Research Article

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Synthetic peptide vaccine for Foot-and-Mouth Disease: synthesis, characterization and immunogenicity

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Abstract

Background: The conjugations of antigenic synthetic peptide sequences with carrier polymers have opened new possibilities for the treatment of diseases. In this study, 135–161 peptide sequence of VP1 capsid protein of Foot-and-Mouth Disease was cross-linked with P(VP-co-AA) copolymer by covalent conjugation using water-soluble carbodiimide at different ratio of components ($\gamma=5, 7, 9, 11, 15$) for the first time in the literature.

Materials and methods: Bioconjugates were characterized by gel permeation chromatography and fluorescence spectroscopy to identify occurrences of the conjugates. After characterization, $\gamma=15$ bioconjugate was determined as optimum conjugate for immunization studies and IC_{50} value is calculated as 1.227 mg/mL. By determining the nontoxic range, indirect ELISA were performed to evaluate the immune response elicited in balb/c mice by either peptide or P(VP-co-AA)-peptide bioconjugates ($\gamma=15$). Two injections were applied to each group and high immune responses were obtained against $\gamma=15$ conjugate compared to free peptide and control.

Results and conclusion: At the end of 9-week, the general pattern of immunoreactivity was acquired as $\gamma=15 >> \text{peptide} > \text{control}$. Peptide formulated in the conjugated form had higher antibody response than free peptide and control

($p < 0.01$, for all in both cases), this conjugate formulation put forward the adjuvant activity of P(VP-co-AA) polymer.

Keywords: bioconjugate; Foot-and-Mouth disease; poly-electrolytes; synthetic peptide; toxicity.

Özet

Amaç: Antijenik özellikli sentetik peptit dizilerinin taşıyıcı polimerler ile konjugasyonu, hastalıkların tedavisi için yeni olanaklar yaratmaktadır. Bu çalışmada literatürde ilk kez, Ayak ve Ağız Hastalığının VP1 kapsid proteininin 135–161 peptit dizisi, farklı bileşen oranlarında suda çözünür karbodiimid kullanılarak P(VP-ko-AA) kopolimeriyle kovalent konjugasyon ile çapraz bağlanmıştır ($\gamma=5, 7, 9, 11, 15$).

Gereç ve Yöntem: Biyokonjugatlar, konjugatların oluşumlarını tanımlamak için jel geçirgenlik kromatografisi ve floresan spektroskopisi ile karakterize edilmiştir. Karakterizasyondan sonra $\gamma=15$ biyokonjugatı immünizasyon çalışmaları için optimum konjugat olarak belirlenmiş ve IC_{50} değeri 1.227 mg/ml olarak hesaplanmıştır. Toksik olmayan aralığın belirlenmesiyle, balb/c farelerinde peptit veya P(VP-ko-AA)-peptit biyokonjugatlarına karşı oluşan immün cevabı değerlendirmek için dolaylı ELISA yapılmıştır ($\gamma=15$). Her gruba iki enjeksiyon uygulanmış ve serbest peptit ve kontrole kıyasla $\gamma=15$ konjugatına karşı yüksek immün yanıt elde edilmiştir.

Bulgular ve Sonuç: 9 haftanın sonunda, immün reaktivitenin genel örüntüsü $\gamma=15 > \text{peptit} > \text{kontrol}$ şeklinde elde edilmiştir. Konjüge formda formüle edilmiş peptit, serbest peptit ve kontrolden daha yüksek antikor cevabı oluşturmuş (her iki durumda da $p < 0.01$), bu konjugat formülasyonu P(VP-ko-AA) polimerinin adjuvan aktivitesini öne çıkarmıştır.

Anahtar Kelimeler: polielektrolitler; Ayak ve Ağız Hastalığı; biyokonjugat; toksisite; sentetik peptit.

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Introduction

Synthetic peptides are promising vaccine candidates in the control of viral diseases [1, 2]. Foot-and-Mouth Disease, an

infectious viral disease [3] in cattle, pig, sheep, goats, and wild cloven-hoofed animals, is caused by foot-and-mouth disease virus (FMDV) [4]. FMDV is the initiative agent of one of the most important animal viral disease in the world. Although its mortality percentage is low, this disease effects amount of livestock, and avoids countries to make international animal trade and use their products. Non-enveloped FMDV has icosahedral symmetry, and it is from the aphthovirus genus of the Picornaviridae family [5]. Its genome is a single stranded RNA molecule of about 8500 nucleotides [6] and has four capsid proteins named as VP1, VP2, VP3 as external, and VP4 as internal [4]. VP1–VP3 are located on the surface and fold as eight-stranded barrels, whereas VP4 is internal and less structurally conserved [7]. FMDV is an alluring model to study the potential of peptide-based synthetic vaccines [8]. Both the fragments of isolated VP1 protein [9] and synthetic peptides [10] induce antibody production [11].

Necessity of cold chain for vaccine stability, reintroduction of the disease by the handling of infectious virus [12] and absence of chemical content for the composition of the viral antigen and the presence of cellular contaminants are prominent disadvantages of conventional vaccines. Next-generation vaccines have been developed by synthetic antigens. However, some of them have poor immunogenicity and inefficient due to the lack of appropriate adjuvants; this handicap can be overcome by the use of harmless and effective immunoadjuvants to induce both humoral and cellular immune responses [13]. The modern definition of an adjuvant contains not only classical immune stimulators but also any aspects of particle size, shape, and surface chemistry that improve vaccine immunogenicity [14].

Technological progress in antigen delivery has led to design the synthetic peptide-based vaccines. The development of these new vaccines has the potential to overcome the problems in current vaccine delivery technologies about major histocompatibility complex (MHC) molecules and immunogenicity [15].

Due to their small size, synthetic peptides have been combined with other carrier molecules such as polymers. Several natural polymers (polysaccharides and their derivatives) and synthetic polymers (polyelectrolytes, polyesters, polyanhydrides, and non-ionic block copolymers) increase the vaccine potential against various infectious diseases by their adjuvant role. Solubility, molecular weight, degree of branching, and the conformation of polymers affect the adjuvant potential of them [16, 17]. Poly(*N*-vinyl-2-pyrrolidone) (PVP) and its copolymers have found wide applications as polymeric material in synthetic vaccines, hydrogels and drug-delivery systems, etc. PVP has low toxicity, high solubility in water/organic solvents,

and ability to bind different kinds of small and large molecules. These positive features have made it a good biocompatible molecule in biotechnological area [18–20]. Copolymerization of *N*-vinyl-2-pyrrolidone (NVP) with acrylic acid (AA) remodels this biocompatible poly(*N*-vinylpyrrolidone-*co*-acrylic acid), P(VP-*co*-AA), copolymer by grafting amidation, or crosslinking for different applications as an adjuvant in vaccine preparation [21]. Several researches are being performed to improve synthetic peptide vaccines against viral pathogens containing FMDV [2, 22–26]. Most of these vaccine studies about FMDV indicate that, 134–158 peptide sequence of VP1 capsid protein has been identified as the major immunogenic site for neutralizing antibodies [2, 9, 23–29]. In this study, the interactions between triptophan (Trp) added 135–161 peptide epitope of VP1 protein and P(VP-*co*-AA) were investigated for the first time. Because there was no study about the conjugation of P(VP-*co*-AA) copolymer with the 135–161 peptide sequence of VP1 capsid protein in the literature. Polymer–peptide covalent conjugates at different component ratios ($n_{\text{peptide}}/n_{\text{polymer}}$) were prepared in the presence of water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (WSC). The characterization of polymer–peptide conjugates were performed with spectroscopic (fluorescence spectroscopy) and chromatographic analyzing methods (VISCOTEC-with Ultraviolet–Visible [UV–vis], refractive index [RI], light scattering [LS], and Viscosity Quadruple Detector Systems). After characterization analyses, cytotoxic and immunological effects of conjugates were performed. The objective of the study was to determine the optimum molar ratio of conjugates for immunological studies. According to the characterization of conjugates and immunological studies, the relationship between immunogenicity and structure of conjugates was analyzed. Highly immunogenic protein–polymer conjugate forming a long-term antibody response was obtained.

Material and methods

Reagents

Sodium hydroxide (NaOH), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate mono basic (NaH_2PO_4), sodium chloride (NaCl), dimethylsulfoxide (DMSO), zinc chloride (ZnCl_2), magnesium chloride (MgCl_2), and glycine were purchased from Sigma Aldrich (St. Louis, USA). Triptophan (Trp) added synthetic peptide representing 135–161 amino acid residues (Trp-Ser-Lys-Tyr-Ser-Thr-Thr-Gly-Glu-Arg-Thr-Arg-Gly-Asp-Leu-Gly-Ala-Leu-Ala-Ala-Arg-Val-Ala-Thr-Gln-Leu-Pro-Ala) of VP1 protein of FMDVs' GH loop was synthesized by Sigma-Aldrich Genosys. The weight-average molecular weight (Mw) of FMDV synthetic peptide was 2.97 kDa. Peptide was retested by semi-preparative HPLC and purity was achieved greater than 95%.

Synthesis of P(VP-co-AA)

P(VP-co-AA) in 3:1 mol ratio was synthesized by free radical polymerization method according to the literature [30] with minor modification. In this synthesis analytical grade reagents, acrylic acid (AA) (Aldrich, Germany), *N*-vinyl-2-pyrrolidone (Fluka), tetrahydrofuran (Riedel-de Haen), ethyl acetate (Fluka), and 4,4'-azobis(4-cyanovaleric acid) (Fluka) were used. Copolymer with 80 kDa weight-average molecular weight (Mw) was obtained.

Synthesis of conjugates

In this study, a multi-step covalent conjugation procedure (Figure 1) [31] was followed to obtain peptide-P(VP-co-AA) conjugates in different molar ratios of peptide ($n_{\text{peptide}}/n_{\text{polymer}} = \gamma = 5, 7, 9, 11, 15$). Water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma E7750) was used as activator. First, P(VP-co-AA) was dissolved in phosphate-buffered saline (PBS) and activation of carboxyl group of acrylic acid was carried out in water (molar ratio of EDC:AA 4:1). EDC was added to the polymer solution (pH 5.0). Then, peptide solution was added on activated polymer solution at room temperature and stirred for 1 h. After, the mixture was stirred for 12 h at 4 °C, and then the pH was adjusted to 7.0 (1 M NaOH). The bioconjugate was purified from the free peptide (unreacted) and low molecular weight compounds (peptide and O-acylisourea intermediate) by centrifugation with Sartorius VIVASPIN (10,000 MW) polyethersulfon (PES) membrane tubes (6 min, +4 °C, 6000 rpm). The bioconjugates were lyophilized and stored at -20 °C.

Size exclusion chromatography analysis of conjugates

Water-soluble P(VP-co-AA)-peptide bioconjugates were analyzed at room temperature using gel permeation chromatography (GPC) with a quadruple detection system consisting of UV, RI, right angle LS, and viscosimetry (VIS) detectors. Aldolase (150 kDa) and human serum albumin (66 kDa) were the standards used to calibrate the column (Shimadzu Shim-Pack Diol-300; 50 × 0.79 cm²). Mobile phase PBS was

at pH 7 and 1.0 mL/min flow rate. Ultrapure water from Millipore MilliQ Gradient system was used to prepare PBS which had 0.05 M phosphate (Na₂HPO₄, NaH₂PO₄) and 0.15 M sodium chloride composition. Buffer solutions were filtered through a 0.45-μm Sartorius RC (cellulose nitrate filter) and degassed before all usages.

Fluorescence spectrophotometry analysis of conjugates

The fluorescence emission spectra of the Trp residues in the 135–161 synthetic peptides and conjugates ($\gamma = 5, 7, 9, 11, 15$) were analyzed in PBS buffer (0.01 M; pH=7.2) using a QM-4/2003 Quanta Master Steady State Spectrofluorimeter (Photon Technology International, Canada) operating in quanta counting mode at an excitation wavelength of 280 nm and an emission wavelength from 290 to 450 nm [32].

In vitro cytotoxicity assay

The cytotoxicity profile of peptide, polymer, and their conjugates was studied by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay which was described by Mosmann [33]. According to MTT assay, alive cells reduce the yellow MTT (Biotium) to purple formazan crystals by dehydrogenase enzyme secreted by the mitochondria of metabolically active cells [34]. L929 fibroblast cell line was used for the cytotoxicity assay. The assay applied briefly, 10⁴ cells/mL were seeded into 96-well plates (Greiner Bio-One GmbH) and kept under 5% CO₂ at 37 °C for 24 h. After the attachments of the cells, they were controlled by microscopy and treated with different compounds resulting in a final volume of 100 μL per well. All compounds (peptide, copolymer, and peptide-copolymer conjugates) treated with the concentrations mentioned above. After 48 h incubation, 50 μL of MTT solution, which was prepared from thiazolyl blue tetrazolium bromide powder (Sigma M5655) (10 mg/mL) was added to each well. After 2–4 h incubation with MTT solution, the formazan needles were formed and 150 μL of 100% DMSO were added to each well and incubated at dark (30 min at room temperature). The same procedure was also conducted without cells for evaluation of interaction of the compounds with the medium. Optical density was

Figure 1: P(VP-co-AA)-peptide conjugation mechanism.

measured at 540 nm wavelength with microplate photometer (ThermoLabsystem, Multiskan Ascent). Cytotoxicity was reflected as mean percentage increase relative to unexposed control $\pm$ standard deviation (SD). Control values were set at 100% cell viability. It is known that IC50 value is the concentration of agent which reduces cell growth by 50% under the experimental conditions. All assays were performed in triplicate. The mitochondrial function was calculated, as a percentage of the control group and considered as 100%. GraphPad Prism (San Diego, USA) was used to perform this analysis. The percentage of cells viability was calculated according to the following equation:

$$\text{Cell viability \%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Immunization

About 21 male BALB/c mice, 20–24 g weighted, 6–8 weeks old were used in all experiments at TUBITAK-Marmara Research Center, Genetic Engineering and Biotechnology Institute (GEBİ) (Gebze, Istanbul, Turkey). Clean and sterile housing conditions in polypropylene cages under controlled photoperiod (12 h light/dark cycle) and humidity (55–60%) were provided. The animals were fed with standard rat pellet chow and tap water ad libitum. All animals were acclimatized for 7 days before the study. The protocols in the ethical treatment guidelines of experimental animals were performed in the experiments. *Animals Ethical Committee of İstanbul University* (No: 22185) approved this study.

The mice were randomly divided into three groups with seven animals in each group. Characterized and purified samples were intraperitoneally injected under ether anesthesia in one-shot immunization to two groups at the same day. NaCl isotonic solution (0.9%) was injected to control group. After first injection, booster immunization was performed on 14th day (Table 1). Serums were collected weekly for 9 weeks through the tail vein for antibody level determinations. The blood samples were collected into sodium citrate microfuge tubes, centrifuged at 2500 rpm to remove red blood cells. Serial dilutions of the serum (1:50 and 1:100) were prepared in PBS. The serum samples were tested with indirect enzyme-linked immunosorbent assay (ELISA).

ELISA method

The indirect ELISA [35, 36] was used to confirm the antibody activities against synthesized 135–161 peptide sequences and its conjugates. 96-well polystyrene plates (GREINER) were coated with 200 ng bovine serum albumin (BSA)–peptide conjugate in parallel to BSA (SIGMA) in 100 μ L PBS and stayed at +4 °C overnight. The plates were washed three times with washing buffer (0.005% Tween-20 [Thermo Fisher Scientific™] in PBS). Then, 0.2% casein in PBS was added to the wells,

and incubated for 1 h at 37 °C followed by washing as above. Each serum was diluted with dilution buffer to 1:100 than 100 μ L diluted serum samples were added to plate. The plate was incubated at 37 °C for 1 h and washed three times with washing buffer. After that, 100 μ L alkaline phosphatase-conjugated goat–antimouse polyvalent (IgG, IgA, and IgM) antibodies (Sigma) in 1:1000 dilution buffer (PBS) was added to each well as a secondary antibodies and incubated for 1 h at 37 °C. The washing step was repeated five times with washing buffer as above. The substrate buffer (1 mM ZnCl₂, 1 mM MgCl₂, 0.1 M Glycine, and pH 10.4) and 1 mg/mL *para*-nitrophenyl phosphate (PNPP) (Thermo Fisher Scientific™) were added. Finally, absorption of the plate was read at 405 nm and antibody amount was determined. This graphics were performed using GraphPad Prism (San Diego, CA).

Statistical analysis

Data were expressed as mean $\pm$ SD. All statistical analyses were performed using GraphPad Prism (San Diego, CA). Non parametric analysis with Tukey's multiple comparisons test was achieved on the data of the biochemical variables to examine differences between the groups. Two-tailed Student's *t*-test was used to analyze statistical differences between the selected two groups. *p*-Value less than 0.05 (*p* < 0.05) was accepted as significant in both tests.

Results

Characterization of conjugates with size exclusion chromatography

A carbodiimid conjugation procedure was followed to obtain peptide–polymer conjugates as described previously [37]. Peptide–polymer conjugates were synthesized at different molar ratios ($\gamma=5, 7, 9, 11, 15$) using water-soluble EDC as a crosslinker. Synthesized conjugates were first characterized by GPC with quadruple detectors. Obtained results gave detailed information about physico-chemical properties of peptide–polymer conjugates. The typical GPC chromatograms of bioconjugates, peptide, and P(VP-*co*-AA) are shown in Figure 2, and GPC peak areas of conjugates depending on $n_{\text{peptide}}/n_{\text{polymer}}$ ratio were given in Figure 3. In all conjugates, the concentration of P(VP-*co*-AA) is constant (1 mg/mL) whereas the concentration of peptide varies. As seen from Figure 2, the peaks eluted at 10.9 and 19–22.5 mL were attributed to peptide and P(VP-*co*-AA), respectively. Also, bioconjugates were identified by one peak close to P(VP-*co*-AA) retention volume. Although the peptide molar ratios in conjugates were increasing ($\gamma=5, 7, 9, 11, 15$), the free peptide peak was not observed in chromatograms. This was because of binding of all peptide molecules to polymer chain. Additionally, the peak areas of conjugates in RI chromatograms (Figures 2B

Table 1: Mice groups of immunization treatments.

Groups	Immunization treatments
1	0.2 mL P(VP- <i>co</i> -AA)-peptide conjugate including 1 mg peptide ($\gamma=15$)
2	0.2 mL peptide solution including 1 mg peptide
3	Control group; 0.2 mL 0.154 M NaCl

Figure 2: GPC chromatograms of free peptide and P(VP-co-AA)-peptide conjugates ($\gamma=5, 7, 9, 11, 15$) obtained from RALS (A), RI (B), UV-vis (C), and viscosity (D) detectors.

and 3) decreased by conjugation of molecules. This situation can be correlated to dn/dc value of bioconjugates. The dn/dc values of molecules are generally related to their chemical composition. Binding of peptide molecules to polymer chain may have reduced the dn/dc value of polymer.

The viscosity of solutions depended on the size and the shape of the molecules. In our study, the viscosity area of conjugates first decreased than slightly increased. However, it can be seen Figure 2D that, the peak areas of all conjugates in viscosity chromatograms were decreased compared to P(VP-co-AA) polymer peak. The same results were also reported in our previous studies [38–40].

In contrast, as seen in Figure 2A, C that, the UV and RALS area of conjugates increased with increasing of peptide concentration used in reactions. Increasing of RALS signal can be correlated to concentration and molecular weight of molecules and increasing in peak area represents the formation of peptide-polymer conjugates.

GPC with quadruple detector results gives comprehensive information about physicochemical properties of P(VP-co-AA), peptide, and P(VP-co-AA)-peptide bioconjugates. Molecular weight, size, and conformational properties of molecules in the solution were calculated from GPC with Quadruple Detector System and OmniSEC 4.1 software program. As shown in Table 2, the increasing of the weight average molecular weight of conjugates indicated that the conjugation process was successfully occurred and the amount of peptide binding was increased by increasing of γ . Similar to molecular weight results, the size of the conjugates (R_h) is increased linearly up to 34.257 nm with increasing of γ . “ a ” in Mark-Houwink equation defines the molecule shape and it changes between 0 and 2. When molecules has like a spherical shape the Mark-Houwink a constant is close to zero [38]. In our study, values of a exponent calculated as 0.797 for polymer and this constant for conjugates increased up to 0.611 until $\gamma=11$ and then decreased to 0.452 at $\gamma=15$.

Figure 3: Peak areas acquired from RI (●), UV (■), RALS (●), and Vis (▲) chromatograms of γ conjugates.

It was observed in our study that, R_h value of polymer was increased with the formation of conjugation and Mark–Houwink a constant of conjugates is decreased when compared with the polymers'. When molecular weight, R_h , and " a " values evaluated, it can be considered that the conjugation in increasing γ caused rise in the radius of conjugates and the formation of more spherical shape.

Characterization conjugates with fluorescence spectrophotometry

Additionally, covalent conjugation mechanism was investigated by fluorescence spectrophotometry (Figure 4) depended on the changes in fluorescence properties of peptide molecules after conjugation. The occurrence of conjugation created important changes in three-dimensional (3D) structures. These changes can be monitored especially by the differences at emission maximum (λ_{max}) of molecules [41]. Thus, in this article, the spectral changes in fluorescence spectrums of peptides and

Table 3: Indirect ELISA analysis values of peptide, $\gamma=15$, and control groups for 9 weeks (each serum was diluted with dilution buffer to 1:100).

Days	Control (I)	Peptide (II)	$\gamma=15$ (III)
7	0.202 ± 0.0014	0.255 ± 0.0015	0.316 ± 0.0015
14	0.208 ± 0.0015	0.267 ± 0.0019	0.400 ± 0.0342
21	0.21 ± 0.0183	0.330 ± 0.0277	0.525 ± 0.0091
28	0.215 ± 0.0082	0.290 ± 0.0082	0.400 ± 0.1253
35	0.201 ± 0.0106	0.280 ± 0.0129	0.300 ± 0.0523
42	0.22 ± 0.0115	0.265 ± 0.0016	0.296 ± 0.0126
49	0.198 ± 0.0036	0.245 ± 0.0016	0.311 ± 0.0116
58	0.185 ± 0.0013	0.240 ± 0.0231	0.303 ± 0.0096
63	0.187 ± 0.0018	0.230 ± 0.0173	0.275 ± 0.0014

Peptide vs. control $p < 0.001$.

Conjugate ($\gamma=15$) vs. control $p < 0.01$.

Peptide vs. conjugate ($\gamma=15$) $p < 0.01$.

conjugates are used for investigation of conformational change caused by conjugation between polymer and peptide. The changes in λ_{max} depend on γ are shown in Figure 4. λ_{max} of conjugates shifted linearly toward blue region from 353 to 332 nm for the lowest molecular ratio of conjugates ($\gamma=5$). This indicated that tryptophan amino acids in peptide sequence were isolated from aqueous solution after conjugation. It was considered that this isolation was occurred as a result of covering of the peptide molecule by the polymer. Due to the peptide molecules interacted with more polymer molecules, the highest λ_{max} quenching (21 nm) was observed especially for $\gamma=5$ conjugates. It is seen in Figure 4 that the shift in wavelength decreases with increasing of $n_{peptide}/n_{polymer}$ ratio.

Peptide/polymer/bioconjugates effect on fibroblast cell viability

MTT assay for peptide, P(VP-co-AA) copolymer, and bioconjugates ($\gamma=15$) were carried out on L929 fibroblast cell viability (Figure 5) for 24 h. The half-maximal inhibitory concentrations (IC_{50}) value on L929 cells were investigated using different concentrations. The IC_{50} of peptide, P(VP-co-AA) copolymer, and bioconjugates ($\gamma=15$) were 1.404, 0.8733, and 1.227 mg/mL, respectively. It was seen that the polymer had more toxic effect than peptide and conjugate at the first 24 h. When copolymer was conjugated with peptide, IC_{50} value of the conjugate was higher than the copolymers'. It could be said that conjugation between copolymer and peptide caused a decrease in copolymers' toxicity. After determining the nontoxic range, experimental animal studies were performed close to the determined IC_{50} value.

Table 2: M_n , M_w , polydispersity index (M_w/M_n), hydrodynamic radius (R_h), and a exponent of Mark–Houwink equation values for P(VP-co-AA)–peptide conjugates.

γ	M_n (Da)	M_w (Da)	M_w/M_n	R_h (nm)	a
5	28.834	169.391	5.875	8.807	0.439
7	907.022	$9.279 \cdot 10^6$	10.23	21.255	0.564
9	$1.361 \cdot 10^6$	$1.477 \cdot 10^7$	10.859	24.62	0.571
11	$3.584 \cdot 10^6$	$2.487 \cdot 10^7$	6.94	33.174	0.611
15	$3.416 \cdot 10^6$	$3.088 \cdot 10^7$	9.038	34.257	0.452
Polymer	9310	21725	2.33	4.45	0.797

Figure 4: Molar ratio effect (γ) on the position of $\lambda_{\max}$ of P(VP-co-AA)-peptide conjugates.

Immunization results

Indirect ELISA were performed to evaluate the immune response elicited in balb/c mice by either peptide or P(VP-co-AA)-peptide bioconjugates at $\gamma=15$ M ratio. The selection of $\gamma=15$ conjugate for vaccination study can be explained in three ways. First, the results obtained from the fluorescence spectrum were examined, the conjugate with $\gamma=15$ molecular ratio that had the highest interaction with water was selected according to its 3D properties. Second, the $\gamma=15$ conjugate contains the highest amount of peptide in its content with the increasing size as the rate of peptide in the conjugate increases. This triggers the immune system [42, 43]. Finally, among all the conjugates, one of the lowest Mark-Houwink “ α ” values was chosen for immunological studies.

Figure 5: Fibroblast cell viability of peptide, polymer, and bioconjugates ($\gamma=15$).

In the immunology study, there were two injections on day 0 (first injection) and on day 14 (booster injection), respectively, and the dynamic of the antibody formation is presented in Figure 6. Peptide specific antibody responses were detected by indirect ELISA method weekly for 9 weeks. The use of peptide highly significantly increased immune response compared to the control for 63 days ($p<0.001$ by Tukey's). Noticeably, peptide formulated in the conjugated form ($\gamma=15$) caused higher antibody response than both the peptide and the control ($p<0.01$ by Tukey's, for all in both cases). This situation was suggested to be due to the adjuvant activity of P(VP-co-AA). It was seen in Figure 6 that highly significant immune responses occurred in the groups injected with free peptide ($p<0.001$) and $\gamma=15$ conjugate ($p<0.01$) according to the control group.

As expected, the first injection with peptide-conjugates ($\gamma=15$) induced significant anti-peptide immune response that was higher than free peptide and the control ($p<0.001$ for all in both cases by t -test). As shown in Figure 6, antibody responses were formulation dependent (peptide or peptide adjuvant conjugate). Additionally, the booster doses improved the immune response for peptide and $\gamma=15$ conjugate and after booster injection on day 14, immune response increased up to 1.5 times on the 21st day. On the 21st day, statistically highly significant ($p<0.001$ by t -test) antibody response was obtained for $\gamma=15$ conjugate. It was 2.5 times higher than the control and 1.5 times higher than the peptide.

As a result of the high standard deviation due to the experimental error on day 35, however, the immune response against to the conjugate was higher than the peptide ($p>0.05$, by t -test) and control groups ($p<0.001$, by t -test), it was not statistically significant according to the peptide.

It was observed that the antibody response against the peptide decreased gradually after 21st day to 63rd day. In contrast, the antibody response against conjugated peptide remained at similar levels between days 35 and 63, due to the adjuvant properties of the copolymer. This was interpreted as the protection of the peptide by the polymer from degradation in the living system for up to 63 days. As a result, the conjugated peptide showed its biological activity more effectively than the free peptide, which was able to trigger the immune system for higher antibody production.

Similar results were obtained our previous study where the immune response of the melanoma peptide antigen was examined together with a polymeric adjuvant. In the study, both peptide polymer complex and conjugate were investigated comparison with peptide, and it was determined that the amount of the antibody levels decreased at the end of the 63rd day [44].

Figure 6: Indirect ELISA results of peptide, bioconjugates ($\gamma=15$), and control group.

* Each serum was diluted with dilution buffer to 1:100. peptide vs. control $p < 0.001$, $\gamma=15$ conjugate vs. control $p < 0.01$ and peptide vs. $\gamma=15$ conjugate $p < 0.01$.

Conclusion

FMDV, an important viral disease, causes livestock economic losses world-wide. Accordingly, effective vaccine development studies against FMDV are still investigating. For example, Lei et al. constructed a vaccine include of two B-cell epitopes (VP1 residue 134–161 and 200–213) and one T-cell epitope (3A residue 21–35) of FMDV. The results in the study showed that the vaccine formulation induces both humoral and cellular immune responses [45]. In another study conducted by Wang et al., a peptide (FMDV 129–169 sequence)-based vaccine was designed for FMDV. The obtained results showed that 20 out of 21 immunised pigs were protected from infectious challenge by FMDV using peptide doses as low as 12.5 μg [2]. As an alternative to conventional vaccines, subunit peptide vaccines require adjuvant due to their weak immunogenic nature. Covalent conjugation of peptide to carrier molecule is one of the highly preferred techniques. Such as in a study, a conjugate of a membrane protein B as an adjuvant (for increasing the stability and solubility of antigen) and B-cell epitopes (residue of VP1 136–162)/T-cell epitope (residue of 3A 21–35) as an antigen were used [23]. The authors observed FMDV-specific antibodies in the serum of mice immunized with peptide protein conjugate. Peptide protein conjugate exhibited high epitope-specific antibody titre, indicating that this conjugate may be a potential candidate as an alternative vaccine against FMDV epidemic variants [23]. All the studies in the

literature showed that, (i) 135–161 peptide sequence of VP1 capsid protein of FMDV has been identified as the immunogenic site for neutralizing antibodies [9, 27–29] and (ii) conjugation of antigenic peptide with adjuvants increases the immunogenic features of synthetic peptides for production of effective vaccine.

As seen in the literature discussion, various adjuvants have been used to increase the immunogenicity of antigenic peptides. However, in the literature, no study was found about the conjugation of P(VP-co-AA) copolymer with the 135–161 sequence of VP1 capsid protein.

Therefore, for the first time in the literature, immunostimulating polymer-peptide conjugates of 135–161 peptide epitope of VP1 protein and P(VP-co-AA) were synthesized to benefit the advantage of the adjuvant feature of the P(VP-co-AA) polymer in this study. The increased immunogenicity of the conjugates relative to the free peptide may provide new possibilities for modeling the artificial carrier polymer containing synthetic peptide epitope for veterinary or clinical vaccination.

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