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EXPERIMENTAL STUDY

From in-silico immunogenicity verification to in vitro expression of recombinant Core-NS3 fusion protein of HCV

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ABSTRACT

BACKGROUND AND OBJECTIVE**:** Hepatitis C virus (HCV) is a serious global health burden. There is no effective vaccine against HCV and new direct acting antivirals (DAAs) are so expensive and virtually unavailable to the public. Therefore, seeking for therapeutic or prophylactic vaccines is exigent and reliever.

METHODS: The secondary and tertiary structures of the recombinant Core-NS3 (rC-N) fusion protein of HCV and its B and T-cells epitopes were evaluated with bioinformatics software. Cloning and in vitro expression of rC-N were performed by pET24a(+) *and E.coli* BL21-DE3 expression host, respectively. The recombinant protein purification was done by affinity chromatography method and then identified by Western blotting using anti-His monoclonal antibody.

RESULTS: The sequences of rC-N protein consist of 1-118 amino acid parts of Core and 1095-1384 amino acids of NS3 were connected by a flexible linker (AAY) with proteasome cleavable site. The expressed and purified 46.7292 kDa rC-N protein had antigenic value up to threshold and conservancy found in this chimeric protein. Ramchandran Plot analysis represented that most residues were fallen in favourable regions. It also interacted with both type I and II major histocompatibility complex (MHC I, II) molecules. The rC-N had antigenic behaviour to create T cell responses.

CONCLUSION: The results indicated that conserved rC-N protein had the ability to induce T-cell-mediated immune responses and it could be utilized as a therapeutic vaccine candidate against HCV *(Tab. 3, Fig. 4, Ref. 40)*. Text in PDF *www.elis.sk.*

KEY WORDS: HCV vaccine, fusion protein, Core, NS3.

Introduction

Hepatitis C virus (HCV) infection remains a leading cause of liver cirrhosis, hepatocellular carcinoma and lymphomas. The recent estimations showed approximately 185 million people, who are infected with this virus, 73 % of them live in middle-income countries (1, 2). However, new direct acting antivirals are over 95–99 % effective against chronic HCV genotype 1 and 2, but they are so expensive (about 63–300000 US $, per a period of treatment, with or without cirrhosis) and inaccessible for all patients, especially for those, who live in low and middle income countries. Therefore, seeking for new drugs and therapeutic or anaphylactic vaccines is exigent (3, 4).

The size of positive sense single stranded RNA genome of HCV is about 9.6 kb and encodes a large poly protein of 3010 to 3033 amino acid residues. The proteins of HCV are arranged along the genome in the following order: N terminal-core-envelope proteins (E1–E2)–p7-nonstructural proteins (NS)–NS2–NS3–NS4A–NS4B–NS5A–NS5B–C terminal (5, 6).

The core protein with 191 amino acids can be divided into three domains on the basis of hydrophobicity: domain 1 (amino acids 1–117), which contains mainly basic residues with two short hydrophobic regions; domain 2 (amino acids 118–174), which is less basic and more hydrophobic and its C-terminus is at the end of p21; domain 3 (residues 175–191), which is highly hydrophobic and acts as a signal sequence for E1 envelope protein (7–9). The sequences of core are the most conserved parts of the hepatitis C genome and have many B cell and T cell epitopes (10).

Non-structural protein 3 contains a serine protease and an RNA helicase. Protease cleaves the genome-encoded polyprotein and inactivates cellular proteins required for innate immunity. Helicase is required for both genome replication and virus assembly. Special characteristics and relatively preserved structure of NS3 makes it

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a suitable target for the development of antiviral therapeutics and vaccine research (7, 8, 11).

Synthetic fusion protein base vaccines are more advanced and applicable in comparison with traditional vaccines. They not only provide opportunities for specific purposes, but also can make ability to exclude deleterious sequences from full-length genome. Also, synthetic protein base vaccines are safer than attenuated or inactivated vaccines. Likewise, HCV culture is difficult and it can be an essential obstacle to traditional vaccine development (12, 13).

The CD4+ and CD8+ T cells immune responses are essential for an effective campaign after infection by hepatitis C virus (14, 15). T cell adoptive transfer therapy or prophylactic vaccine based on Core and NS3 proteins is suitable to induce both CD4+ and CD8+ T cell responses against HCV (16–17).

Objectives

The present study aimed to immunoinformatic analysis, modelling and simulation of conserved and immunogenic regions in core and NS3 genes from HCV 1a genotype by using online bioinformatics tools, thereafter, expression and purification of this projected construct to produce recombinant Core-NS3 (rC-N) fusion protein for its potential application as therapeutic vaccine candidate. By the next considerations, the chimeric produced protein will be evaluated solitarily or besides some adjuvants in the future.

Materials and methods

Sequence analysis of recombinant Core, NS3 fusion protein (rC-N)

In order to do *in-silico* translation of a hepatitis C recombinant fusion protein to obtain amino acid sequences in high levels of CTL epitopes, some parts of core and ns3 gene sequences, corresponding to genotype 1a were derived from the national centre of biotechnology information (hhtp://www.ncbi.nlm.nih.gov). These two peptides were analysed for conserved domains in (NCBI-Conserved Domains) http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=111760.

Physicochemical properties of both fragments were computed using ProtParam online tool (http://web.expasy.org/protparam/) (18), while a flexible linker with proteasome cleavable site was predicted by Rankpep (http://bio.dfci.harvard.edu/RANKPEP) and paproc (http://paproc.de) to obtain final rC-N sequence.

Secondary, tertiary structures and disulfide connectivity of rC-N

Secondary structure prediction and calculating the number of α-helix, β-sheets, turns random coils, and extended strands of rC-N protein were carried out using **S**elf-**O**ptimized **P**rediction **M**ethod with **A**lignment **(SOPMA)** (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and Jpred (http://www.compbio.dundee.ac.uk/jpred/) tools (19, 20).

Disulfide connectivity of the rC-N protein was analysed using **DiA**minoacid **N**eural **N**etwork **A**pplication (**DiANNA**) tool, which predicts cysteine conditions of proteins that have small number of disulfide bonds (21). To get informed of cys-cys connection is valuable in perception the secondary and tertiary structure of protein, because it has an important role in fold permanence.

The **I**terative **T**hreading **ASSE**mbly **R**efinement (**I-TASSER**) online server program, which creates three dimensions (3D) models besides their confidence score (C-Score) was used in order to get tertiary structure prediction. C-score is typically in the range of (–5 to 2), where a C-score of higher value signifies a model with a high confidence and vice-versa (22). Five top patterns were presaged and the best model among them was selected for structural analysis. The pattern was selected by three indicators: C-score, DFIRE2 energy profile (23) and stereo chemical qualities. Stereo chemical analysis of obtained 3D protein models was performed by PROCHECK, ERRAT, VERIFY 3D programs for structural analysis, which was authenticated by **S**tructure **A**nalysis and **VE**rification **S**erver (SAVES) (http://nihserver.mbi.ucla.edu/SAVES) and ramachandram plot assessment (RAM-PAGE) (mordred.bioc.cam.ac.uk/~rapper/rampage.php) (24–27).

Prediction of T-cell epitopes from rC-N

T-cell epitope regions of rC-N were predicted using the web servers SYFPEITHI (http://www.syfpeithi.de/), ProPred-I (http://www.imtech.res.in/raghava/propred1/) and IEDB (http://tools.iedb.org). The cut-off score was adjusted to≥18 for SYFPEITH (28, 29). The outcomes revealed a classified list of nine amino acid sub-strands of the proffered antigen sequence for affinity computations (30).

Prediction of rC-N antigenicity

The antigenicity scores of all the predicted epitopes were examined by VaxiJen v2.0 online antigen prediction tool (http://www.ddg-pharmfac.net/vaxijen/). Epitopes having antigenic score > 0.5 were selected as antigenic. VaxiJen v2.0 allows antigen assortment based on the physicochemical qualities of proteins without the use of sequence alignment (31).

Cloning and expression of rC-N protein

The corresponding rC-N fragment (Core 1-118 aa, AAY linker and NS3, aa 1095-1387), was synthesised using Biomatik company (Canada) and was inserted into BamH I and EcoR I restriction sites of the pET24.a (+) vector, a T7 promoter based plasmid (Invitrogen, USA) and the recombinant construct was transformed into E. coli BL21-DE3 expression system (Invitrogen, USA). Then, induction was carried out with 1 mM isopropyl B-D-thiogalactopyranoside (IPTG) (SinaClon bioscience Co, Iran) at 37 °C for 4 hours. Finally, lysate of bacteria expressing rC-N protein was analysed using Poly Acrylamide Gel Electrophoresis on 12% sodium dodecyl sulfate (SDS-PAGE).

Protein purification under native condition

The recombinant rC-N fusion was purified in native condition using Affinity chromatography method on a nickel-nitrilotriacetic acid (Ni-NTA)-agarose column (Qiagen, Germany) according to the manufacturer procedure. Briefly, the lysate of bacteria expressing rC-N protein was solubilised by lysis buffer containing 2.5 mM imidazole and after 1hour incubation on ice and sonication,

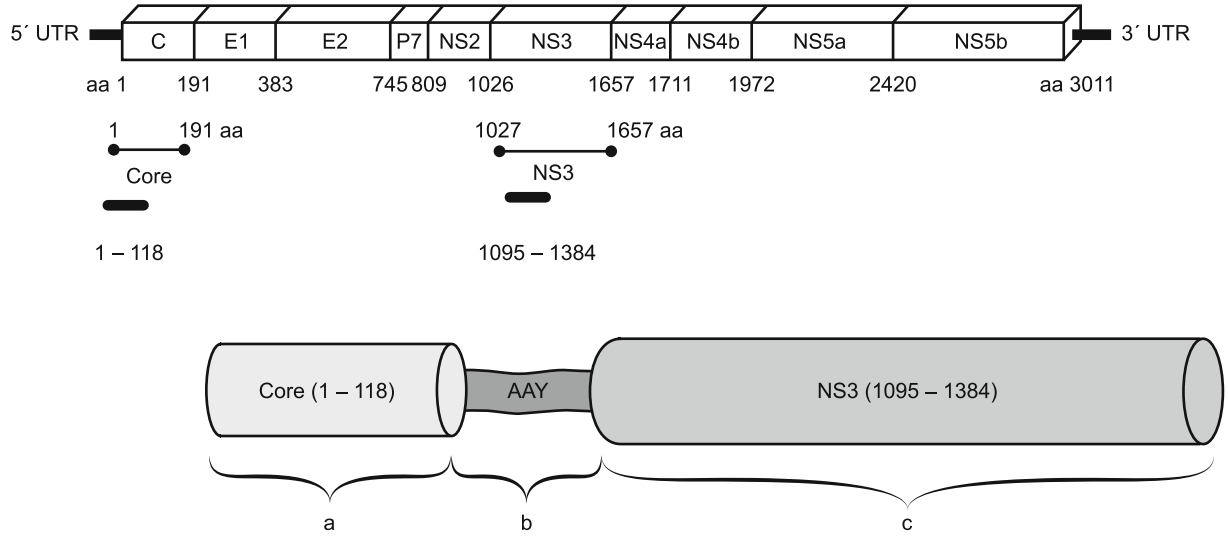

Fig. 1. (A) Schematic representation of the arrangement of core and NS3 in HCV full genome. (B) Recombinant Core-NS3 (rC-N) covering sequences from: a; The 1-118 amino acid parts of Core [Domain 1 (D1)], b; spacer. c; 1095-1384 amino acids of NS3.

the lysate was applied to the Ni-NTA column and following three times washing steps by washing buffer (50 mM imidazole), the purified rC-N was eluted by elution buffer (250 mM imidazole). To remove a high concentration of imidazole, evaluation sample was subsequently exchanged to PBS by overnight dialysis (cut off: 10 KDa, Sigma, USA) at 4°C. and finally protein concentration was measured by Bradford method according to standard protocols.

SDS-PAGE and Western blotting analysis

The purified rC-N protein was analysed by SDS-PAGE and western blotting. The bacterial pellets were suspended in a loading buffer, heated for 5 minutes at 95 °C, and 25 µL of sample was subjected to 15 % SDS-PAGE gel. Western blotting was used in order to verify the purified rC-N protein. In this stage, after purification, the sample was separated by the SDS-PAGE and transferred on nitrocellulose membrane. The membrane was incubated with the conjugated His-tag antibody (Roche, Germany) for 1 hour at room temperature and developed by 3,3'- Diaminobenzidine (DAB) solution (Bio-Rad USA).

Results

Position of rC-N in HCV full genome and its arrangement

The schematic arrangement of the rC-N protein (Domain 1 from Core, truncated middle region of NS3 that consists of some conserved parts of protease and helicase of NS3 and AAY linker between them) is indicated in Figure 1.

Physiochemical, Sequences and structural analysis of rC-N protein

Physiochemical characterization of the rC-N consisted of 437 amino acids with an estimated molecular weight of 46.7292 kDa and theoretical isoelectric point of 10.05, with the calculated grand average hydropathicity of –0.392, which indicates rC-N is soluble and hydrophilic in nature. The total number of positively charged residues (Arg+ Lys) in the polypeptide was 51, and 27 of negatively charged residues (Asp+ Glu). The protein also contains 4 disulfide bridges (Tab. 1).

Secondary structure of the rC-N protein showed that random coil makes the 55.61 % of the protein while sheets, helices and turn contribute 24.71 %, 11.67 % and 8.01 of the structure, respectively (Fig. 2A).

Tertiary structure of the rC-N protein was predicted using the I-TASSER online server and the best predicted structure with the maximum confidence score (C-Score:–1.57) was selected from a set of top models (Figs 2B and 2C).

Prediction of T-cell epitopes

T-cell epitope regions of rC-N were predicted using the web servers SYFPEITHI (http://www.syfpeithi.de/), ProPred-I (http://www.imtech.res.in/raghava/propred1/) and IEDB (http://tools.iedb.org). The cut-off score was adjusted to ≥ 18 for SYFPEITH (22, 23). The outcomes revealed a classified list of nine amino acid sub-strands of the proffered antigen sequence for affinity computations (24).

Epitopes belonging to T cell epitopes were revealed on MHC I and MHCII. We used ProPred-I, IEDB and SYFPEITHI servers for the prediction of T-cell epitopes, with 3 (H-2Kd, H-2Dd and H-2Ld) alleles for MHC class I, and 2 (H2-IAd and H2-IEd) alleles

Tab. 1. Predicted disulfide bonds.

Position of Predicted bonds	Sequence of predicted bonds
105–168	YGNEGCGWAGW – RSLTPCTCGSS
170–363	LTPCTCGSSDL – IICDECHSTDA
216–360	GGPLLCPAGHA – YDIIICDECHS
230–350	FRAAVCTRGVA – LADGGCSGGAY

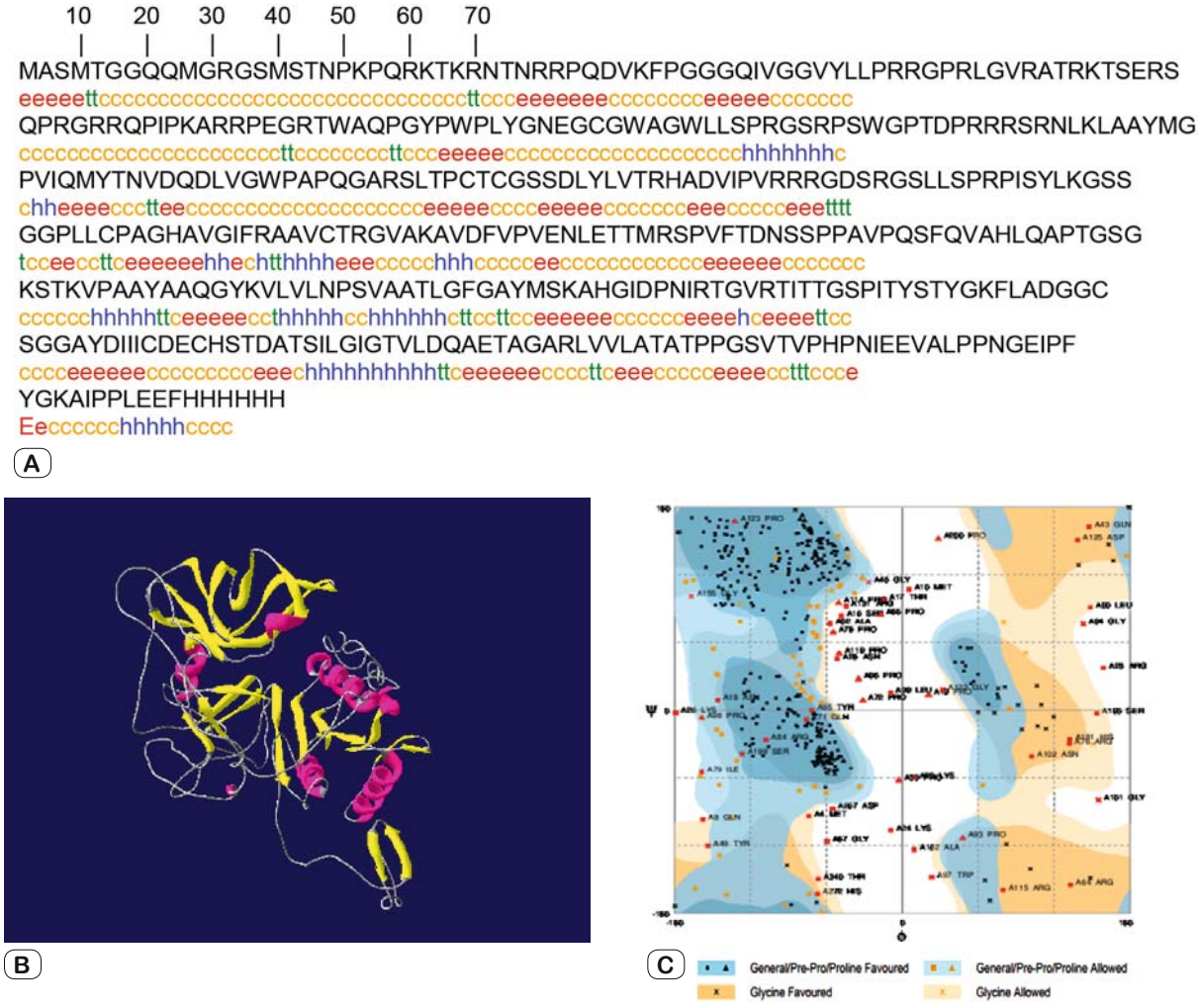

Fig. 2. Sequence and structural analysis of rC-N protein. (A) Secondary structure of the protein with respect to protein sequence and (B) Predicted 3D model rC-N protein obtained from I-TASSER. Image was produced using the Swiss PDB viewer with colours identification as; helices, pink; beta sheets yellow; and all others grey. (C) Ramachandran plot is showing validation of protein structure using the phi and psi angles distribution in the protein. The Ramachandran plot shows that 74.9 % of amino acid residues from modelled structure were incorporated in the favoured regions of the plot. Thirteen point one percentages of the residues were in allowed regions of the plot and 12.0 % of residues in outlier regions.

for MHC class II. The epitopes with top score were predicted for MHC class I and II (Tabs 2 and 3). For MHC class I, 5 epitopes (FYGKAIPPL, HPNIEEVAL, RGPRLGVRA, PRGRRQPIP and LLPRRGPRL) showed antigenicity score greater than antigenicity threshold (0.5).

Evaluation of the recombinant protein

The pET24a+ expression vector harbouring rC-N gene was confirmed using a restriction analysis (Fig. 3). Expression of fusion rC-N gene in *E.coli* BL21-DE3 resulted to a C-terminally 6x His-tagged protein that provided a simplified purification process. Evaluation of rC-N protein using SDS-PAGE method indicated that the produced protein after IPTG induction was corresponding to 46.7292 kDa fusion protein (Core 1-118 aa, AAY linker and NS3, aa 1095-1387). The homogenous protein band under native condition was purified and the protein size was determined about 47 KDa in accordance to the calculated theoretical value by compute MW/pI tool (www.us.expasy.org) (Fig. 4).

Discussion

Current vaccines against HCV couldn't produce robust and broadly cross-reactive $CD4^+$, $CD8^+$T-cells and neutralizing antibody (NAb) responses and couldn't prevent the virus completely. Therefore, as a realistic goal, a vaccine should prevent the progression of HCV infection to chronic and persistent infection by immunotherapy (32). Up to now, there isn't any licensed therapeutic vaccine against HCV. With the improvement of bioinformatics, computational analysis and prediction of genomic and post-genomic structures of antigens became an applied approach in the first step

Tab. 2. *In silico* analysis predicted MHC class I epitopes with 9 mer for rC-N fusion protein.

Starting position	Peptide	Allele	Antigenicity score
311	**AYMSKAHGI**	**H-2K^d**	**0.1959**
145	**MYTNVDQDL**	**H-2K^d**	**0.1030**
337	**TYSTYGKFL**	**H-2K^d**	**0.1231**
420	FYGKAIPPL	H-2K^d	0.7316
412	**LPPNGEIPF**	**H-2L^d**	**0.3514**
260	**SPPAVPQSF**	**H-2L^d**	**0.3523**
404	HPNIEEVAL	H-2L^d	1.1654
92	**QPGYPWPLY**	**H-2L^d**	**-0.2133**
50	LLPRRGPRL	H-2D^d	0.6801
190	**RGDSRGSLL**	**H-2D^d**	**0.0181**
54	RGPRLGVRA	H-2D^d	1.2561
139	**MGPVIQMYT**	**H-2D^d**	**–0.1951**
72	PRGRRQPIP	H-2K^d	1.6553
425	IPPLEEFHH	**H-2K^d**	**0.0789**
175	LYLVTRHAD	**H-2D^d**	**0.4932**

Non-Antigens are shown in bold face.

for vaccine design (33). Investigations showed the numerous *in silico* studies had examined the effects of various vaccines on pathogenic viruses, and indicated that the vaccines targets are mostly proteins (34). Therefore, in order to induce potent and firm immunological responses, specially cellular type, core and NS3 as two highly conserved immunogenic antigens of HCV with multi- specific $CD8^+$ and $CD4^+$ T-cell responses were selected. These specifications make them attractive for peptide-based therapeutic vaccine design (35). But, full length of NS3 by protease and helicase enzymatic activities may disturb the APCs activities and reduce sufficient immune responses (36). Moreover, C- terminal domain of core is essential for processing of HCV, and previous studies showed a suppression of immune response by full length of core in murine model (37, 38), and also, both terminals of core HCV have

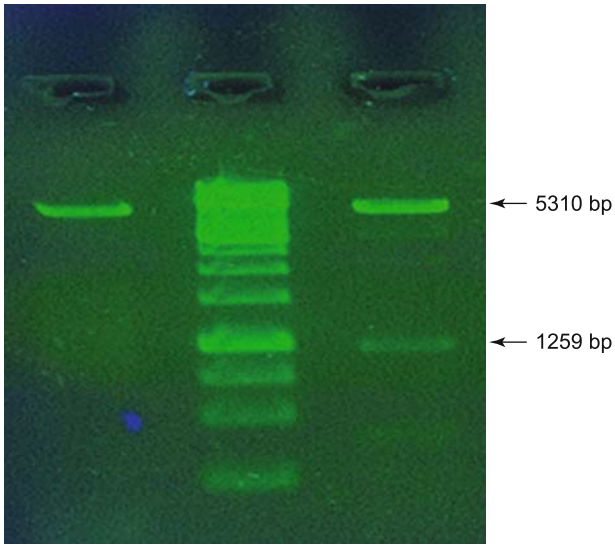

Fig. 3. Restriction analysis of the recombinant plasmid by *BamH* I and *EcoR*. Lane 1: undigested plasmid, Lane 2: DNA ladder marker (1Kb), Lane 3: The corresponding DNA fragment of digested recombinant plasmid (rC-N gene: 1259 bp and the vector: 5310bp).

Tab. 3. *In silico* analysis predicted MHC class II epitopes with 15-mer, for rC-N fusion protein.

Starting position	Peptide	Allele	Antigenicity score
279	SGKSTKVPAAYAAQG	H2-IA^D	0.6573
291	**AQGYKVLVLNPSVAA**	**H2-IA^D**	**0.2886**
383	**TAGARLVVLATATPP**	**H2-IA^D**	**0.3801**
176	YLVTRHADVIPVRRR	H2-IA^D	1.5777
235	AKAVDFVPVENLETTA	H2-IA^D	1.4968
267	SFQVAHLQAPTGSGK	H2-IA^D	0.6072
282	**STKVPAAYAAQGYKV**	**H2-IA^D**	**0.3827**
378	**LDQAETAGARLVVLA**	**H2-IA^D**	**0.3252**
264	VPQSFQVAHLQAPTG	H2-IA^D	0.4150
280	GKSTKVPAAYAAQGY	H2-IA^D	0.5660
297	LVLNPSVAATLGFGA	H2-IA^D	0.9593
299	LNPSVAATLGFGAYM	H2-IA^D	1.0267
303	VAATLGFGAYMSKAH	H2-IA^D	0.9851
373	GIGTVLDQAETAGAR	H2-IA^D	0.5933
380	**QAETAGARLVVLATA**	**H2-IA^D**	**0.3065**
129	RSRNLKLAAYMGPVI	H2-IA^D	0.9641
256	**TDNSSPPAVPQSFQV**	**H2-IA^D**	**0.4598**
143	**IQMYTNVDQDLVGWP**	**H2-IA^D**	**–0.3124**
207	**KGSSGGPLLCPAGHA**	**H2-IA^D**	**–0.0795**
338	**YSTYGKFLADGGCSG**	**H2-IA^D**	**0.0147**
375	**GTVLDQAETAGARLV**	**H2-IA^D**	**0.3267**
388	LVVLATATPPGSVTV	H2-IA^D	0.5535
396	PPGSVTVPHPNIEEV	H2-IA^D	0.5115
95	YPWPLYGNEGCGWAG	H2-IA^D	0.5449
33	PQDVKFPGGGQIVGG	H2-IA^D	–0.0265
118	RPSWGPTDPRRRSRN	H2-IE^D	1.7912
311	AYMSKAHGIDPNIRT	H2-IE^D	0.6769
92	**QPGYPWPLYGNEGCG**	**H2-IE^D**	**–0.0432**
21	**PQRKTKRNTNRRPQD**	**H2-IE^D**	**0.1123**
47	GVYLLPRRGPRLGVR	H2-IE^D	1.2585
179	TRHADVIPVRRRGDS	H2-IE^D	1.3969
226	RAAVCTRGVAKAVDF	H2-IE^D	0.7941
55	GPRLGVRATRKTSER	H2-IE^D	1.3117
73	**RGRRQPIPKARRPEG**	**H2-IE^D**	**0.1051**
104	GCGWAGWLLSPRGSR	H2-IE^D	0.6286
153	LVGWPAPQGARSLTP	H2-IE^D	0.7407
184	**VIPVRRRGDSRGSLL**	**H2-IE^D**	**0.4811**
43	QIVGGVYLLPRRGPR	H2-IE^D	0.5810
46	GGVYLLPRRGPRLGV	H2-IE^D	0.8929
107	WAGWLLSPRGSRPSW	H2-IE^D	0.6516
222	**VGIFRAAVCTRGVAK**	**H2-IE^D**	**–0.6039**
417	**EIPFYGKAIPPLEEF**	**H2-IE^D**	**0.4868**
13	GSMSTNPKPQRKTKR	H2-IE^D	1.0111
16	STNPKPQRKTKRNTN	H2-IE^D	0.9401
145	**MYTNVDQDLVGWPAP**	**H2-IE^D**	**–0.1459**

Non-Antigens are shown in bold face.

autoimmune sequences that are deleterious for vaccine activity (39). Then we used the middle region of NS3, containing some parts of helicase and protease of NS3 and first domain of core without C terminus of core in order to design and construct a recombinant fusion protein as an efficient candidate for HCV therapeutic vaccine.

Herein, *in silico* analysis was applied in computational biotechnology for rational verification and designing of rC-N protein for HCV vaccine development. Accordingly, a new fused construct of hepatitis C peptides including core (1-118) and NS3 (1095-1384) was designed, constructed, expressed and purified as an antigen. A protease-sensitive linker (AAY) was placed between them, in order to optimize the site of fission to facilitate the processing of this bipartite fusion protein and decrease altered bioactivity of the

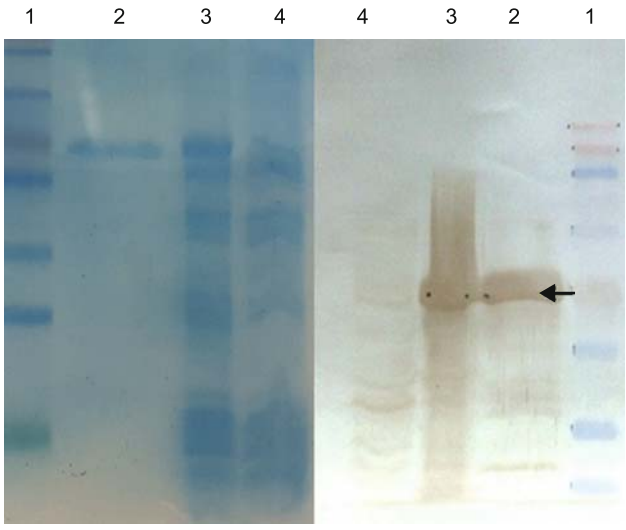

Fig. 4. The profile of rC-N fusion protein by A: SDS PAGE and B: Western blotting for confirmation of expression and purification. Lane1: Pre-stained Protein ladder (9-170 kDa). Lane 2: purified rC-N (46.7292 kDa band indicated by arrow in fig B). Lane 3: Induced lysate by IPTG. lane 4: Uninduced lysate.

protein moieties by the juxtaposition of the epitopes (40). This protein has four disulfide bridges (Table 1), which render extracellular stability in its structure. Ramachandran plot analysis through RAMPAGE of the predicted model resulted in the favoured region and showed that most of the residues are in the allowed regions of the plot, then proving validity of the model.

The I-TASSER online server was used to provide the most accurate structure. The C-Score is an evaluation of the statue of the predicted models by I-TASSER and is calculated based on the importance of threading template alignments and the proximity parameters of the structure assembly reproductions (21). The value of C-score was in the acceptable confidence (–1.57).

The MHC class I presented by target cells along with MHC class II epitopes presented by APCs with antigenic value up to threshold and conservancy predicted in this chimeric protein. Pursuant to aforementioned predictions and results, rC-N has antigenic behaviour to create CD4+, CD8+ T cell responses. The present fusion protein is a scaffold for therapeutic vaccine. This study showed the capability of the rC-N in virtual environment, but it will be challenged in solitude or with different adjuvants in vivo to improve immune responses in BALB/c mice, with regard to further evaluation to produce firstly an effective cellular, and or humoral immunity.

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