmed infection cases in some countries, WHO declared a "public health emergency of international concern (PHEIC) [1, 2]. Some members of the Coronaviridae family result in mild respiratory disease in humans. However, the number of coronavirus strains such as severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and the Middle East respiratory syndrome-associated coronavirus (MERS-CoV) cause severe respiratory diseases in the human population, SARS disease, and MERS disease, respectively [3-5]. Coronaviruses (CoVs) genome is single-stranded RNA (~26–32 kb in length) and positive-sense. Human CoVs are enveloped viruses that belong to the Coronaviridae family. The structural proteins of mature coronaviruses are the spike, envelope (E), membrane (M), and nucleocapsid (N). The spike protein plays essential roles, including receptor recognition, virus attachment, and entry into human cells by attaching angiotensin-converting enzyme 2 (ACE2) as an entry receptor [6-8]. According to the increasing spread of COVID-19 infection, the design and development of an effective vaccine or antiviral drugs against this infection are crucial. The development of conventional (traditional) vaccines has some difficulties such as cost, need to lots of time from months to years for development and in vitro culture of the target virus, concerns about biological safety, and instability in diverse storage conditions [9-12]. In contrast, designing of new vaccine approaches such as multi-epitope vaccines (MEVs) that contain B cell epitopes, cytotoxic T lymphocyte (CTL), and helper T lymphocyte

epitopes (HTL) by *in silico* approaches (bioinformatics tools) overcome the problems mentioned above. These multi-epitope vaccines have some benefits compared to others, including cost-effectiveness, ~~high~~ specificity, and stability in different temperature conditions [13-16]. According to recent advances in sequencing methods, access to the sequences of many pathogenic genomes, and the availability of protein sequence databases, bioinformatics-based approaches have attracted much attention from researchers. In the beginning, the selection of suitable target antigens and the prediction of the immunodominant B-cell and T-cell epitopes is a very significant step in the development of an ideal multi-epitope vaccine [17-20]. In this context, the bioinformatics tools can play an essential role in the prediction of the epitopes. The predicted immunodominant epitopes should activate an effective response against a targeted infection. Thus, it seems that a suitable designed multi-epitope vaccine can be an ideal approach for the prevention and treatment of infections such as this emerging infection [21-25]. The incorporation of some adjuvants such as Toll-like receptor (TLR) agonists could be a suitable strategy against the low immunogenicity of multi-epitope vaccines. As a class of the pathogen recognition receptors (PRR), TLRs are conserved type I transmembrane receptors expressed on the immune and non-immune cells. TLRs respond to specific molecules of pathogens known as pathogen-associated molecular patterns (PAMPs) that lead to adjust the induction of more effective immune responses [26-29]. Bacterial cell wall flagellin (fliC), as TLR5 agonist, can activate both innate and adaptive immune systems by interacting with the TLR5, which has been used as an effective adjuvant in different candidate vaccines [30-32].

On the other hand, various studies showed that spike protein is a valuable target for developing vaccines and therapeutic approaches against COVID-19 infection [33-37]. Therefore, this study aimed to use different bioinformatics methods to design a potential multi-epitope vaccine consists of several T-cell, B cell epitopes derived from the S protein of SARS-CoV-2 as the target antigen. Further, fliC was used as a biologic adjuvant in the final construct to increase vaccine immunogenicity.

2. Materials And Methods

2.1. Retrieving of SARS-COV-2 spike protein and flagellin sequences. In the first stage of the research, the amino acid sequence of Spike protein of SARS-COV-2 and flagellin (fliC) protein from Salmonella enterica serovar Typhimurium (S. Typhimurium) were retrieved from the UniProt database (uniport.org) (P0DTC2, Q66PQ5, respectively).

2.2. Prediction of B-cell epitopes. The identification of B-cell epitopes as potential antigens recognized by B-cell lymphocytes' surface receptor leads to generating a specific immune response. Thus, B-cell epitopes play a key role in vaccine design. The BCPREDS server [38] allows users to choose the method for predicting B-cell epitopes among several developed prediction methods. Herein, the length of 14-mer B-cell epitopes and specificity of 75 % were selected.

2.3. Prediction of CTL epitopes. The freely-accessible NetCTL 1.2 server [39] was used for CTL epitopes prediction. This method integrates prediction of peptide MHC class I binding, proteasomal C terminal

cleavage, and TAP (Transporter Associated with Antigen Processing) transport efficiency. The server allows for predictions of CTL epitopes restricted to 12 MHC class I supertype. This study has chosen the A1 supertype and threshold value set at the default score (0.75) to predict CTL epitopes.

2.4. HTL epitopes prediction. The amino acid sequence of S protein subjected to NetMHCII 2.3 Server [40] for screening HTL epitopes with 15-mer length. This server using an artificial neuron network predicts binding of MHC II epitopes for human (HLA-DR, HLA-DQ, HLA-DP) and mouse MHC class II alleles (H-2). Herein, we selected mouse H2 class II alleles (H-2-IAb, H-2-IEd, and H-2-IAd). The prediction values are inferred from the IC50 values and as a percentile-rank to a set of random natural peptides. Strong and weak binding peptides are illustrated in the output. An IC50 values <50 nM, <500 nM and <5000 nM indicates high-affinity, intermediate affinity and low affinity, respectively.

2.5. Multi-epitope vaccine candidate construction. The selected epitope candidates, including high-scoring CTLs, high-affinity HTLs epitopes, and B-cell epitopes, were joined together using AAY and GPGPG linkers [41] to produce the multi-epitope vaccine (MEV) construct. Flagellin (FliC) as an adjuvant was chosen and was included in the N-terminal region of the MEV construct by an EAAAK linker [41].

2.6. Interferon-gamma (IFN-γ) inducing epitope prediction. One of the signatures of the innate and adaptive immune systems is interferon-gamma (IFN-γ), which has an antiviral function, immune regulation, and anti-tumor activity. IFN-γ cytokine, as the major arm of the Th1 response, has a crucial role in the control of intracellular pathogens such as viruses. IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope/scan.php) [42] developed to predict and design IFN-gamma inducing MHC class II binder peptides from target proteins to design an effective subunit vaccine. The Support Vector Machine (SVM) hybrid algorithms and Motif were used to predict IFN-γ epitopes. This server is based on a dataset including IFN-γ inducing and non-inducing MHC class-II binder, which has the potential to activate T-helper cells.

2.7. Prediction of antigenicity of the designed vaccine construct. To analyze antigenicity of multi-epitope vaccine construct (contain the flagellin adjuvant sequence), two servers including Vaxijen 2.0 (Vaxijen 2.0 is freely available online at the URL: http://www.jenner.ac.uk/VaxiJen) [43] and ANTIGENpro (ANTIGENpro is integrated into the SCRATCH suite of predictors available at http://scratch.proteomics.ics.uci.edu) [44] were used. The VaxiJen 2.0 server base is on auto- and cross-covariance (ACC) transformation of target protein sequences into uniform vectors of principal amino acid properties. The prediction approach used in VaxiJen v2.0 is alignment-free and based on the protein's various physicochemical properties. The threshold value of Vaxijen 2.0 was set at 0.5. ANTIGENpro server for checking protein antigenicity applies protein antigenicity microarray data and illustrate an antigenicity index. It was estimated that the ANTIGENpro server's accuracy to be 76%, based on cross-validation experiments and using the combined dataset.

2.8. Allergenicity prediction of designed vaccine construct. AllerTOP V2.0 (http://www.ddg-pharmfac.net/AllerTOP) [45] and Algpred (Available at: http://webs.iiitd.edu.in/raghava/algpred) [46] servers were used to predict the allergenicity nature of the

multi-epitope vaccine construct. In AllerTOP V2.0 server, various methods are employed to classify allergens such as amino acid E-descriptors, auto- and cross-covariance transformation, and the k nearest neighbors (kNN) machine learning. In this method, an accuracy of 85.3% has been presented at 5-fold cross-validation. Algpred allows the prediction of allergens based on the similarity of known epitope with any region of the protein. In AlgPred a systematic attempt has been made to integrate various approaches to predict allergenic proteins with high accuracy. In this study, a hybrid approach of the server, using a combined method (SVMc + IgE epitope + ARPs BLAST + MAST), was used to predict allergen.

2.9. Different physicochemical properties and solubility analysis of vaccine construct. Different physicochemical parameters of MEV such as amino acid composition, theoretical pI, Grand Average Hydropathy, Stability Profiling, Instability Index, Molecular Weight, Half-Life, and Aliphatic Index, were determined using ProtParam online server (http://web.expasy.org/protparam/) [47]. SOLpro was used for solubility analysis of MEV. SOLpro is integrated into the SCRATCH suite of predictors available at http://scratch.proteomics.ics.uci.edu [48]. SOLpro predicts if the protein is soluble or not (SOLUBLE/INSOLUBLE) and gives the corresponding probability (≥ 0.5).

2.10. Secondary structure prediction of vaccine construct. The PSIPRED server was used for our vaccine protein secondary structure prediction. PSIPRED, a web-based freely-accessible online server (http://bioinf.cs.ucl.ac.uk/psipred/)[49], is based on primary amino acid sequences input in a precise manner. In this method, a very rigorous cross-validation approach use to evaluate the method's efficiency. PSIPRED 3.2 server combines two feed-forward neural networks that analyze output obtained from PSI-BLAST (Position-Specific Iterated - BLAST).

2.11. Tertiary structure prediction. Three different servers, including I-TASSER, SWISS-MODEL, and Phyre2, were used to achieve the best 3D structural models. In the following, Phyre2 software was selected. Phyre2 is available at http://www.sbg.bio.ic.ac.uk/phyre2 [50]. This server is one of the most widely used protein structure prediction servers, which uses advanced remote homology detection methods to build 3D models.

2.12. 3D structure validation. Given the importance of model validation and to find the possible errors in primary 3D structure models, three tools were used for model validation. ProSA-web at https://prosa.services.came.sbg.ac.at/prosa.php, ERRAT server at http://nihserver.mbi.ucla.edu/ERRATv2/ [51] and PROCHECK's Ramachandran plot analysis at https://servicesn.mbi.ucla.edu/PROCHECK/ [52] were utilized. The overall quality of a specific input structure calculates using ProSA-web [53] and is presented as a quality score. ProSA-web provides an easy-to-use interface to the program ProSA which is frequently used in protein structure validation. The ProSA-web score is shown in a plot, including the z-score of experimentally determined structures deposited in PDB. ERRAT program is used for verifying protein structures determined by crystallography. This program is based on the statistics of non-bonded atom-atom interactions in the reported structure.

PROCHECK's Ramachandran plot program checks the stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry.

2.13. Discontinuous B-cell epitope prediction in final MEV construct. ElliPro (http://tools.iedb.org/ellipro/) server [54] was employed to predict conformational B-cell epitopes from the validated 3D structure. ElliPro is a new web-tool for predicting antibody epitopes based on the geometrical properties of protein structure. ElliPro considers a score to each output epitope defined as a PI (Protrusion Index) value averaged over epitope residues. In the method, the protein's 3D shape is approximated by several ellipsoids; thus, the ellipsoid with PI = 0.9 would include within 90% of the protein residues, with 10% of the protein residues being outside of the ellipsoid.

2.14. Molecular docking of designed vaccine with TLR-5 receptor. The interaction patterns of the final MEV construct as a ligand with the TLR5 receptor (PDB ID: 3J0A) were analyzed by using ClusPro 2.0 server (https://cluspro.org) [55]. ClusPro is a web-based server for the direct docking of two interacting proteins. Three computational steps including (1) rigid-body docking, (2) RMSD based clustering of the 1000 lowest energy structures, and (3) the removal of steric clashes by energy minimization performs using ClusPro software.

2.15. Fast simulations of flexibility of the docked vaccine complex. The CABS-flex webserver was used for fast simulations of the TLR5 designed complex vaccine flexibility. A protein structure in the PDB format is the only data needed for input (or a protein PDB code). Here, as the input for the quick flexibility simulation, the selected docked TLR5-vaccine complex was used. Protein flexibility, contact map and root-mean-square fluctuations (RMSFs) of atoms within a protein complex are provided on this server. RMSF simulation of all amino acid residues in a given protein is shown by the CABS-flex server in a nanosecond duration [56].

2.16. In silico cloning and codon optimization of MEV construct. The Java Codon Adaptation Tool (JCat) (http://www.prodoric.de/JCat) [57] was used for codon optimization and reverse translation of the MEV construct sequence to express the MEV in a suitable expression vector. Here, an E. coli (strain K12) host was selected to express the final MEV sequence, and other options such as rho-independent transcription termination, prokaryote ribosome binding site, and restriction enzyme cleavage sites were included. To ensure the high-level protein expression, two JCat output indexes include the codon adaptation index (CAI), and percentage GC content can be used. CAI supply data on codon usage biases; the CAI score is optimal at 1.0, but a score above 0.8 (> 0.8) is also good. Usually, the optimal GC content range between 30–70% is favorable effects on translational and transcriptional efficiencies. Also, to clone the final MEV sequence in E. coli pET-28a (+) vector, two restriction enzyme sites containing XhoI and NcoI were included at N and C-terminals of the target sequence, respectively. The optimized MEV sequence (with restriction sites) was inserted into the pET-28a (+) vector using the SnapGene tool to certify MEV expression.

3. Results

3.1. Protein sequence retrieval. In this research, the extracellular domain of spike glycoprotein (from SARS-COV-2) amino acid sequence (without signal sequence) was chosen as virus target antigen and then was subjected to in silico analysis to predict potential B cell, CTL, and HTL epitopes. Flagellin (fliC) protein sequence (5-143 amino acid) from Salmonella typhimurium as an agonist of TLR5 was selected as an adjuvant for raising the efficacy of vaccines.

3.2. Linear B-cell epitope prediction. Linear B-cell epitopes with 14 amino acid length, in S protein of SARS-COV-2, were selected for the final vaccine construct. Linear B-cell epitopes with the highest score were shown in **Table 1**.

3.3. Helper T Lymphocytes (HTL) epitopes prediction. Prediction of high scored MHC-II epitopes (as HTL epitopes) for mouse H2 class II alleles were performed using NetMHCII 2.3 web server based on their IC50 scores. Finally, for generating the final MEV construct, several ten high score HTL epitopes were selected (**Table 2**).

3.4. Cytotoxic T Lymphocyte (CTL) epitopes prediction. Some 37 CTL (9-mer) epitopes were predicted for S protein using NetCTL 1.2 server set at the default threshold. Of these, eight high-scored CTL epitopes were selected in the final multi-epitope vaccine construct (as listed in **Table 3**).

3.5. Design and construction of multi-epitope vaccine candidate sequence. To generate the vaccine candidate sequence, selected high-scoring CTLs, high-affinity HTLs epitopes, and B-cell epitopes were linked to each other using suitable linkers (GPGPG, and AYY). Domains D0/D1 of flagellin as an adjuvant was added at the N-terminal of multi-epitope construct sequence using EAAAK linker to improve the vaccine's immunogenicity construct. UniProt database (http://www.uniprot.org/) was used to achieve the flagellin domain sequences. Also, at the C-terminal of the multi-epitope construct sequence, six histidine amino acid residues (as a His-tag) were included for the purification process in the future. Finally, a vaccine construction with 536 amino acid residues was designed. The schematic diagram of the final vaccine construct is indicated **(Fig 1).**

3.6. Physiochemical parameters and solubility prediction. The theoretical isoelectric point value (pI) and Molecular weight (Mw) of the final protein were predicted 6.07 and 55.26 kDa, respectively. The half-life was calculated to be 30 hours in mammalian reticulocytes (in vitro) >20 hours in yeast (in vivo) and >10 hours in Escherichia coli (in vivo). GRAVY and aliphatic index were estimated at -0.343 and 65.00, respectively. An instability index (II) with a 21.67 score was estimated. The vaccine protein was calculated to be soluble upon expression with a solubility score of 0.557194 in an *E. coli* host.

3.7. IFN-γ inducing epitope prediction. Identification of the IFN-gamma inducing epitopes from MHC-II binding epitope fragments in the final vaccine construct was performed using the IFNepitope server. The numbers of 5 epitopes were selected as IFN-gamma inducing epitopes (**Table 4**).

3.8. Antigenicity and allergenicity of the vaccine construct. The antigenicity of the whole vaccine sequence (including the adjuvant sequence) was estimated by the VaxiJen 2.0 and ANTIGENpro servers

to be 0.7751 with a bacteria model at a threshold of 0.5 and 0.931486, respectively. The allergenicity prediction results using both the AllerTOP v.2 and AlgPred servers indicated that the constructed vaccine sequences be non-allergenic.

3.9. Secondary structure prediction. Based on PSIPRED data, the prediction of the secondary structure of the final protein vaccine was estimated to contain 47.26 % alpha-helix, 5.74% beta-strand, and 47% coil **(Fig 2).** The application of the predicted secondary structure is to the refinement of the tertiary structure of a protein.

3.10. 3D structure homology modeling and validation. Various servers were implemented for 3D structure modelings such as Phyre2, SWISS-MODEL, and I-TASSER. Based on prior validation results, c3k1hA_.20 model from Phyre2 server (**Fig 3**) was selected as the best model to follow the protein construction process. In the selected model, the ProSA z-score was -2.95 **(Fig 4A**), and ERRAT, the overall quality factor of the selected model was 100% **(Fig 4B).** The PROCHECK's Ramachandran plot analysis of the selected model revealed that 94.8%, 5.2%, and 0.0% of residues are located in favored, allowed, and outlier regions, respectively **(Fig 4C).**

3.11. Conformational (Discontinuous) B-cell epitopes prediction. Conformational epitopes play an essential role in the humoral response. High-rank residues as conformation epitopes were determined in the 3D model of the final MEV construct. Here, discontinuous peptides with a value of 0.7 or higher were chosen (**Table 5**). Additionally, the predicted discontinuous epitopes in the 3D structure of the final multi-epitope protein are illustrated **(Fig 5).**

3.12. Molecular docking of designed vaccine with TLR5. Molecular interaction between the final MEV model and TLR5 was determined using the ClusPro server. The docked structure with the lowest energy score was selected as the best-docked complex. Therefore, based on global free energy, the best possible docked complex and the highest binding affinity (total free energy -992.4) was chosen. The best-docked model of MEV and TLR5 complex is shown (Fig 6). PyMOL 1.1eval was used for analysis and visualization of the docked complex.

3.13. Flexibility of the docked complex.

In this study, we have analyzed the flexibility of the designed vaccine by CABS- Flex 2.0 with 50 cycles' simulation at 1.4 °C temperature. Our complex structure has achieved a high level of fluctuations in the residue positions 247, 615, 21, 611 and 615, 5.15 Å, 4.32 Å, 3.41 Å and 3.42 Å, respectively (Fig. 7a). The CABS-Flex 2.0 software also provides 10 different models based on the parameters selected for the first model, such as structural heterogeneity, optimum free energy, and highly stable configuration. Stable protein complex structure following fast flexibility simulations using CABS-Flex 2.0 has been shown (Fig. 7b).

3.14. In silico cloning and codon optimization of MEV construct.

Reverse translation and codon optimization of nucleotide sequence was done using the JCAT tool for efficient protein expression in *E. coli*. In this study, parameters like codon adaptive index (CAI) and GC content of our optimized nucleotide sequence were 0.93 and 55.59, respectively. These parameters were represented as a good adaptation, which permitted the high rate of expression of the MEV to construct in *E. coli* K12. Finally, the optimized sequence (1608 nucleotides) was cloned into a pET28a expression vector using *XhoI and NcoI* restriction sites. A poly histidine-tag (6xHis-tag) at the C-terminus of the multi-epitope protein was incorporated for purification purposes **(Fig 8).**

Discussion

Currently, the Wuhan Novel Coronavirus 2019 (2019-nCoV) or (COVID-19) disease is now widespread in all countries and has become a global pandemic that has led to death in infected people [58]. Although efforts are ongoing, no specific antiviral drug or vaccine has been introduced to control the COVID-19 disease to date. Today, researchers are conducting extensive studies to develop multi-subunit vaccines as one of the most effective vaccination methods because of their benefits [59, 60]. Therefore, in this study, we use bioinformatics methods to design a potential candidate epitope-based vaccine against the SARSCoV-2 based on the spike protein, one of the major antigenic proteins SARSCoV-2 and viral entry into the host cell [3]. For this purpose, we first selected surface glycoprotein (S protein) encoded by SARSCoV-2 as the target antigenic protein for further analysis. T cells (helper T cells and cytotoxic T cells) are the primary mediators of cell-mediated immunity. In this way, antigens are identified by cytotoxic T cells (CTLs), whereas helper T cells activate B cells, macrophages, and even cytotoxic T cells. In intracellular pathogens such as viruses, cellular immunity identifies and destructs infected cells by secreting antiviral cytokines and creating a life-long immunity [18, 61, 62].

Furthermore, in the following, prediction of B-cell and T-cell possible epitopes from S protein was done to construct the potential multi-epitope vaccine (MEV) to induce both cell-mediated and humoral response. Then, to construct the MEV, the predicted epitopes fused using suitable linkers (AAY and GPGPG linkers) as specialized spacer sequences. The AAY linkers play a role in increase epitope presentation and remove junctional epitopes. In the GPGPG linkers, these types of linkers can cause stimulate T-helper responses and conformational dependent immunogenicity of helper and antibody epitopes [63-66]. Also, to overcome the low immunogenicity challenge of these vaccines, D0/D1 domains of flagellin protein from S. Typhimurium bacteria were used as an adjuvant to stimulate immune system responses effectively [27, 32]. D0/D1 domains fused at the N-terminal region of the multi-epitope sequence using the EAAAK linker. The EAAAK linker helps decrease adjuvant interference part with other protein segments by effective separation and improves the expression and bioactivity of the target fusion protein [41]. Finally, a candidate vaccine with a length of 536 amino acids, including some linear B-cell, CTL, and HTL epitopes fused to the adjuvant sequence, was constructed. Moreover, we predicted three interferon-γ (IFN-γ) epitopes in the final multi-epitope construct. IFN-γ secretion has been shown to stimulate innate immune responses and directly eliminate viral replication [67-69]. Therefore, it seems that IFN-gamma inducing epitopes can be influential in vaccine development. MEV was predicted to be antigenic with the probability of antigenicity 0.900656 and non-allergenic. This means that our MEV has potentially the

ability to produce a robust immune response without an allergic reaction, so make it a potent vaccine. The analysis of the physicochemical properties of the structure was as follows: Molecular weight (MW) of the MEV construct was 55650.26 kDa. The theoretical pI value was 6.07, indicating that MEV is acidic. The amount of light absorbed in a particular wavelength can be explained as an extinction coefficient index for a particular compound. The extinction coefficient of the construct was 78620M-1 cm-1. The instability index (II) score computed 21.67 which, indicated the candidate vaccine protein as a stable protein (II of >40 indicates instability) [47]. The aliphatic index of a protein is related to the relative volume occupied by aliphatic amino acids (alanine, valine, isoleucine, and leucine) in the protein side chains. It may be regarded as a positive factor for the increase of the thermostability of globular protein. The aliphatic index of the construct was 65.00, which was shown as a thermostable protein. The GRAVY (Grand Average of Hydropathy) value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. The positive and negative values represent the hydrophobic and hydrophilic properties of a compound, respectively. Our designed construct had the GRAVY value of -0.343 that predicted construct is hydrophilic protein [47]. The half-life predicts the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. ProtParam tool predicted the half-life of our construct in the following; 30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo), and >10 hours (Escherichia coli, in vivo). For many biochemical and functional evaluations of recombinant proteins, the solubility overexpression in the *E. coli* host is necessary for the efficient purification process in later stages [47]. Here, the solubility upon overexpression of our multi-epitope protein construct predicted (by SOLpro server) with probability 0.557194, which indicates the overexpression of our multi-epitope protein in *E. coli*, insoluble form[48].

In this research, the analysis of the secondary structure using the PSIPRED method illustrated that our protein construct composed predominantly of alpha-helical (47.26%), 47% coils, and 5.74% of the amino acids strand formation**.** In this study, the tertiary structure of the vaccine construct was obtained by various servers (SWISS-MODEL, phyre2, and I-TASSER). The validation process, to identify the potential errors and improve the quality of the predicted 3D model was done by ProSA-web, RAMPAGE and ERRAT servers. Validation data showed that the c3k1hA_.20 model generated by the phyre2 server was selected as a final 3D model. Validation output indicated that the selected model (c3k1hA_.20) had a high quality and did not require a refinement process. According to the Ramachandran plot, most of the residues are located in the favored (94.8%) and 5.2% in allowed regions. *In vivo*, it expected that interaction between designed MEV constructs with the TLR5 on professional antigen-presenting cells (APCs), inducing a potentially protective immune response against the virus. For this proposal, immune interaction between the designed MEV construct and TLR5 receptor was performed using Cluspro server. Cluspro presented dozens of models, which docked models scored based on hydrophobicity, geometry, and electrostatic complementarity of protein surfaces. Here, we were selected the best possible docked model between hydrophobicity models. So, the docked structure with the lowest energy score (-992.4) was selected as the best-docked complex**.** In this research, CABS-Flex 2.0 software was used for MD simulation. CABS-Flex presents the stable arrangement of the TLR5-designed vaccine complex. Based on the root mean square fluctuation (RMSF) values (using CABS Flex 2.0), the fluctuation of the individual amino acid residues as

described. The highest RMSF value and the lowest value indicates more fluctuation and low fluctuation of our complex structure during the simulation process, respectively. Fluctuations in the structure of the MEV indicate its high flexibility and validate it as a potential structure of the vaccine [56]. The codon optimization process was performed to reach high-level expression and translation efficacy of our multi-epitope protein in *E. coli* (strain K12). Here, CAI value (0.93) and GC content (55.59%) parameters were obtained, which showing a possible higher expression of the protein vaccine within the *E. coli* K-12 system [70, 71]. Finally, the pET-28a vector including the MEV sequence, was constructed to efficiently and effectively encode the MEV protein in the *E. coli* cells. Based on the results of this study, to develop our candidate vaccine against COVID-19, we recommend that validation assays containing *in vitro* and *in vivo* analysis be performed in the future.

Conclusion

Nowadays, the Wuhan Novel Coronavirus 2019 outbreaks worldwide and has become a global problem. Unfortunately, due to a lack of antiviral drugs and any vaccine against COVID-19, morbidity and mortality increase. Therefore, the prevention and control of this infection are very mandatory. This study tried to make a multi-epitope vaccine (MEV) against the Wuhan Novel Coronavirus 2019 using immunoinformatics methods. Thus, in addition to performing fewer experiments and errors, saving time and costs, *silico* methods can be used to design and develop safe and potential MEV vaccines. Here, different computational tools were used to design MEV based on immunogenic epitopes (B and T cells) from the antigenic spike [1] protein of COVID-19. In this work, study results revealed that our vaccine construct might confer proper immunogenic responses. Furthermore, *in vitro* and *in vivo* studies are necessary to ensure the proposed vaccine's efficacy and safety.

Declarations

Conflict of Interest

The authors have no conflicts of interest to declare.

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References

1. Zhang Y-Z, Holmes EC. A genomic perspective on the origin and emergence of SARS-CoV-2. Cell. 2020.
2. Bhatnager R, Bhasin M, Arora J, Dang AS. Epitope based peptide vaccine against SARS-COV2: an immune-informatics approach. Journal of Biomolecular Structure and Dynamics. 2020:1-16.

3. De Wit E, Van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. Nature Reviews Microbiology. 2016;14(8):523.
4. Su S, Wong G, Shi W, Liu J, Lai AC, Zhou J, et al. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends in microbiology. 2016;24(6):490-502.
5. Ziebuhr J. Current topics in microbiology and immunology. Curr Top Microbiol Immunol. 2005;287:57-94.
6. Djomkam ALZ, Ochieng'Olwal C, Sala TB, Paemka L. Commentary: SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Frontiers in Oncology. 2020;10.
7. Kim D, Lee J-Y, Yang J-S, Kim JW, Kim VN, Chang H. The architecture of SARS-CoV-2 transcriptome. Cell. 2020.
8. Schoeman D, Fielding BC. Coronavirus envelope protein: current knowledge. Virology journal. 2019;16(1):1-22.
9. Amanat F, Krammer F. SARS-CoV-2 vaccines: status report. Immunity. 2020.
10. Dhama K, Sharun K, Tiwari R, Dadar M, Malik YS, Singh KP, et al. COVID-19, an emerging coronavirus infection: advances and prospects in designing and developing vaccines, immunotherapeutics, and therapeutics. Human vaccines & immunotherapeutics. 2020:1-7.
11. Pandey SC, Pande V, Sati D, Upreti S, Samant M. Vaccination strategies to combat novel corona virus SARS-CoV-2. Life Sciences. 2020:117956.
12. Srivastava S, Kamthania M, Kumar Pandey R, Kumar Saxena A, Saxena V, Kumar Singh S, et al. Design of novel multi-epitope vaccines against severe acute respiratory syndrome validated through multistage molecular interaction and dynamics. Journal of Biomolecular Structure and Dynamics. 2019;37(16):4345-60.
13. Abdelmageed MI, Abdelmoneim AH, Mustafa MI, Elfadol NM, Murshed NS, Shantier SW, et al. Design of a Multiepitope-Based Peptide Vaccine against the E Protein of Human COVID-19: An Immunoinformatics Approach. BioMed Research International. 2020;2020.
14. Bojin F, Gavriliuc O, Margineanu M-B, Paunescu V. Design of an epitope-based synthetic long peptide vaccine to counteract the novel China coronavirus (2019-nCoV). 2020.
15. Enayatkhani M, Hasaniazad M, Faezi S, Guklani H, Davoodian P, Ahmadi N, et al. Reverse vaccinology approach to design a novel multi-epitope vaccine candidate against COVID-19: an in silico study. Journal of Biomolecular Structure and Dynamics. 2020:1-16.
16. Li L, Sun T, He Y, Li W, Fan Y, Zhang J. Epitope-based peptide vaccine design and target site characterization against novel coronavirus disease caused by SARS-CoV-2. bioRxiv. 2020.
17. Ahmed SF, Quadeer AA, McKay MR. Preliminary identification of potential vaccine targets for the COVID-19 coronavirus (SARS-CoV-2) based on SARS-CoV immunological studies. Viruses. 2020;12(3):254.

18. Baruah V, Bose S. Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-nCoV. Journal of medical virology. 2020;92(5):495-500.
19. Bhattacharya M, Sharma AR, Patra P, Ghosh P, Sharma G, Patra BC, et al. Development of epitope-based peptide vaccine against novel coronavirus 2019 (SARS-COV-2): Immunoinformatics approach. Journal of medical virology. 2020;92(6):618-31.
20. Chen W-H, Strych U, Hotez PJ, Bottazzi ME. The SARS-CoV-2 vaccine pipeline: an overview. Current tropical medicine reports. 2020:1-4.
21. Chen C, Huang H, Wu CH. Protein bioinformatics databases and resources. Bioinformatics for Comparative Proteomics: Springer; 2011. p. 3-24.
22. Florindo HF, Kleiner R, Vaskovich-Koubi D, Acúrcio RC, Carreira B, Yeini E, et al. Immune-mediated approaches against COVID-19. Nature nanotechnology. 2020;15(8):630-45.
23. Kardani K, Bolhassani A, Namvar A. An overview of in silico vaccine design against different pathogens and cancer. Expert Review of Vaccines. 2020;19(8):699-726.
24. Mukherjee S, Tworowski D, Detroja R, Mukherjee SB, Frenkel-Morgenstern M. Immunoinformatics and Structural Analysis for Identification of Immunodominant Epitopes in SARS-CoV-2 as Potential Vaccine Targets. Vaccines. 2020;8(2):290.
25. Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO, Rosales-Mendoza S. An overview of bioinformatics tools for epitope prediction: implications on vaccine development. Journal of biomedical informatics. 2015;53:405-14.
26. Bhardwaj N, Gnjatic S, Sawhney NB. TLR agonists: Are they good adjuvants? Cancer journal (Sudbury, Mass). 2010;16(4):382.
27. Gupta SK, Deb R, Dey S, Chellappa MM. Toll-like receptor-based adjuvants: enhancing the immune response to vaccines against infectious diseases of chicken. Expert review of vaccines. 2014;13(7):909-25.
28. Kumar S, Sunagar R, Gosselin E. Bacterial protein toll-like-receptor agonists: a novel perspective on vaccine adjuvants. Frontiers in immunology. 2019;10:1144.
29. Toussi DN, Massari P. Immune adjuvant effect of molecularly-defined toll-like receptor ligands. Vaccines. 2014;2(2):323-53.
30. Cui B, Liu X, Fang Y, Zhou P, Zhang Y, Wang Y. Flagellin as a vaccine adjuvant. Expert review of vaccines. 2018;17(4):335-49.
31. Gries CM, Mohan RR, Morikis D, Lo DD. Crosslinked flagella as a stabilized vaccine adjuvant scaffold. BMC biotechnology. 2019;19(1):48.
32. Hajam IA, Dar PA, Shahnawaz I, Jaume JC, Lee JH. Bacterial flagellin—a potent immunomodulatory agent. Experimental & molecular medicine. 2017;49(9):e373-e.
33. Chen Z, Wherry EJ. T cell responses in patients with COVID-19. Nature Reviews Immunology. 2020;20(9):529-36.

34. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell. 2020.
35. Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z. Immunological considerations for COVID-19 vaccine strategies. Nature Reviews Immunology. 2020;20(10):615-32.
36. Oany AR, Emran A-A, Jyoti TP. Design of an epitope-based peptide vaccine against spike protein of human coronavirus: an in silico approach. Drug design, development and therapy. 2014;8:1139.
37. Samrat SK, Tharappel AM, Li Z, Li H. Prospect of SARS-CoV-2 spike protein: Potential role in vaccine and therapeutic development. Virus Research. 2020:198141.
38. Cui J, Li F, Shi Z-L. Origin and evolution of pathogenic coronaviruses. Nature Reviews Microbiology. 2019;17(3):181-92.
39. Liu W, Morse JS, Lalonde T, Xu S. Learning from the past: possible urgent prevention and treatment options for severe acute respiratory infections caused by 2019-nCoV. Chembiochem. 2020.
40. Skwarczynski M, Toth I. Peptide-based synthetic vaccines. Chemical science. 2016;7(2):842-54.
41. Lucchese G. Epitopes for a 2019-nCoV vaccine. Cellular & molecular immunology. 2020;17(5):539-40.
42. Walls AC, Park Y-J, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. Cell. 2020.
43. Dorosti H, Eslami M, Negahdaripour M, Ghoshoon MB, Gholami A, Heidari R, et al. Vaccinomics approach for developing multi-epitope peptide pneumococcal vaccine. Journal of Biomolecular Structure and Dynamics. 2019;37(13):3524-35.
44. Eslami M, Nezafat N, Negahdaripour M, Ghasemi Y. Computational approach to suggest a new multi-target-directed ligand as a potential medication for Alzheimer's disease. Journal of Biomolecular Structure and Dynamics. 2019;37(18):4825-39.
45. Livingston B, Crimi C, Newman M, Higashimoto Y, Appella E, Sidney J, et al. A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. The Journal of Immunology. 2002;168(11):5499-506.
46. Saadi M, Karkhah A, Nouri HR. Development of a multi-epitope peptide vaccine inducing robust T cell responses against brucellosis using immunoinformatics based approaches. Infection, Genetics and Evolution. 2017;51:227-34.
47. Chen X, Zaro J, Shen WC. Fusion protein linkers: effects on production, bioactivity, and pharmacokinetics. Fusion Protein Technologies for Biopharmaceuticals: Applications and Challenges. 2013:57-73.
48. Bao Y, Liu X, Han C, Xu S, Xie B, Zhang Q, et al. Identification of IFN-γ-producing innate B cells. Cell research. 2014;24(2):161-76.
49. Kak G, Raza M, Tiwari BK. Interferon-gamma (IFN-γ): exploring its implications in infectious diseases. Biomolecular concepts. 2018;9(1):64-79.

50. Lee AJ, Ashkar AA. The dual nature of type I and type II interferons. Frontiers in immunology. 2018;9:2061.
51. Gasteiger E, Hoogland C, Gattiker A, Wilkins MR, Appel RD, Bairoch A. Protein identification and analysis tools on the ExPASy server. The proteomics protocols handbook: Springer; 2005. p. 571-607.
52. Magnan CN, Randall A, Baldi P. SOLpro: accurate sequence-based prediction of protein solubility. Bioinformatics. 2009;25(17):2200-7.
53. Kuriata A, Gierut AM, Oleniecki T, Ciemny MP, Kolinski A, Kurcinski M, et al. CABS-flex 2.0: a web server for fast simulations of flexibility of protein structures. Nucleic acids research. 2018;46(W1):W338-W43.
54. Atapour A, Mokarram P, MostafaviPour Z, Hosseini SY, Ghasemi Y, Mohammadi S, et al. Designing a fusion protein vaccine against HCV: an in silico approach. International Journal of Peptide Research and Therapeutics. 2019;25(3):861-72.
55. Atapour A, Negahdaripour M, Ghasemi Y, Razmjuee D, Savardashtaki A, Mousavi SM, et al. In silico designing a candidate vaccine against breast cancer. International Journal of Peptide Research and Therapeutics. 2020;26(1):369-80.
56. EL-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. Journal of Molecular Recognition: An Interdisciplinary Journal. 2008;21(4):243-55.
57. Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, Nielsen M. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. BMC bioinformatics. 2007;8(1):424.
58. Jensen KK, Andreatta M, Marcatili P, Buus S, Greenbaum JA, Yan Z, et al. Improved methods for predicting peptide binding affinity to MHC class II molecules. Immunology. 2018;154(3):394-406.
59. Dhanda SK, Vir P, Raghava GP. Designing of interferon-gamma inducing MHC class-II binders. Biology direct. 2013;8(1):30.
60. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC bioinformatics. 2007;8(1):4.
61. Magnan CN, Zeller M, Kayala MA, Vigil A, Randall A, Felgner PL, et al. High-throughput prediction of protein antigenicity using protein microarray data. Bioinformatics. 2010;26(23):2936-43.
62. Dimitrov I, Bangov I, Flower DR, Doytchinova I. AllerTOP v. 2—a server for in silico prediction of allergens. Journal of molecular modeling. 2014;20(6):2278.
63. Sharma N, Patiyal S, Dhall A, Pande A, Arora C, Raghava GP. AlgPred 2.0: an improved method for predicting allergenic proteins and mapping of IgE epitopes. Briefings in Bioinformatics. 2020.
64. McGuffin LJ, Bryson K, Jones DT. The PSIPRED protein structure prediction server. Bioinformatics. 2000;16(4):404-5.
65. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nature protocols. 2015;10(6):845-58.
66. Colovos C, Yeates TO. Verification of protein structures: patterns of nonbonded atomic interactions. Protein science. 1993;2(9):1511-9.

67. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. Journal of applied crystallography. 1993;26(2):283-91.
68. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic acids research. 2007;35(suppl_2):W407-W10.
69. Ponomarenko J, Bui H-H, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC bioinformatics. 2008;9(1):514.
70. Kozakov D, Hall DR, Xia B, Porter KA, Padhorny D, Yueh C, et al. The ClusPro web server for protein–protein docking. Nature protocols. 2017;12(2):255.
71. Grote A, Hiller K, Scheer M, Münch R, Nörtemann B, Hempel DC, et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic acids research. 2005;33(suppl_2):W526-W31.

Tables

Start-end position	Sequence	Score
1067	YVPAQEKNFTTAPA	0.988
596	SVITPGTNTSNQVA	0.982
14	QCVNLTTRTQLPPA	0.963
114	TQSLLIVNNATNVV	0.951
516	ELLHAPATVCGPKK	0.934

Table 1. List of the final B-cell epitopes from S protein of SARS-CoV-2 by BCPREDS server.

Allele	HTL epitopes	1-log50k(aff)	affinity(nM)	%Rank
H-2-IAd	QQLIRAAEIRASANL	0.5960	79.2	0.50
H-2-IAd	TQQLIRAAEIRASAN	0.5958	79.3	0.60
H-2-IAb	QMAYRFNGIGVTQNV	0.5904	84.1	0.40
H-2-IAb	QEKNFTTAPAICHDG	0.5899	84.6	0.40
H-2-IAb	MQMAYRFNGIGVTQN	0.5857	88.5	0.40
H-2-IAb	DSSSGWTAGAAAYYV	0.5849	89.3	0.40
H-2-IAb	MAYRFNGIGVTQNVL	0.5772	97.0	0.40
H-2-IAb	SSSGWTAGAAAYYVG	0.5771	97.1	0.40
H-2-IAb	GDSSSGWTAGAAAYY	0.5771	97.1	0.40
H-2-IAd	YVTQQLIRAAEIRAS	0.5776	96.6	0.70

Table 2. List of the high score HTL epitopes from S protein of SARS-CoV-2 by NetMHCII 2.3 Server.

Position	CTL epitopes Sequence	Prediction score	MHC binding affinity
851	LTDEMIAQY	3.6616	0.7953
244	HTSSMRGVY	3.1128	0.6735
590	TSNQVAVLY	3.0758	0.6559
347	CVADYSVLY	2.5759	0.5348
719	KTSVDCTMY	2.3795	0.4908
732	STECSNLLL	2.3492	0.5136
182	NIDGYFKIY	1.9606	0.3921
146	YSSANNCTF	1.9531	0.3975

Table 3. List of the high-scored CTL epitopes from S protein of SARS-CoV-2 by NetCTL 1.2 server.

Result	Position	Epitope	Score
Positive	Epitope_1	FPSVYAWERKKISNC	1
Positive	Epitope_3	YNYKYRYLRHGKLRP	1.0637672
Positive	Epitope_4	LIRAAEIRASANLAA	0.6887053

Table 4. IFN-gamma inducing epitopes predicted by IFNepitope server.

No.	Residues	Number of residues	Score
1	A:M1, A:I2, A:N3, A:T4, A:N5, A:S6, A:L7, A:S8, A:L9, A:L10, A:T11, A:Q12, A:N13, A:N14, A:L15, A:N16, A:K17, A:S18, A:Q19, A:S20, A:A21, A:L22, A:G23, A:T24, A:A25, A:I26, A:E27, A:R28, A:L29, A:S30, A:S31, A:G32, A:L33, A:R34, A:I35, A:N36, A:S37, A:A38, A:K39, A:D40, A:D41, A:A42, A:A43, A:G44, A:Q45, A:A46, A:I47, A:A48, A:N49, A:R50, A:F51, A:T52, A:A53, A:N54, A:I55, A:K56, A:N147, A:E148, A:N149, A:G150, A:T151, A:I152, A:H536, A:H537	64	0.858
2	A:S190, A:F191, A:R193, A:G194, A:V195, A:Y196, A:Y197, A:G198, A:P199, A:G200, A:P201, A:G202, A:G203, A:K204, A:I205, A:A206, A:D207, A:Y208, A:N209	19	0.738
3	A:T327, A:A407, A:A408, A:E409, A:I410, A:R411, A:A412, A:S413, A:A414, A:N415, A:G416, A:P417, A:G418, A:P419, A:G420, A:Q421, A:Q422, A:L423, A:I424, A:R425, A:A426, A:A427, A:E428, A:I429, A:R430, A:A431, A:S432, A:A433, A:N434, A:L435, A:A436, A:A437, A:Y438, A:L439, A:E442, A:A448, A:A449, A:Y450	38	0.712

Table 5. Conformational epitopes of the designed MEV as predicted by the ElliPro server.

Figures

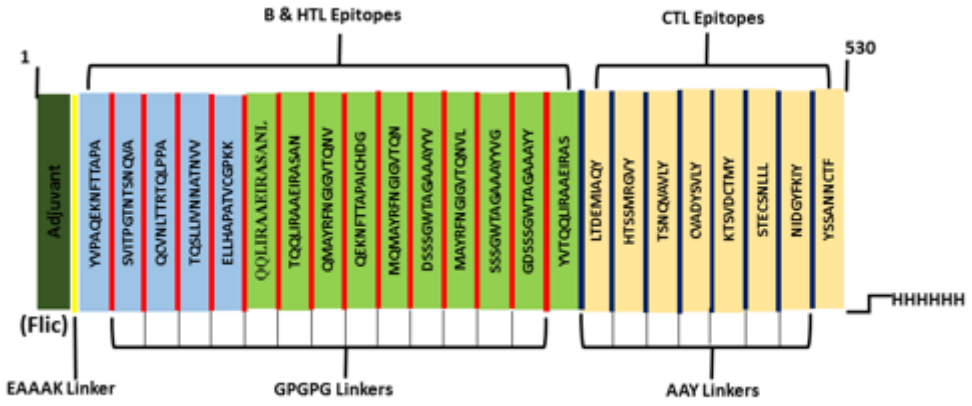

Figure 1

The schematic diagram of the final MEV construct. The constructed sequence containing an adjuvant [40] at the N-terminal end linked with the multi-epitope sequence using an EAAAK linker (yellow). GPGPG linkers (red) are used to link B epitopes and HTL epitopes. AAY linkers (blue) are included for CTL epitopes. A 6x-His tag is placed at the C-terminal for purification and identification purposes.

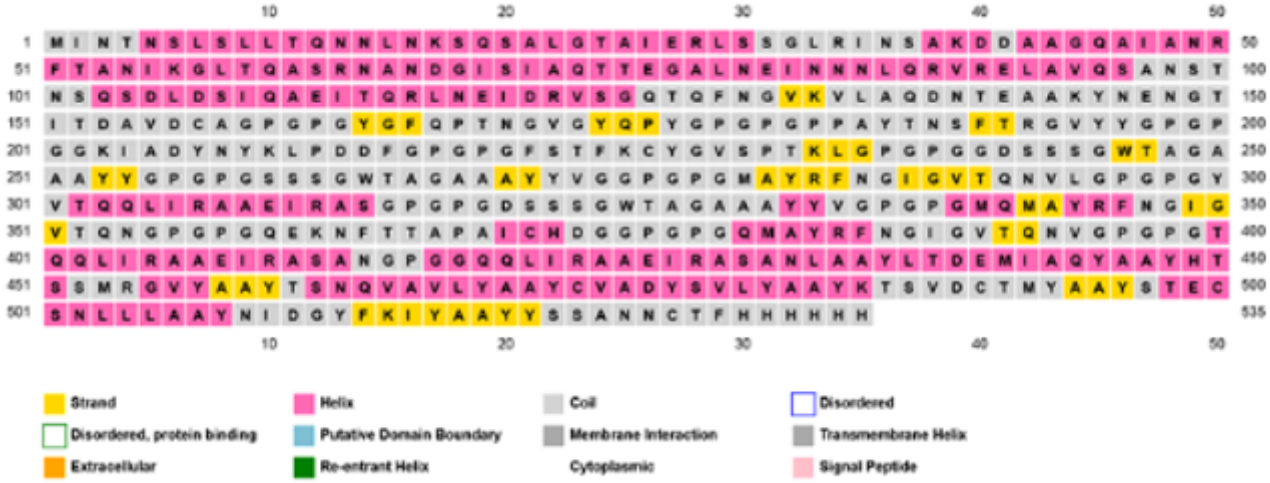

Figure 2

Graphical presentation of the secondary structure of the MEV vaccine, which includes alpha-helices (67.0%), beta strands (5.74%), and coils (47%).

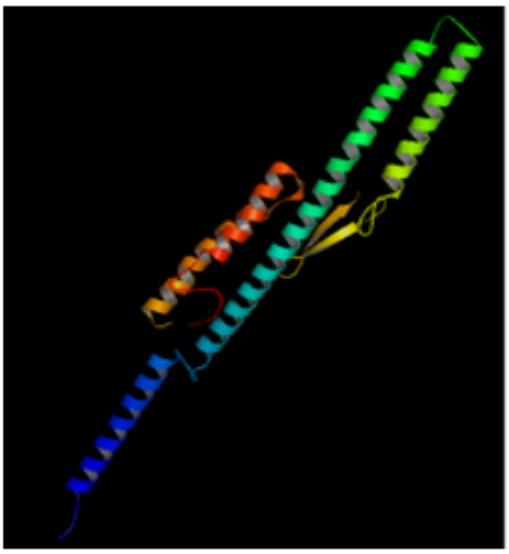

Figure 3

The final 3D model of the multi-epitope vaccine.

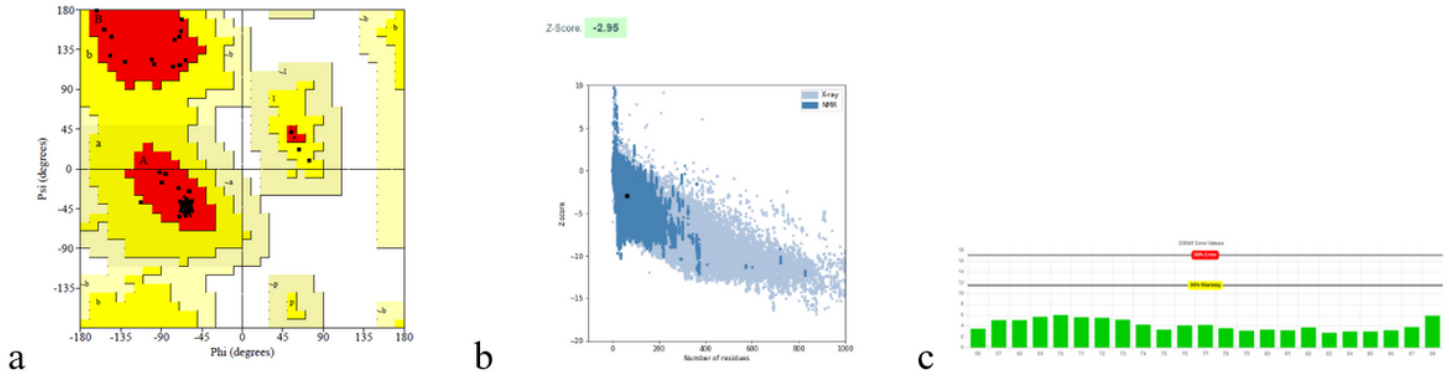

Figure 4

(a) PROCHECK's Ramachandran plot shows that the residues are located in the favored (94.8%) and 5.2% in allowed regions (b) ProSA Z-score plot indicate a −3.45 score that is in the range of native protein conformation (c) ERRAT plot with a score of 100, respectively.

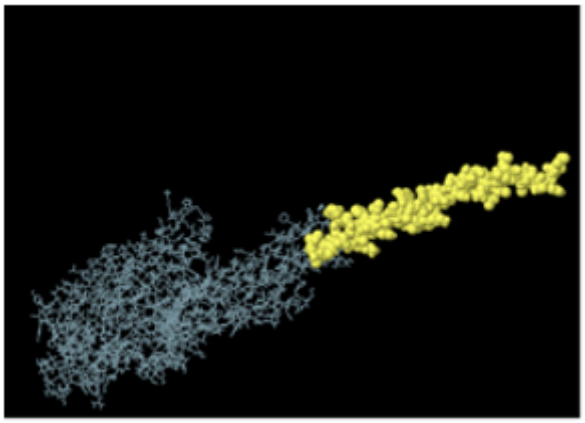

Figure 5

a 3D illustration of the conformational B-cell epitopes predicted in the final MEV.

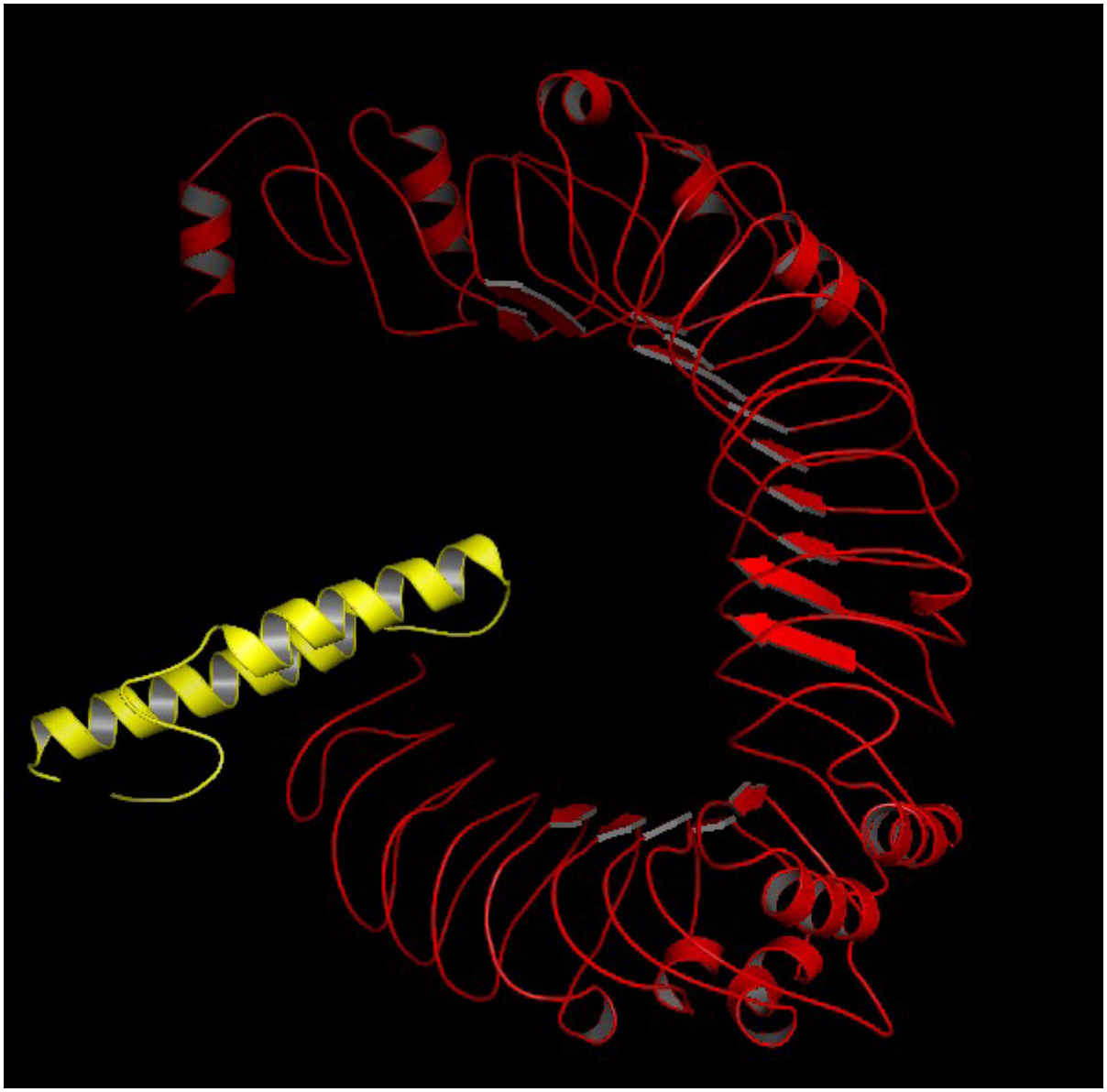

Figure 6

Docking model of the TLR5 molecule and designed multi-epitope protein obtained by the ClusPro server.

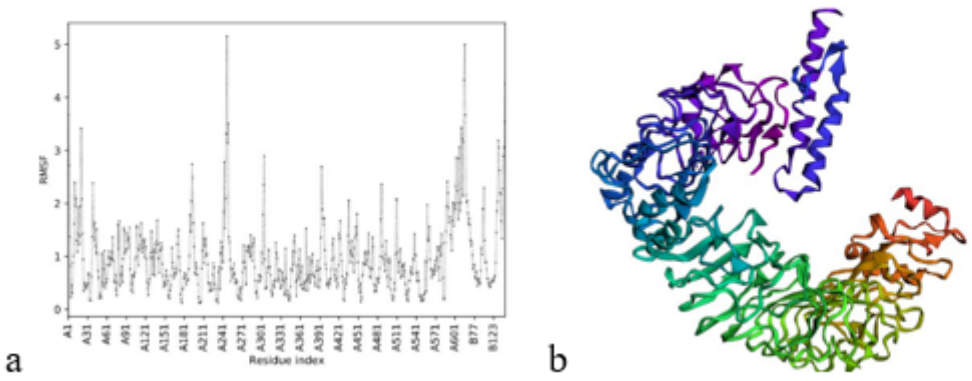

Figure 7

(a) During the fast simulations, the Root Mean Square Fluctuation (RMSF) plot of the protein complex illustrates the fluctuations of MEV residues. A high degree of fluctuation was recognized to be 5.15 Å and 4.32 Å in the residue positions 247 and 615 (b) The complex structure of stable protein after fast flexibility simulations using the CABS-Flex server.

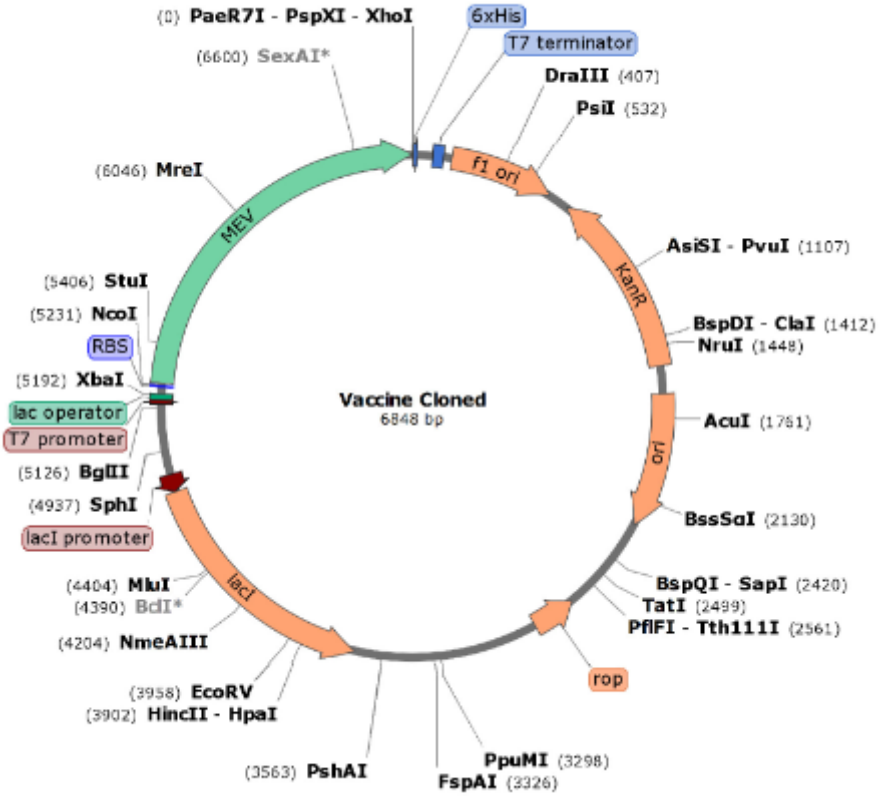

Figure 8

In silico cloned of multi epitopes sequence into pET28a (+) vector. Green colored semicircle showing multi epitopes sequence and Yellow-colored semicircles indicating backbone of pET28a (+) vector.