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INVESTIGATING NATURAL MUTAGENESIS OF THE **H1N1** VIRUS AND RELATIONSHIPS BETWEEN ITS MUTANTS AND VIRULENCE OF THE VIRUS USING A COMPUTATIONAL APPROACH*

ISTRAŽIVANJA PRIRODNE MUTAGENEZE VIRUSA INFLUENCE **H1N1** I ODNOS IZMEĐU VIRULENTNOSTI TIH MUTANATA I VIRUSA KORIŠĆENJEM MATEMATIČKO FIZIČKOG MODELA*

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Abstract

In this paper we investigated the natural mutagenesis of the influenza H1N1 virus, the relationship between its mutants and virulence of the virus using a physico-mathematical approach, the Resonant Recognition Model and time-frequency analysis. In particular, we analysed the relationships between two surface proteins, hemagglutinin (HA) and neuromidase (NA), because the virulence of the influenza virus depends on the compatibility of HA with NA. We used a frequency domain presentation to distinguish between the selected mutated and non-mutated HA sequences. The results obtained show that the RRM approach is an efficient tool for predicting functional mutations in HA proteins. The possibility of predicting the positions of glycosylations sites in HA proteins is an important objective in studying their functional behaviour and thus, understanding the virulency of the H1N1 virus. Here we also employed time-frequency transformation to predict the locations of N-glycans in the selected HA protein sequence. The findings of this study indicate that the signal processing techniques presented and discussed here are efficient tools that can be successfully applied for structure-function analysis of proteins associated with the influenza H1N1 virus and theoretical allocation of glycosylation sites within the selected HA protein sequence.

Key words
Influenza H1N1 virus, hemagglutinin, amino acid, signal processing, glycasylation, time-frequency analysis

Ključne reči
Influenza H1N1 virus, hemaglutinin, amino kiseline, obrada signala, glikozilacija, vremenska i frekventna analiza.

INTRODUCTION

Influenza A viruses circulate in a wide range of avian and mammalian hosts. B virus genomes consist of eight separate segments shown in figure 1 [1]. Each segment codes for a functionally important protein: (i) Polymerase B2 protein (PB2); (ii) Polymerase B1 protein (PB1); (iii) Polymerase A protein (PA); (iv) Haemagglutinin (HA); (v) Nucleocapsid protein (NP); (vi) Neuraminidase (NA); (vii) Matrix protein (M1 and M2); and (viii) Non-structural protein (NS1). The influenza A and influenza B viruses have the ability to exchange these segments, creating new genetically different viruses. Haemagglutinin (HA) is a glycoprotein presented at the outer surface and regarded as the main influenza virus antigen. HA serves as a receptor by binding to sialic acid (N-acetylneuraminic acid) and induces penetration of the interior of the virus particle by membrane fusion.

New epidemic influenza A strains arise every 1 to 2 years via selected point mutations within HA proteins. These new variants are able to elude human host defences which lead to no lasting immunity against the virus, neither after natural infection nor after vaccination [2]. HA determines the host range, antigenicity and the pathogenicity of the viruses. The HA protein is an important target for diagnostics and used to

designate the subtype of the virus. Currently sixteen different hemagglutinins and nine neuraminidases have been identified. The majority of these viral subtypes are found in waterfowl, with only a few combinations being found in humans and swine. Thus, there is a great interest in analysing the development of the influenza virus and particularly its adjustment to different host cells. One of the most important objectives is to understand how the virus can modify itself to move from animals to humans, leading to a new perspective on prophylactic measures for this and future pandemics.

At present the method for viral entry into host cells, other than respiratory cells, remains unclear. The hemagglutinin and neuraminidase proteins are important targets for diagnostics and used to designate the subtype of the virus. It is known that the compatibility of neuraminidase with hemagglutinin characterises the virulence of the influenza virus. In a virulent virus the mutated hemagglutinin requires compensatory mutations in the neuraminidase protein to maintain its virulence [3,4]. It was found that that viral fitness and virulence are reduced in influenza viruses resistant to neuraminidase inhibitors [5]. Studying mutations in HA proteins may explain the pantropic nature of influenza virus. Therefore, in this computational study we aimed to investigate protein cleavage and interaction/recognition between HA and NA.

NA acts as a receptor-destroying enzyme by catalysing the removal of sialic acids from viral and cellular components. It was shown that NA activity promotes the release of progeny viruses from host cells and prevents virion aggregation [6]. The importance of a functional HA and NA match for productive infection was also suggested indicating that changes in HA receptor-binding activity, occurring during adaptation to a new host, are accompanied by concomitant changes in the NA sequence [7,8]. It was reported that the N-glycans at the tip of HA appear to be potent regulators of virus growth in cell culture. In addition, it was revealed that this effect was dependent on the nature of the accompanying viral NA [6]. The nature and extent of glycosylation of HA have been implicated in altering its receptor binding properties and in the emergence of viral variants with enhanced cytopathogenicity and virulence and in masking its antigenic sites [9]. Therefore, the possibility of predicting the positions of glycosylations sites in HA proteins is an important objective in studying their functional behaviour and thus, understanding the virulency of the H1N1 virus.

Signal processing methods have been successfully used in structure-function analysis of proteins and DNA. In this study we applied signal processing methods for retrieval of informational content about protein biological activity contained in HA protein primary structures. We performed a cross-spectral analysis of HA and NA for each epidemic H1N1 to study the mutual relationship between these functionally important antigenic glycoproteins using the Resonant Recognition Model (RRM) [10,11]. By using this approach we showed that changes in the intensity of a common peak in corresponding frequency spectra can correlate to changes in the level of virulence. We also utilized the RRM for analysis of possible interactions between HA and other functionally important proteins involved in the process of viral entry into the host cell. We then studied how the point mutations in the selected HA protein can affect its spectral function, thus showing that the presented computational approach can assist in distinguishing between the mutated and non-mutated HA proteins. In addition, we used Smoothed Pseudo Wigner Ville distribution (SPWV) to define the functionally important regions in the HA protein (glycosylation sites) and compared our computational findings with the experimentally obtained data. Results of this study revealed that the employed signal processing tools can be used successfully in studying the virulence of HA proteins.

MATERIALS AND METHODS

Resonant Recognition Model (RRM)

It is generally recognised that the relationship between the structure and biological function of a protein and its ability to bind to a specific ligand, can be enunciated in terms of a multistage process which involves specific biorecognition, chemical binding and energy transfer. In order to define the topological and biorecognition processes which underpin biological functions, it is necessary to decode the informational content of the amino acids sequence of a protein. Distribution of periodical patterns and tandem repeats of residues in protein and DNA sequences determine structural and functional characteristics of the molecules. The Resonant Recognition Model (RRM) [10-13] is one attempt to identify the selectivity of protein interactions within the amino acid sequence. The RRM allows investigation of the periodicity of structural motifs with defined physicochemical characteristics, which determine biological properties of protein and DNA sequences. Once these characteristic motifs (frequencies) for a particular protein function is identified, it is possible then to utilize the RRM approach to predict the amino acids in the protein sequence, which mostly contribute to this frequency and thus, to the observed function, as well as to design de novo peptides having the desired periodicities [14,15]. The RRM is able to determine a protein's functional and structural information by analysis of its primary sequences using signal processing methods.

In the RRM, a protein's primary structure is presented as a numerical series by assigning to each amino acid a physical parameter value relevant to the protein's biological activity. Our previous investigations [10-13,16-19] as well as studies of other authors [20] have shown that the best correlation are achieved with parameters related to the energy of delocalized electrons from each amino acid. These findings can be explained by the fact that electrons delocalized from a particular amino acid have the strongest impact on the electronic distribution of energy in the entire protein. In this study the energy of delocalized electrons, calculated as the Electron Ion Interaction Potential (EIIP) [21-23] of each amino acid residue, was used. By assigning to each amino acid the EIIP value we convert the original protein sequence into a numerical sequence. This resulting numerical series represents then the distribution of free electron energies along the protein molecule. The numerical sequences obtained are analysed using Discrete Fourier Transform (DFT) in order to extract information pertinent to the biological function. As the average distance between amino acid residues in a protein sequence is about 3.8 Å, it can be assumed that the points in the numerical sequence derived are equidistant. For further numerical analysis the distance between points in

these numerical sequences is set at an arbitrary value d=1. Peak frequencies in the amplitude cross-spectral function define common frequency components of the two sequences analysed. To determine the common frequency components for a group of protein sequences, we have calculated the absolute values of multiple cross-spectral function coefficients M, which are defined as follows [10,11]:

$$|M_n| = |X_{1,n}| \cdot |X_{2,n}| \Lambda \; |X_{M,n}| \mathrm{K} \; n = 1,2,\mathrm{K} \; N/2 \qquad (1)$$

Peak frequencies in such a multiple cross-spectral function denote common frequency components for all sequences analysed. The presence of a distinct peak frequency in a cross-spectral function implies that all of the analysed sequences within the group have one frequency component in common. From previous studies [11] a fundamental conclusion was drawn: each specific biological function of a given protein or DNA is characterized by a single frequency. It was also shown that proteins and their interacting targets (receptors, binding proteins, inhibitors) display the same characteristic frequency in their interactions. Further research in this direction has lead to the conclusion that interacting molecules have the same characteristic frequency but opposite phases at that frequency [11,12]. Thus, the RRM characteristic frequencies represent a proteins general functions as well as a mutual recognition between a particular protein and its target (receptor, ligand, etc.). As this recognition arises from the matching of periodicities within the distribution of energies of free electrons along the interacting proteins, it can be regarded as resonant recognition.

"Hot spot" amino acids prediction in terms of the RRM and 3-D protein structures

It is known that proteins can express their biological function when they achieve a certain active 3D conformation. By identifying the characteristic frequency of a particular protein, it is possible to predict which amino acids in the sequence predominantly contribute to that particular frequency and consequently to the observed function [10-12]. Since the characteristic frequency correlates with the biological function, the positions of amino acids that are mostly affected by the change of amplitude at the particular frequency can be defined as "hot spots" for the corresponding biological function. The strategy for this prediction includes the following steps:

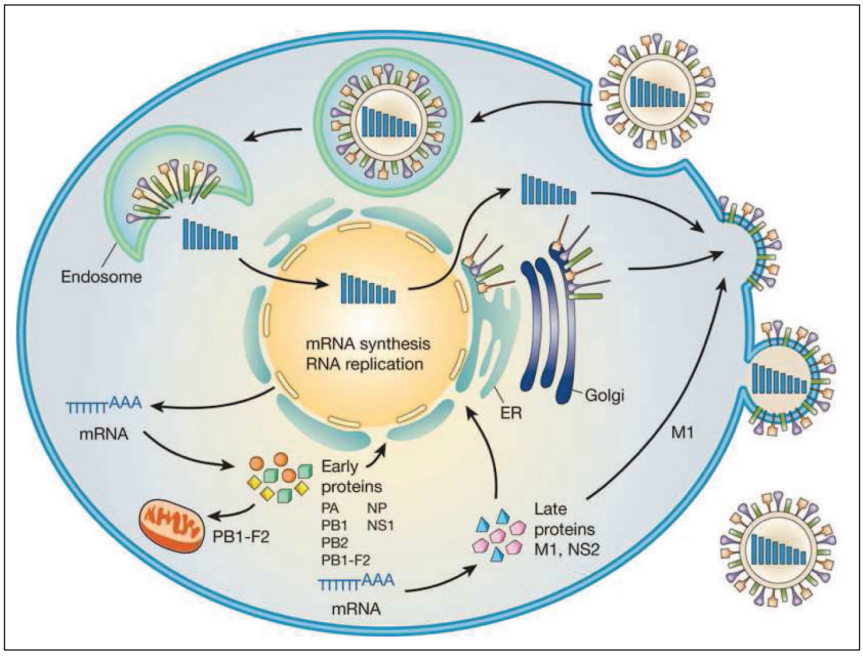
- To determine the unique characteristic frequency for the specific biological function by multiple cross-spectral analysis for the group of sequences with the corresponding biological function.
- To alter the amplitude at this characteristic frequency in the particular numerical spectrum. This involves minimization of a number of amino acids that do not affect the amplitude at the particular characteristic frequency.
- To derive a new numerical sequence from the modified spectrum using Inverse Fourier Transform (IFT). In a newly derived numerical series each term is different from those terms in the original series. By using the tabulated EIIP values we can translate the numerical sequence into the new amino acid sequence.

The procedure described was used in a number of protein examples: hemoglobins, myoglobins, lysozymes, chymotrypsins, glucagons, TNFs, EGFs, FGFs, etc. [11]. These examples have shown that such predicted amino acids denote residues crucial for protein functions. Moreover, these "hot spot" amino acids are found spatially clustered in the protein's 3D structure in and around the protein active site. Therefore, it was proposed that these specific amino acids play a crucial role in determining the structure of the active site and possibly, the active structure of the whole molecule.

Time-frequency analysis

The main disadvantage of signal analysis based on the standard Fourier Transform is that the information about frequency characteristic along the series is hidden, and we can obtain only an averaged time and frequency content of the analysed signal. In the last 20 years the time-frequency distribution methods have become powerful alternative tools for signal analysis. A time-frequency transform presents energy distribution of a signal over the time and frequency domains. In this study we applied the time-frequency signal processing technique to structure-function analysis of HA proteins aiming to demonstrate how the signal's energy is distributed over the two dimensional time-frequency space. By incorporating the Smoothed Pseudo Wigner Ville distribution (SPWV) in the standard RRM approach we intend to overcome the problem of non-localization events currently present in the model and improve the RRM predictive capabilities and accuracy for investigation of a proteins' physical characteristics.

The Wigner quasi-distribution was initially introduced to replace the classical phase-space distribution in statistical physics with a corresponding quantum analogue [24]. Von Neumann [25] established a method where two non-simultane-



Picture 1. Schematic diagram of viral entry of the H1N1 influenza virus in the host cell [1].

ously measurable quantum mechanical quantities, such as the coordinates and momentum, can be measured simultaneously with a limited precision. He also showed that all measurements, with limited accuracy, can be replaced by the absolutely accurate measurements of other quantities, which are related to their non-simultaneously measurable quantities. Although due to the uncertainty principle, the concept of phase space in quantum mechanics is somewhat problematic, various functions which bear some resemblance to true phase-space distribution functions of non-quantum world were introduced. They proved to be useful not only as calculation tools, but also provided insights into the relations between classical and quantum mechanics [25]. The first of such functions was introduced by Wigner [24] to study quantum corrections in classical statistical mechanics. It is now known as the Wigner function. It may be shown [24,25] that the phase space distribution, which is produced in simultaneous measurements of position and momentum, can be represented as a convolution of the Wigner function of considered quantum state and the Wigner function of the filter state, which represents a measuring apparatus.

In general, Wigner-Ville distribution (WVD) describes the frequency content changes as a function of time. The distribution is the actual energy intensity of various frequency components of the signal at a given position along the protein assuming that the average distance between amino acids is set at an arbitrary value d=1. In practical calculations, convolution of the signal generates the cross term that represents interference of the signals, and consequently decreases significantly the resolution of the signal. A number of methods have been developed to reduce the cross-term [26]. In this investigation we replaced the WVD by the Smoothed Pseudo Wigner-Ville distribution (SPWVD), where some window functions are convolved with the WVD to restrain and decrease the effect of the interference terms. Supposing EIIP*[i]*, i=1,2..N is the numerical sequence of the Electron Ion Interaction Potentials of amino acids along the polypeptide chain, then the SPWVD of EIIP*[i]* is given by [26]:

$$S(t,f)=\int_{-\infty}^{\infty} h(\tau)\int_{-\infty}^{\infty} g(s-t)z(s+\tau/2)z(s+\tau/2)^{*}\,ds\,e^{-j2\pi\nu\tau}d\tau \quad (2)$$

In discrete form the SPWVD can be calculated as:

$$W(n,m)=\frac{1}{2}N\sum_{k=-N+1}^{N+1}|h(k)|\sum_{p=-M+1}^{M-1}g(p)z(n+p+k)z^{*}(n+p-k)e^{-\frac{2i\pi km}{M}} \quad (3)$$

Where h(k) and g(p) represent an independent frequency and time smoothing, respectively. In this study we used Gauss filters as the smoothing functions. These are defined as:

$$h(k)=e^{(-k^2/2\sigma)/(\sigma\sqrt{2\pi})} \quad \text{and} \quad g(p)=e^{(-p^2/2\sigma)/(\sigma\sqrt{2\pi})} \quad (4)$$

where s is standard deviation , and k and p are the average values in the frequency and distance sets. The resulting SPWVD could be shown in a t-f plane as a contour plot according to the values of S(t,f), which represents the distribution of the signal energy in the space domain. By choosing the standard deviation of the Gaussian functions h and g, we are practically balancing between the resolution in frequency and space domain interferences. In this paper we employed the SPWVD to predict the locations of glycosylation sites in the selected HA protein sequence.

RESULTS

The binding of the influenza virus H1N1 to the host cell as well as its cleavage from the binding site at the host cell are essential for the viral entry. Cleavage is the functional role of viral neuraminidase [27]. Here we performed a cross-spectral analysis of HA and NA for H1N1 epidemic. We aimed to test the hypothesis that the most virulent H1N1 influenza virus has to have similar changes in HA and NA. Ultimately the change of the intensity of a common peak can correspond to the change in level of virulence. The RRM was used to investigate the structure-function relationship of HA and NA proteins. The characteristic frequencies of sixteen HA protein sequences was identified at f1=0.282 and less significant at f2=0.296 (figure 2). The presence of only one prominent peak with a significant signal intensity/amplitude in the cross-spectral function implies that all of the analysed sequences within the group have this frequency component in common. This frequency presents a characteristic feature of the selected HA protein and thus, corresponds to their common biological activity, e.g binding to and enter into host cells during virus infection process Similarly, the characteristic frequency of sixteen NA proteins was identi-

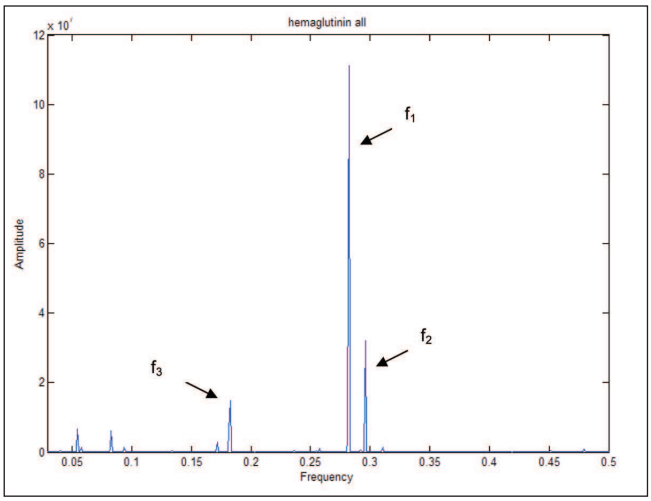


Figure 2. Multiple cross-spectral function of the sixteen Hemagglutinin protein sequences. The prominent peak(s) denote common frequency components. The abscissa represents the RRM frequencies and the ordinate is the amplitude (normalised signal intensity).

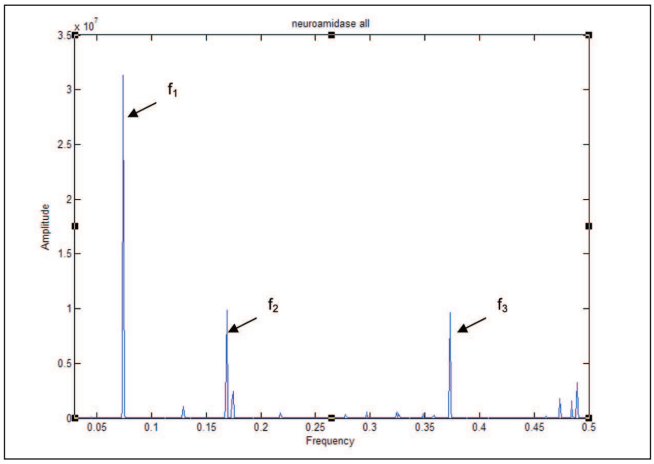


Figure 3. Multiple cross-spectral function of sixteen Neuramidase protein sequences. The prominent peak(s) denote common frequency components. The abscissa represents the RRM frequencies and the ordinate is the amplitude (normalised signal intensity).

fied at f1=0.074 (less significant are at f2=0.169 and f3=0.373). This determined frequency is a common feature of all NA protein sequences analysed, and correspond to their common biological function, e.g. preventing virion aggregation. The multiple cross-spectral function of the analysed NA proteins is shown in figure 3.

Once the characteristic frequencies of the selected HA and NA proteins were determined, we could identify the amino acid residues in the HA and NA sequences, which predominantly contributed to a protein's specific characteristic frequency, by applying an inverse resonant recognition model procedure. The mutated HA protein sequence (gi|516371|emb|CAA40728.1| Influenza A virus (A/swine/Hong Kong/1/1974(H1N1), NCBI Database) was studied and the "hot spots" analysis was carried out. Results revealed that the amino acids mostly contributing to the HA characteristic frequency f1=0.282 were identified to be at the following positions: G157, G242, G254, F259, F272, G277, G279, G301, and F309. According to the RRM concepts these "hot spots" will be spatially clustered in the protein's 3D structure in and around the HA protein active site. The same procedure was applied to analysis of the selected NA sequence (gi|254692675|emb|CAO82699.1| Influenza A virus (A/swine/Cotes d'Armor/016007/2005(H1N1), NCBI Database). The computationally determined "hot spot" amino acids mostly contributing to the characteristic frequency of sixteen NA proteins f1=0.074 are predicted to be located at: G247, G260, F317, G326, G347, G353, G359, G367, G393, G406, and G412.

We also analysed the mutual relationships between various proteins functionally important for the viral infection of a host cell: (i) Polymerase B2 protein (PB2); (ii) Polymerase B1 protein (PB1); (iii) Polymerase A protein (PA); (iv) Haemagglutinin (HA); (v) Nucleocapsid protein (NP); (vi) Neuraminidase (NA); (vii) Matrix protein (M1 and M2); and (viii) Non-structural protein (NS1). We used the RRM to determine the characteristic frequencies for their mutual interactions. The flow chart diagram presented below (figure 4) summarises the relationships between the analysed proteins in a spectral domain and present the life cycle of the H1N1 Influenza virus. In particular, each block of the flowchart represents a specific intracellular and/or extracellular biological process (interaction) involved various viral proteins. By using the RRM, we identified the characteristic frequencies that correspond to each biological process (interaction). The calculated frequencies are given in figure 4.

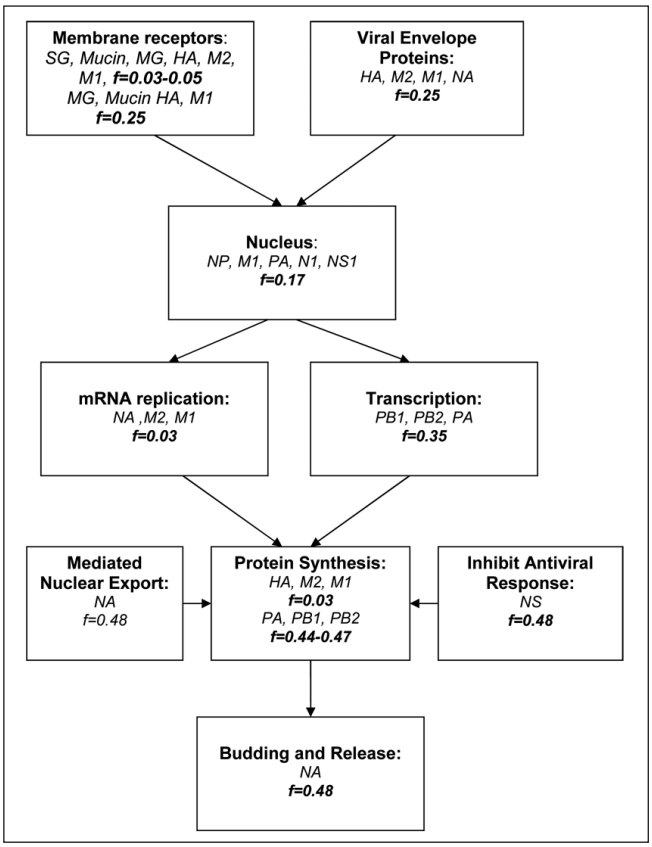


Figure 4. Flow chart showing the mutual interactions between the functionally important proteins involved in H1N1 virus entry into host cell.

In addition, we used a frequency domain presentation to distinguish between the selected mutated and non-mutated HA sequences. The cross-spectral functions of twelve non-mutated and twelve mutated HA protein sequences are shown in figures 5 and 6 respectively. From these figures we can observe that the same HA characteristic frequency (identified at f1=0.282) is on both spectral functions presenting non-mutated (figure 5) and mutated (figure 6) HA proteins. However, these frequencies have different amplitudes (signal intensities). This finding implies that a substitution of only one amino acid at the position of D222G leads to a significant change in the spectral characteristics of the respected analysed sequence. This in turn can lead to changes in affinity of binding of HA to sialic acid. Thus, the result obtained shows that the RRM approach is an efficient tool for prediction of functional mutations in HA proteins. This

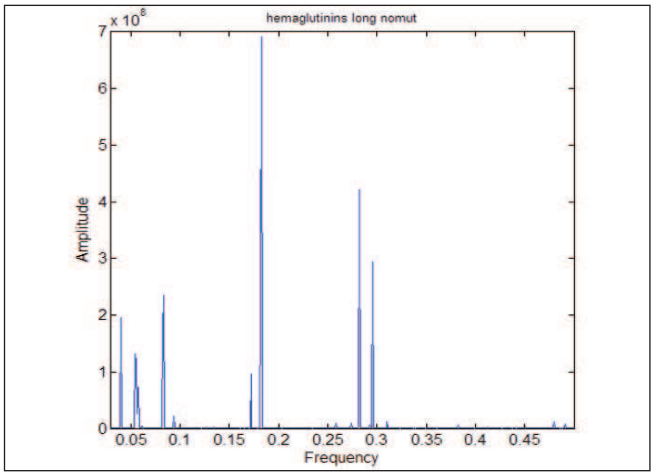


Figure 5. Multiple cross-spectral function of twelve non-mutated hemagglutinin protein sequences.

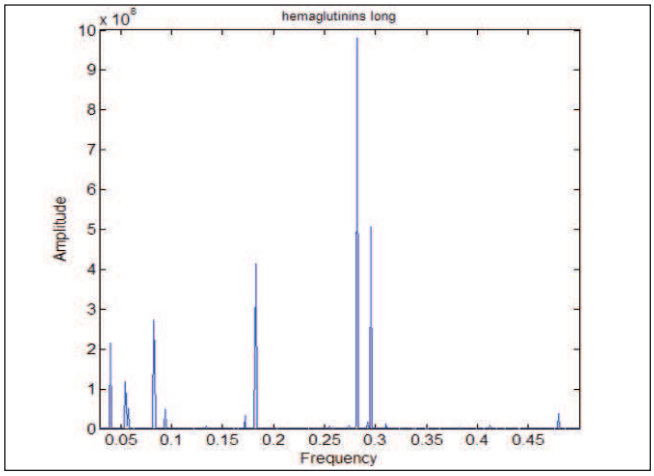


Figure 6 Multiple cross-spectral function of twelve mutated hemagglutinin protein sequences.

finding can be useful in development of therapeutic drugs for treatment of H1N1 influenza virus.

It was shown that glycosylation sites at certain positions on the HA of influenza A viruses, isolated from various animals and humans, are highly conserved and therefore, appear to be essential for the formation and/or maintenance of functional HA [9]. Computational prediction of the positions of N-glycans within the analysed HA protein will contribute greatly to elucidating the functional activity of the HA protein. In this study we applied the SPWVD (using time-space analogy) for the analysis of structural and functional content of the selected HA protein sequence. Thus, the "spatial component" or the position of specific amino acids along the protein chain can be represented in the time-space plane. Influenza A virus (strain A/Duck/Alberta/35/1976 H1N1) was selected as a protein example here. As can be observed from figure 7 in the time-space plane, there are high energy areas corresponding to the HA protein characteristic frequency f1=0.282. These areas are located at the following amino acid positions: 25-40, 100-115, 180-205, 301-307, and 482-496. These computational predictions were compared with the available structural data obtained using the method for isolation and identification of individual glycosylation sites [9]. Evidently, the results of computational analysis correspond closely with the experimental findings (NCBI Database, Taxonomic identifier 352520). The results obtained reveal that the presented SPWVD method is a rational approach which can be successfully applied for theoretical allocation of glycosylation site positions within the selected HA protein sequence.

CONCLUSION

In this study we demonstrated that our computational models, the RRM and SPWVD, can be used efficiently to model and retrieve the structure-functional contents of the analysed surface glycoproteins HA and NA. We also analysed protein-protein recognition and interaction between the various proteins associated with the viral entry of infuenza A H1N1 into the host cell. We also showed with examples of the selected HA and NA proteins that it is possible to predict the positions of "hot spot" amino acids functionally important for the biological activities of the analysed proteins using the RRM approach. Furthermore, we demonstrated that by using the SPWVD and applying it to the analysis of the selected primary sequence of the HA protein, we are able to identify/predict the specific N-glycans positions of the HA protein which are known as HA glycosylation sites, which in turn leads to better understanding of HA functionality and virulency of the influenza virus.

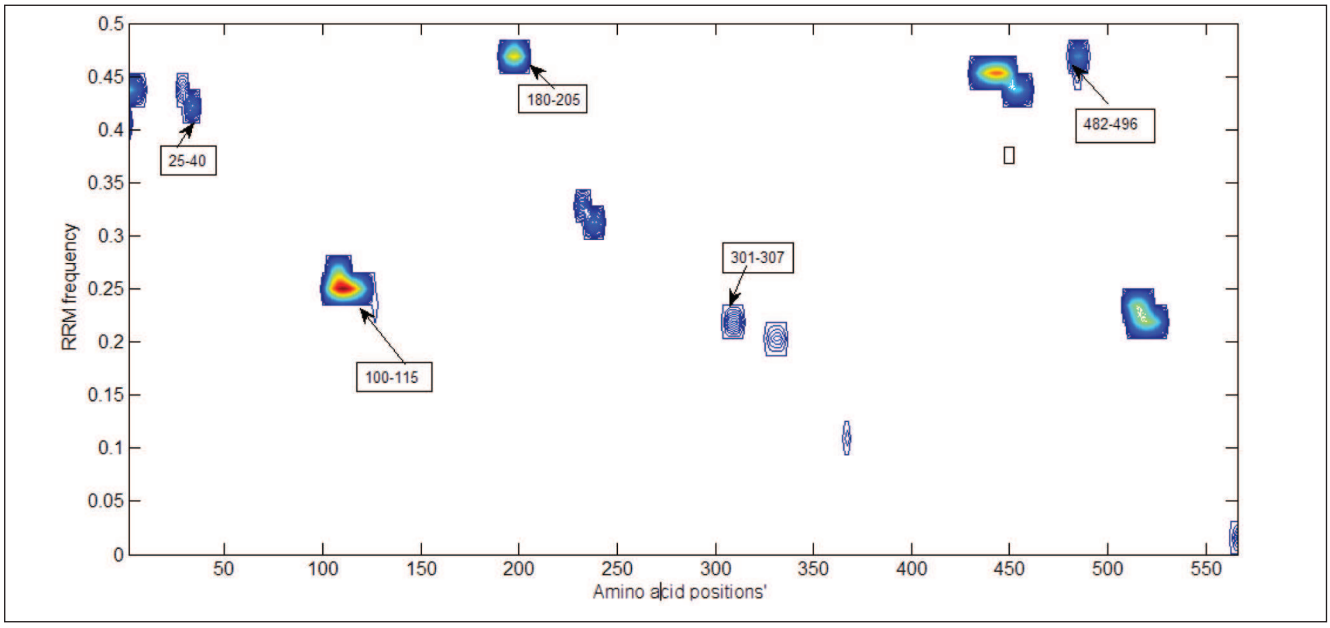


Figure 7. Time-space representation of the HA Influenza A virus (strain A/Duck/Alberta/35/1976 H1N1) analysed using the SPWVD method. High energy areas correspond to the positions of the glycosylations sites within the HA protein sequence.

Apstrakt

U ovoj studiji istraživali smo pojavu prirodnih mutacija virusa influence H1N1. Takođe je posmatrana relacija mutanata i njihove virulentnosti koristeći fizičko matematički pristup (Resonant Recognition Model i vremensko-frekventnu analizu). Kao specijalni slučaj mi smo analizirali relaciju dva površinska proteina: hemaglutinina (HA) i neuroamidase (NA) zbog njihovog uticaja na virulentnost H1N1 virusa. Za razdvajanje mutiranih od nemutiranih HA proteina koristili smo prezentaciju u frekventnom domenu.

Dobijeni rezultati pokazuju da je RRM efikasan alat za predviđanje funkcionalnih mutacija u HA proteinu. Moguænost predviđanja pozicije mesta glikozilacije na HA proteinima je važan cilj u studiji njihovog funkcionalnog ponašanja a samim tim za razumevanje virulentnosti H1N1 virusa. U ovom radu je takođe primenjena vremenska i frekventna analiza za predviđanje N-glukana u prethodno izabranom HA proteinu. Nalazima u ovoj studiji potvrđuju se efikasnost tehnike za obradu signala koji su obradjivani u ovom radu i pokazano je da ista tehnika može da se uspešno primeni za strukturno-funkcionalnu analizu proteina asociranih sa influencom H1N1.

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