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SYNTHESIS AND CHARACTERIZATION OF ANTIGENIC PEPTIDE OF SHEEP POX DISEASE

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ABSTRACT

The aims of this study are to synthesize the Sheep-Pox Diseases' 92-118 antigenic peptide sequence epitope belonging to P32 major protein by microwave supported solid phase peptide synthesis method, characterize the synthesized peptide and bind it covalently to polyacrylic acid polymer (PAA). This polymer showing adjuvant properties, peptide-polymer conjugates synthesize with constant peptide ratios and various polymer ratios and characterize the peptide-polymer conjugate in order to prepare a vaccine prototype for the aforementioned disease is another purpose of the study.

Keywords: *sheep-pox disease, solid phase peptide synthesis, bioconjugation*

1. INTRODUCTION

Sheep-Pox is an extremely contagious disease caused by Sheep-Pox Virus (SPPV) belonging to Capripoxvirus genus. SPPV is a big (>200 nm) virus with a linear, double stranded 150 kb sized genome. SPPV is a notifiable disease by definition of the World Health Organization (WHO) [1-3].

Vaccination is the most economic and efficient way to control Sheep-Pox Disease and increase ovine productivity in endemic countries. Even though many animals benefit from live and inactive vaccines against Sheep-Pox Disease, studies aimed at developing a new generation of vaccines are still underway [4, 5]. Peptide vaccines, which is a type of new generation vaccine system, have no disadvantages that classic vaccines exhibit and have been on the agenda of academics in the past few years[6, 7].

The basis of the new generation of synthetic peptides is the synthesis of peptides that constitute antigenic regions of pathogen spreading and link synthesized peptides to carriers of polymers and proteins with adjuvant properties through various procedures aimed at obtaining a strong immune response [8-10]. Traditionally, the synthesis of determined antigenic peptide epitope to be used in the new generation of synthetic vaccines is grouped into two categories. These include the classical peptide solution synthesis method and the solid phase peptide synthesis method. Even though classical methods are still relevant in pharmaceutical industry due to short-term peptide series synthesis, the very technique is slow, problematic and time consuming. Solid phase peptide synthesis (SPPS) method allows to get larger and more complex peptides synthesized with high purity and yield [11-13].

Solid phase peptide synthesis consists of 3 basic steps as follows: removal of protection, activation of carboxyl group and formation of peptide bond. These steps are called de-protection, activation and coupling respectively and observed at all amino acids until the desired peptide series is obtained. In the last step, the protective group of final amino acid added can be removed (final de-protection) to get the N- end freed. The application of microwave in peptide synthesis goes back to 1992, and the microwave-backed peptide synthesis has become one of the most commonly used methods in research works studying the peptide chemistry for synthesizing both routine and complex peptides. Throughout the peptide synthesis, the

microwave energy is the only one to radiate during the reaction within a specified time limit or until the environment reaches a certain temperature. The vibrating property of the microwave prevents the formation of aggregations that arose from intervening of chains and deletions caused by absence of added amino acids on the long peptide chain growing on top of resin [11, 14-16].

In the paper, antigenic peptide epitope of P32 major proteins' 92-118 peptide sequence of the stated Sheep-Pox Disease [17] was synthesised via microwave-backed solid phase peptide method while purification and characterization were performed with the help of LC-MS, GPC, Zeta Sizer and Fluorescence Spectroscopy. In order to obtain water soluble conjugates which can be used as vaccine prototype, the synthesised peptide was covalently bounded to an adjuvant PAA polymer via cross-linker, so the obtained peptide-polymer bioconjugates were described through the use of GPC, Zeta Sizer and Fluorescence analysis.

2. MATERIAL-METHOD

Material

Wang resin charged with Lys which is required for the synthesis of synthetic 92-118 peptide sequence *(EAKSSIAKHFSLWKSYADADIKNSENK*, NH_2-Glu - Ala - Lys - Ser - Ser - Ile - Ala - Lys - His - Phe - Ser - Leu - Trp - Lys - Ser - Tyr - Ala - Asp - Ala - Asp - Ile - Lys - Asn - Ser - Glu - Asn - Lys –COOH) belonging to P32 major antigen of Sheep-Pox Virus used in the study. Note that amino acids and linker reagents were obtained from Nova Biochem and DMF, DCM, TFA, HOBt, HCTU, DIEA, NMP while Fmoc-protected amino acids obtained from Sigma Aldrich. The carrier Polyacrylic Acid (PAA) polymer, as well as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) obtained from Sigma; disodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide (NaOH), sodium chloride (NaCl) obtained from Fluke; sodium hydrogen phosphate (NaH_2PO_4) and hydrochloride acid (HCl) obtained from Riedel-de Haën. Ultra-pure water obtained from Millipore MilliQ Gradient system was used as solvent for any solution prepared.

Peptide synthesis

The antigenic property of synthetic 92-118 peptide sequence belonging to P32 major antigen of Sheep-Pox Disease is referred to in the literature[17].

The synthesis of the antigenic peptide in this study was performed with a microwave-backed solid phase peptide synthesis device (Liberty (CEM, USA)). The synthesis of the peptide was carried out with microwave-backed SPPS technique inside DMF, using 2-(6-chloro-1H-benzotriazole-1-yl)–1,1,3,3–tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole (HCTU/HOBt) as an activator, N, N-diisopropylethylamine/N-methyl–2-pyrrolidinone (DIEA/NMP) as an activator base and piperidine as a de-protector (DEP). Also, Wang resin was inflated inside DMF prior to the synthesis.

Removal of peptide from resin (cleavage)

In order to deprive the peptide of the solid support, the obtained resin-based peptide was slowly mixed inside the cleavage cocktail [(TFA/thioanisole/EDT/water), (87,5/5/2,5/5 mL, (v/v)] for 3 hours at room temperature with the help of mixer. Cold ether (-20°C) was used for precipitation and removal of peptide. After the peptide was centrifuged, the precipitation procedure was repeated and the final precipitate washed and then dried.

Peptide purification and description

The synthesised peptide was analysed and purified by means of preparative HPLC (Shimadzu) and Shimadzu PRC-ODS C-18 column 30 x 2.1 column with UV detector.LC-MS system (Shimadzu LC MS 2010 EV). Electro Spray Ionizing (ESI) probe and Teknokroma Tracer Exel 120 ODS-A, C-18 column (20 cm length and 0.21 cm inlet diameter) were used to describe the peptide.

The 92-118 sequenced synthetic peptide belonging to P32 major antigen of Sheep Pox

Virus (*EAKSSIAKHFSLWKSYADADIKNSENK)* is composed of 27 amino acid residues. The 13th hydrophobic and non-polar amino acid Tryptophan (Trp) absorbs UV radiation at 280 nm.s' which gives the sequence a fluorescent property. Other hydrophobic and non-polar amino acids of the sequence --- Phenylalanine (Phe) and Tyrosine (Tyr) absorb UV radiation similar to Trp; however, their absorbance values and quantum yield proved to be lower than that of Trp. Note that with its' -OH group, Tyr contributes to the Hydrogen bonds that contribute to peptide formation. All of three aromatic amino acid mentioned above absorb UV radiation at 260-280 nm wavelengths with the double conjugate bonds they have to arise from a specific peak for each of the amino acids. Amino acids which include highly hydrophobic side chains such as Alanine (Ala) and Leucine (Leu) cluster with each other in an aqueous solution and help the peptide to get stabilized with hydrophobic interactions. Sequences that polar side chains follow such as Serine (Ser), Asparagine (Asn) raise the solubility of the peptide because of their hydrophilic properties which allow them to get dissolved in water better as compared to other amino acids. Also, the hydroxyl group of Serine and amide group of Asparagine form Hydrogen bonds with water which is crucial for peptides of three dimensional structure. Given the covalent modification of the peptide sequence and the conjugation mechanism, the most crucial amino acids are as follows: Aspartic acid (Asp), Glutamic acid (Glu), Histidine (His) and Lysine (Lys). It should be noted that 5 Lys amino acid on a peptide sequence not only provides the protein of hydrophilic property with their -NH$_2$ functional groups but these groups also allows the peptide to get outwards and thus allow it to interact with water molecules. Note that Asp and Glu side chains contain -COOH functional groups whose pKa values are so low (pKa$_{Asp}$ =3.86, pKa$_{Glu}$=4.25) and charges are protected when they join the structure of the peptide even at the physiological pH (-). With their high polarity and solubility, the amino acids are located on outer regions of the peptide and contribute to ionic and hydrogen bond formations. Imidazole group that HIS carries can both donate and accept H$^+$. The pKa value of imidazole ring (pKa=6.0) on the side chain is very close to the physiological pH which provides HIS of amino acid with greater buffering effect[18, 19].

The isoelectric point and net charge at pH=7 values for the synthesized peptide had been measured with Innovagens' peptide calculator which read 9.52 and 1.1 respectively. It revealed that the peptide is highly water soluble and its hydrophilic and hydrophobic amino acid residues are given in Fig. 1.
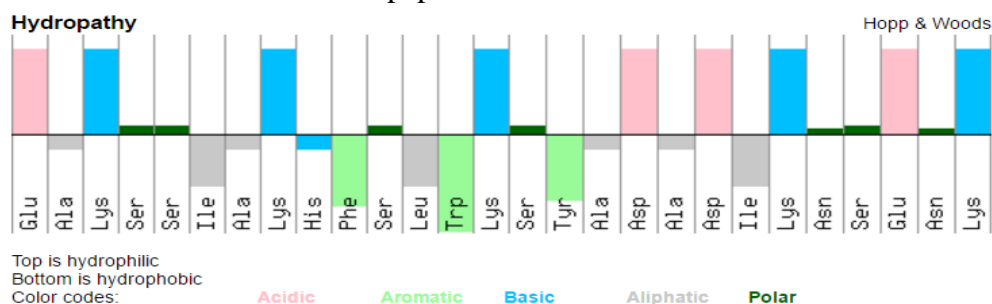

Fig.1. Schematic Representation of Hydrophilic and Hydrophobic Amino Acid Residues

Peptide-Polymer Bioconjugate Synthesis

In this study, PAA-peptide bioconjugates were synthesised with constant peptide concentrations and increasingly polymer concentrations (n$_{PAA}$/n$_{peptide}$= 0.022; 0.044; 0.066) using 1-ethyl-3-(3-dimethyl- obtained. Dry EDC at 2:1 ratio (EDC:PAA) was added on top of the solution to activate the aminopropyl) of carbodiimide cross binder. PAA polymer was dissolved in 0.1M phosphate buffer solution at pH=5, and this value was set after the solution had been over and 3 different polymer concentrations carboxyl groups of the polymer, then the solution had been mixed for an hour at room

temperature. Following the activation of the carboxyl groups of the polymer, an appropriate concentration of ready synthetic peptide dissolved in 0.1M phosphate buffer solution was added to the polymer solution, and the blend had been mixed overnight at 4°C with magnetic mixer. The pH values of mixtures were set at 7.0 and the blend had been mixed for another 4 hours at 4°C. Bioconjugates' varied molar ratios ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066) were calculated as per the equation 2.1[20].

$$n= c.NA/M \qquad 2.1$$

Symbols' representations are as follows: M is a molecular weight of the component, NA is an Avagadros' Number and c is a concentration of the component in terms of mg/mL.

Characterizations of Peptide and Peptide-Polymer Conjugate with Size Exclusion Chromatography (SEC)

Size exclusion chromatography with triple detector (refractive index (RI) 660 nm), Right Angle Light Scattering (RALS, 670 nm) detector and Ultraviolet (UV,280 nm) detector were used in the peptide and peptide-polymer bioconjugate analysis. Peptide, polymer and peptide-polymer bioconjugate were separated by means of Shim-Pack Diol 300 column (500 mm length, 7.9 mm inlet diameter). Polymer standards with a known molecular weight and size were used for column calibration. Chromatographic separation was performed at room temperature with 1.0 mL/min flow rate.

Phosphate buffered saline (PBS) was prepared with ultra-pure water, 50 mM phosphate and 150 mM sodium chloride and then used as mobile phase. NaN_3 was added to the mobile phase in order to prevent contaminations. This phase was also filtered through 0.45 µm Millipore cellulose nitrate filter and degassed before use.

Characterizations of Peptide and Peptide-Polymer Conjugate with Fluorescence Spectroscopy

Fluorescence emission spectra of peptide, polymer and peptide-polymer conjugate were obtained with the help of QM-4/2003 Quanta Master Steady State Spectrofluorometer (Photon Technology International, Canada). Analyses were carried out in quanta counting mode with a slit gap of 3 nm for excitation and emission monochromators. Excitation was performed in 270 nm and spectra measured between 280-500 nm's.

Particle Size Analysis of Peptide and Peptide-Polymer Conjugates

Particle size analyses of peptide, PAA and polymer-peptide conjugates were performed with Dynamic Light Scatter Technique with Zetasizer Nano ZS (Malvern Instruments, UK) equipped with 4.0 mV He-Ne laser lamp (633 nm) at 25°C with ± 0.1 °C sensitivity. The solvent had a viscosity value of 0.08872 cP, a refractive index of 1.330 and all of the samples were run through 0.20 µm filter (Sartorius) prior to analysis. All of the analyses were retried three times.

3.RESULT AND DISCUSSION

Synthesis and Characterization Results of P-32 Major Protein and 92-118 Sequenced Peptide Series

Molecular weight of the synthesised peptide was analysed by means of LC-MS apparatus which gave a value close to the theoretical Mw=3068.35 molecular weight. GPC UV Chromatogram of the peptide given in Fig. 2.a shows that the peptide left the column at 23rd minute having a single peak. It can be seen

that the peptide shows fluorescence properties due to Tryptophan (Trp,W), Tyrosine (Tyr, Y) and Phenyl Alanine (Phe, F) in its structure from the λ_{max} value on the Fluorescence Spectrum of the peptide given in Fig. 2.b. From the size graph measured in Zeta Sizer apparatus given in Fig. 3 it follows that the size of the peptide is 4.1 nm.s' bearing in mind the Z-ave value.
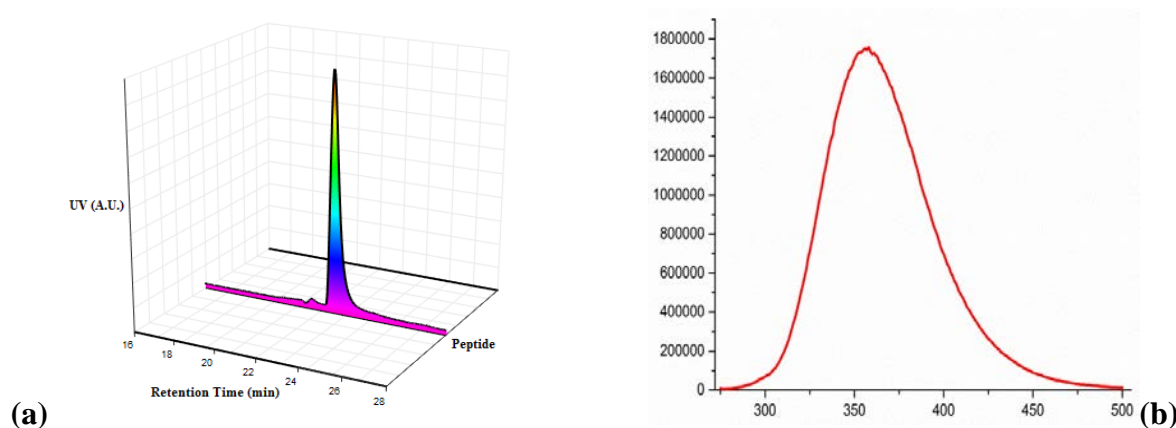
(a)

(b)

Fig. 2. a) GPC-UV Chromatogram of the Peptide**; b)** Fluorescence Spectrum of the Peptide
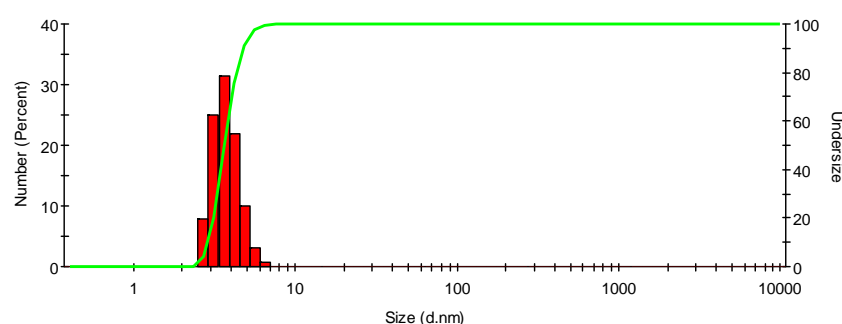

Fig. 3. Size Graph of the Peptide

Peptide-Polymer Conjugation Results and Discussion GPC Measurements

UV and RALS Chromatograms obtained from GPC measurements of peptide, PAA and peptide-polymer conjugates in various concentrations ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066) are given in Fig. 4.a and 4.b respectively. It can be seen from the chromatogram obtained at RALS detector which is dependent on molecular weight that PAA gives a peak at 10th minute term and the weak peak obtained at UV chromatogram belongs to PAA. It is obvious from the UV chromatogram of the peptide that the peak leaves relatively later than PAA polymer, at 23rd minute. Due to its low molecular weight the peptide shows no peak at RALS chromatogram.
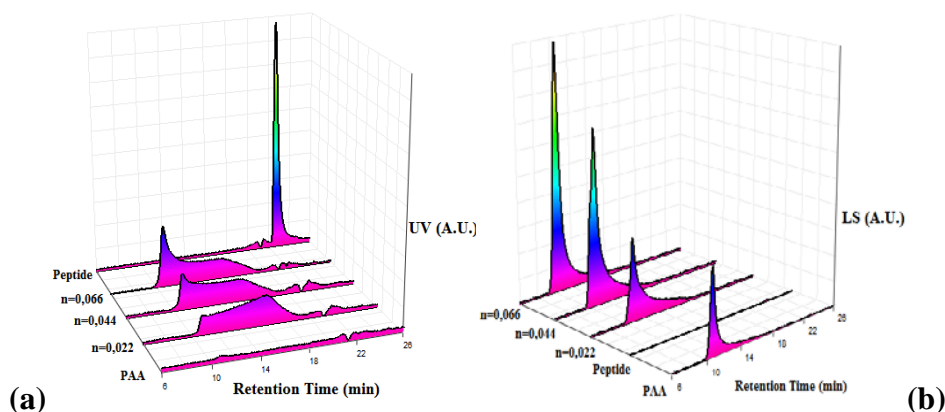

(a)

(b)

Fig. 4.a) GPC-UV chromatogram **b)** GPC-RALS chromatogram of peptide, PAA and PAA-peptideconjugates ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066)

Bioconjugates with varying ratios ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066) were synthesised by constant peptide and increase of polymer concentrations. The polymer and conjugates leaving the column as is seen in Fig. 4.a, it shows comparative UV chromatograms obtained from GPC analyses of peptide, PAA and peptide-PAA conjugates. Changes in the peak area with the ratios of components are shown in Fig. 5. It is obvious that the peak area rises as the $n_{PAA}/n_{peptide}$ increases. A trail on the UV chromatograms of the PAA polymer can be seen which is caused by high PDI value of the polymer. It is believed that peptide may be bound when forming conjugates with polymer, for it
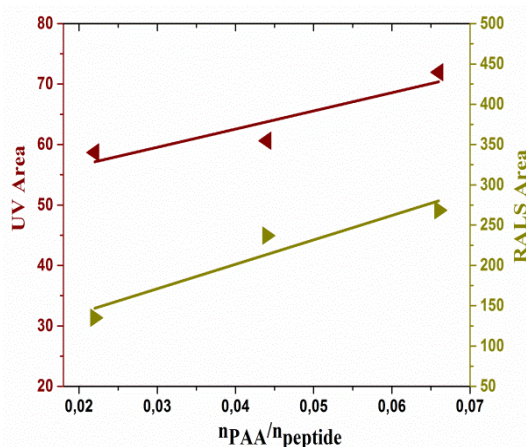
contains both -NH$_2$ and -COOH functional groups. Using both RALS and UV chromatograms and literature [21], one can assume that these peptide oligomers may be bound on the PAA polymer.

As the GPC-UV chromatogram of bioconjugates is observed, it can be seen that no peaks were obtained at a moment where peptide gave peaks. Note that every peptide molecule was used during conjugation reaction. From the RALS chromatograms of bioconjugates given in Fig. 4.b it is obvious that conjugates give peaks at the moment where PAA gave peaks and changes in the peak area with varying $n_{PAA}/n_{peptide}$ ratio can be seen in Fig. 5.

Fig. 5. GPC-UV and RALS peak areas of bioconjugates ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066)

Data obtained from GPC chromatograms show that peptide-polymer bioconjugates were formed successfully.

Particle Size Measurements

Particle size measurements of polymer, peptide and polymer-peptide bioconjugates are given in Fig.6. Sizes of the bioconjugates are expected to be greater than those of free polymer and free peptide, as well as

components that form the conjugate by binding them via covalent bonds. Z-Ave values for every bio-conjugate ratio are given in Fig.6 which says that these values are greater than that of Z-Ave value of free polymer. A comparison of Z-Ave values of the bio-conjugates reveals a linear increase of Z-Ave values in the polymer concentration. This means that Z-Ave values obtained from Zeta Sizer are consistent with data obtained from GPC-RALS chromatograms.
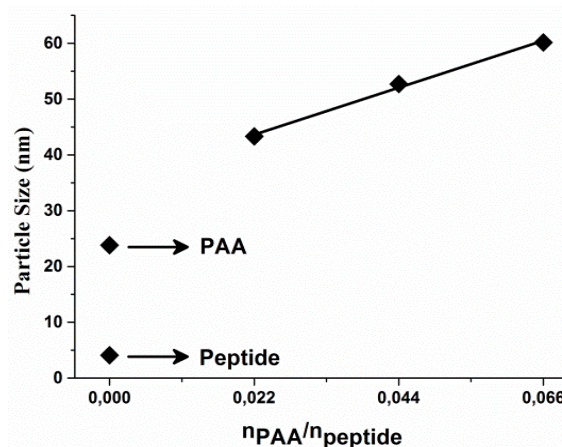
Fig.6. Particle size of peptide, PAA and bioconjugates (n_{PAA}/n_{peptid}=0.022; 0.044; 0.066)

Fluorescence Measurements

Fluorescence spectra of the peptide and conjugates with various ratios ($n_{PAA}/n_{peptide}$= 0.022; 0.044; 0.066) are given in Fig. 7. It follows from Fig.2.b that the peptide has a high fluorescence intensity. The literature [22, 23] says that a decrease in the fluorescence intensity and/or a shift at I_{max} value is expected to stem from interactions occurring between the peptide and the polymer molecules during bioconjugation reactions. Fig. 7 demonstrates clearly a drop in fluorescence intensity with increasing polymer concentration. In the peptide-polymer conjugate, fluorescent sensitive regions on the peptide get in between the polymer clusters to reduce the fluorescence intensity. Furthermore; the shift in the I_{max} value as compared to peptide is another evidence supporting that the conjugation was successful.
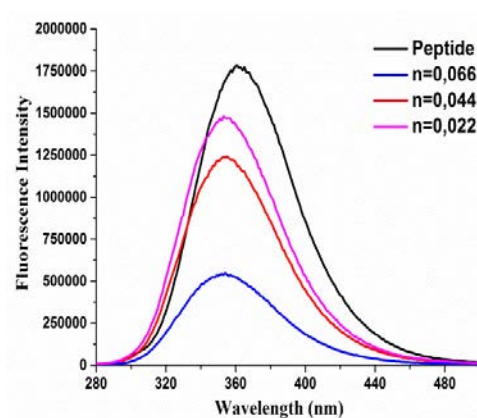

Fig. 7. Fluorescence spectra of bioconjugates with different ratios; $n_{PAA}/n_{peptide}$=0.022 (I_{max}=353 nm), $n_{PAA}/n_{peptide}$=0.044 (I_{max}=355 nm), $n_{PAA}/n_{peptide}$=0.066 (I_{max}=354 nm) in comparison with equivalent amounts of peptide (I_{max}=361 nm) in bioconjugates.

4. CONCLUSION

In the paper, a research into 92-118 showed that the synthetic peptide of P32 major protein from Sheep-Pox Disease Virus was synthesised with solid phase supported by microwave peptide synthesis method and purified with preparative HPLC. The purified peptide was analysed with the help of LC-MS, GPC, ZetaSizer and Fluorescence Spectroscopy. The analysed peptide had later been conjugated with PAA polymer in the presence of EDC cross binder with the following ratios; $n_{PAA}/n_{peptide}$= 0.022, 0.044, 0.066 which were later analysed with the help of GPC, ZetaSizer and Fluorescence Spectroscopy.

All of the analyses performed are consistent with each other to indicate that the synthesis of the peptide and the conjugation of this peptide with PAA polymer in forming a bioconjugate were successful. The results obtained from this experiment are believed to pave the way to the development of synthetic peptide vaccine for Sheep-Pox Disease.

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СИНТЕЗ И ХАРАКТЕРИСТИКА АНТИГЕННОГО ПЕПТИДА У ОВЕЦ, БОЛЬНЫХ ОСПОЙ

Зейнеп МУСТАФАЕВА

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Синтезирован и охарактеризован антигенный пептидный эпитоп против болезни оспы овец с последовательностью 92-118 основного протеина Р32 в твердой фазе под влиянием микроволн, и ковалентным связыванием синтезированного пептида к полимеру полиакриловой кислоты; определены его характеристики и разработаны пептидно-полимерные конъюгаты, которые могут быть использованы в качестве прототипа вакцины против болезни оспы овец.

Ключевые слова: *твердофазный синтез пептидов, биоконъюгация, антигенный пептидный эпитоп, полимер полиакриловой кислоты.*