ChemPubSoc Europe

DOI: 10.1002/slct.201901375

Chemistry SELECT Full Papers

Biological Chemistry & Chemical Biology

Rabies Virus 31D Peptide-[P(VP-co-AA)] Conjugates: Synthesis, Characterization and Cytotoxicity Evaluation

Pelin Pelit Arayici,*[a] Tayfun Acar,[a] Burcu Ucar,[a] Mesut Karahan,[b] Belkis Atasever Arslan,[c] and Zeynep Mustafaeva[a]

The prevention of rabies disease, which has been the subject of many pieces of researches since ancient times, has become possible by vaccination. Therefore, new generation vaccine systems should be developed to achieve more effective, accessible and reliable vaccines than conventional vaccines against this disease. In this study, modificated Rabies virus 31D antigenic peptide epitope was synthesized by microwave supported solid phase peptide synthesis method and the synthesized peptide was characterized. Bioconjugates of the antigenic peptide epitope with poly (N-Vinyl-2-pyrrolidone-co-acrylic acid) that a biocompatible polymer was synthesized synthetically were synthesized at different ratios in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Characterization of bioconjugates was performed with Gel Permeation Chromatography and ZetaSizer. Cytotoxicity test was carried out using ECV304 human epithelial cells to determine the biocompatibility of peptide, polymer and bioconjugates. It is thought that the produced biocompatible synthetic peptide-polymer based rabies vaccine systems will contribute greatly to the vaccination studies.

Introduction

Rabies has been known since ancient times, today is a zoonotic viral disease that is responsible for the 59 000 human deaths worldwide annually. Of all infectious diseases, the highest rate is one of the infections with mortality rate. Ninety-nine percent of human rabies cases are caused by dog bites and the disease is almost deadly after symptoms begin. Although rabies is fatal, it can be prevented by appropriate vaccination in humans and animals.[1]

The rabies virus (RABV), which affects the central nervous system, consisting of five gene nucleoproteins. The relatively small RNA genome of the virus (~ 12 kb) encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and viral RNA polymerase (L).[2] The Rabies virus N protein as the important antigen responsible for the stimulation of T-helper (Th) cells that cross-react among rabies and rabies-related viruses.[3] In our study, 31D peptide sequences the T cell epitope of the Rabies nucleoprotein, was synthesized and characterized.[4]

Since the past, many different vaccines have been developed against rabies. Research along with biotechnological advances is the basis for current studies to develop more effective and safer vaccine production technologies. For this purpose, preliminary studies for the production of a new generation of peptide vaccines against rabies was investigated in this study.[5]

In the development of the current vaccines, it is important to use only antigenic fragments of the pathogen that can induce long-term protection against the pathogen. Thus, synthetic peptide-based vaccines are the potential future of vaccination.[6] The advantages of peptide-based vaccines are (i) easily synthesized by SPPS devices in a short time, less costly, reproducible, fully chemical synthetic approach; (ii) eliminates the risk of biological contamination with antigens; (iii) usually do not require "cold chain"; (iv) generally water soluble and (v) avoiding allergenic and/or reactogenic responses.[6a,7] In addition to the advantages, peptides alone are weakly immunogenic and the addition of adjuvant in the vaccine formulation is necessary to generate a stronger immune response.[7–8] Choosing a safe and effective adjuvant is essential for peptide-based vaccine design.[8a,9]

The term adjuvant is derived from the Latin word adjuvare, which means "help" or "boost". It has an important role in enhancing the immunization-induced ability of the vaccine by contributing to an effective immune response to a related antigen.,[10,11] Polyacrylic acid (PAA) is a high molecular weight anionic polymer from weak synthetic polyelectrolytes which can be used as adjuvants. Use of N-vinyl-2-pyrrolidone as a copolymer increases the biocompatibility of acrylic acid [P (VP-co-AA)], necessary for its application as an adjuvant in vaccine preparation.[12] In the literature, Skwarczynski et al. have also

[a] P. P. Arayici, T. Acar, Dr. B. Ucar, Dr. Z. Mustafaeva
Bioengineering Department
Chemistry and Metallurgy Faculty
Yildiz Technical University,
İstanbul 34220, Turkey.
E-mail: parayici@yildiz.edu.tr
[b] Dr. M. Karahan
Biomedical Devices Department
Vocational School of Health Services
Uskudar University, Istanbul 34662, Turkey.
[c] Dr. B. A. Arslan
Department of Molecular Biology and Genetics,
Faculty of Engineering and Natural Sciences,
Uskudar University, Istanbul 34662, Turkey.

Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.201901375

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conducted a vaccine delivery system study on polyacrylate dendrimers which is the first reported self-adjuvanting peptide-polymer conjugates.[13] In our previous study,[2] we researched a model for the synthesis of conjugates with PAA. In this study, it is expect that the synthesis of more biocompatible peptide-[P (VP-co-AA)] bioconjugates may be the basis of larger potential vaccine candidates for rabies.

Conjugation reactions are one of the most important methods for the delivery of small molecule antigens. The synthesis of bioconjugates by conventional methods take place by the activation of macromolecules by functional groups in aqueous solution. Carbodiimides (CDI), used to mediate the formation of amide bonds between carboxyl and amine groups are classified as a zero length crosslinking agents. 1-Ethyl-3-(3-dimethyl amino- propyl) carbodiimide (EDC) plays an active role in binding polymers with the peptide. Basically, the EDC mechanism based on modification of the carboxyl groups of the polymers by EDC and to covalently link the amino groups of the peptides by forming an amide bond to the activated polymer groups.[12c,14]

In our study, [P(PV-co-AA)] polymer and conjugates of rabies disease 31D peptide (WAVYTRIMMNGGRLKRC) were synthesized via EDC mechanism and their biocompatibility was evaluated on ECV human epithelial cells. The results show that it appears the conjugates were successfully synthesized and to be biocompatible for using in vivo tests. As a result of the evaluations, the conjugates were successfully synthesized as biocompatible.

Results and Discussion

Efforts to develop a more effective, cheaper and safer vaccine against rabies virus disease have gained momentum with the development of new generation vaccine systems. The basis of this study is the synthesis of the rabies virus 31D peptide epitope with antigenic properties and the [P(VP-co-AA)] polymer, a biocompatible polymeric adjuvant. The basis of this study was the synthesis of the antigenic rabies virus 31D peptide epitope with a biocompatible adjuvant [P (VP-co-AA)] polymer. Vaccine prototype formulations were provided by conjugation of different proportions of peptide and polymer. Biocompatibility rates were selected based on the results of cytotoxicity studies performed before in vivo testing.

Synthesis and Characterization Results of 31D Peptide

Figure 1a and 1b which respectively belongs to the synthesized peptide and purified peptide represent the LC-UV chromatograms of the rabies virus protein antigenic 31D peptide epitope synthesized by microwave assisted SPPS. While the purity of the synthesized peptide was 83%, the purified peptide (73 mg) was obtained as a white solid with $\geq$99% purity (preparative HPLC conditions: mobile phase A (H_2O containing 0.1% TFA) and B (CH_3CN containing 0.1% TFA), gradient elution (0-5 min, 20% B; 5–15 min, 20–40% B; 15–30 min, 40–80% B; 30–35 min, 80–20% B), flow rate: 14 mL/min). The identity of purified peptide was verified by FT-IR and mass spectrometry.[15] FT-IR: υ (cm^{-1}) = 1639, 3338; LC-ESI-MS (m/z): calculated for $C_{89}H_{147}N_{29}O_{21}S_3$, 2055.51; observed 2056.23 $[M + H]^+$.

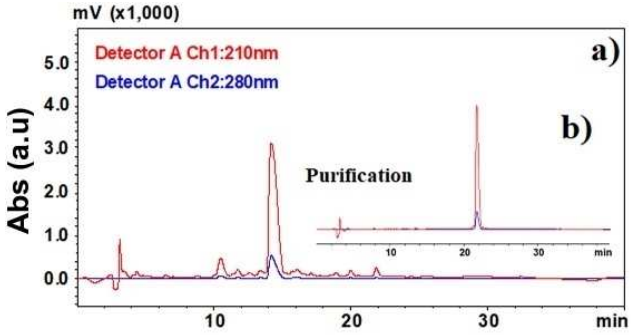

Figure 1. Figure 1 a) LC-UV chromatogram of the synthesized peptide b) LC-UV chromatogram of the peptide after purification. Intensity given in arbitrary unit (a.u)

The spesific 31D peptide was synthesized by various modifications. A purification procedure for the peptide was developed and peptide was characterized by LC–MS system. In Figure 1b, it is seen that the peptide is successfully synthesized in high purity (min 99%). Based on our experience in previous similar studies, this purity is sufficient for the development of a vaccine prototype. Likewise, in the study of Moyle et al., vaccination studies were carried out with using similar purity peptides.[6b,16] Additionally, with the microwave-assisted SPPS, it was obtained peptide with higher purity and chromatography peaks with high-resolution indicating the high reaction yield. The results presented for the linear peptide will be the source data for the peptide which can be used as a vector in different carrier systems in the future.

Characterization of [P(VP-co-AA)]

[P(VP-co-AA)] copolymer was synthesized by free radical polymerization. FT-IR, GPC and NMR analyzes were performed for the characterization of the synthesized polymer.

The average molecular weight (Mw) and polydispersity index (Mw/Mn) of the synthesized copolymer was calculated as 50 kDa and 2.201 respectively with using GPC analysis results that were given in the supplementary file at Figure S2, Figure S3, Figure S4. The functional group information of the polymer was detected by the FT-IR spectrum given in Figure S5 and discussed in detail at the supplementary file. NMR spectra was presented on Figure S6.

Gel permeation chromatography (GPC) analysis of peptide, polymer and bioconjugates

The GPC-UV comparative chromatograms of polymer and peptide-polymer bioconjugates were given in Figure 2a. In the UV chromatogram, the [P(VP-co-AA)] has very low UV absorption at 280 nm, it is shown at 12. minutes. The pure peptide appears to gives a peak at 23. minutes in the UV chromatogram of the peptide in Figure 2b. The rabies virus 31D peptide gave

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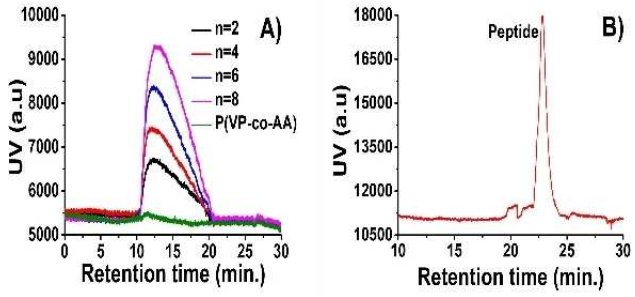

Figure 2. GPC-UV chromatogram of a) polymer and bioconjugates ($n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$) b) pure peptide. Intensity given in arbitrary unit (a.u)

a strong peak in the UV chromatogram at 280 nm because of the peptide epitope containing tryptophan and tyrosine amino acids.

Figure 2a also demonstrates the GPC UV chromatograms; as the amount of the polymer was kept constant and the peptide amounts of the conjugates were increased, the growth of the peaks was observed linearly. Although the polymer has low UV absorption, increasing of the peaks were observed by binding of the peptide to the polymer. Also, when the UV chromatograms of the bioconjugates were considered, for each ratio no peak was observed at the 23. minute that the peptide was eluted. This indicates that not unconjugated peptide is present in all ratios and that the entire peptide has been bound to the polymer for all n ratios. The synthesis of high rates of bioconjugates using larger amounts of peptide may be considered, but at this stage it is necessary to take into account the toxicity because the peptide may cause toxicity after a certain concentration.

Figure 3 shows the UV and RALS area values obtained by the GPC chromatograms of peptide, polymer and bioconjugates. Light scattering signal depends on the molecular weight and concentration of molecule. As the ratio of bioconjugates increases, it presents a parallel increase in UV area values. The pure peptide has comparatively less peak area obtained from RALS chromatogram due to its low molecular weight. The UV area values in Figure 3 show the increase in areas. As the ratios increase, the areas are in parallel with the ratios.

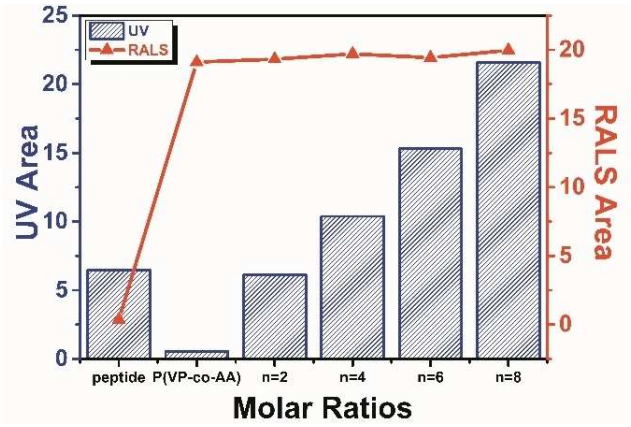

Figure 3. GPC-UV and RALS peak areas of bioconjugates ($n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$)

Increase in the RALS signal can be associated with an increase in the molecular weight of the molecules. Pure peptide has a weak signal compared to polymer strong RALS signal. When the RALS area values are compared, there are no major changes in the size of the molecules obtained by polymer-peptide binding. As the peptide had a small molecular weight, the amount of peptide added to the polymer did not change dramatically. Both GPC UV and RALS results show us that peptide polymer conjugates were successfully synthesized.

Size, PDI and zeta potential values of peptide, polymer and bioconjugates

The size and PDI values of the peptide, [P(VP-co-AA)] and $n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$ bioconjugates were given together in a single graph in Figure S7. The mean particle size increased with increasing conjugation rate, while PDI values of the conjugates remained lower than the polymer. The zeta potential values of the peptide, [P(VP-co-AA)] and $n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$ bioconjugates synthesized by the EDC method were measured in the Zetasizer. Zeta potential values of peptide, polymer and bioconjugates were given comparatively in Figure 4. While the zeta potential of peptide and polymer

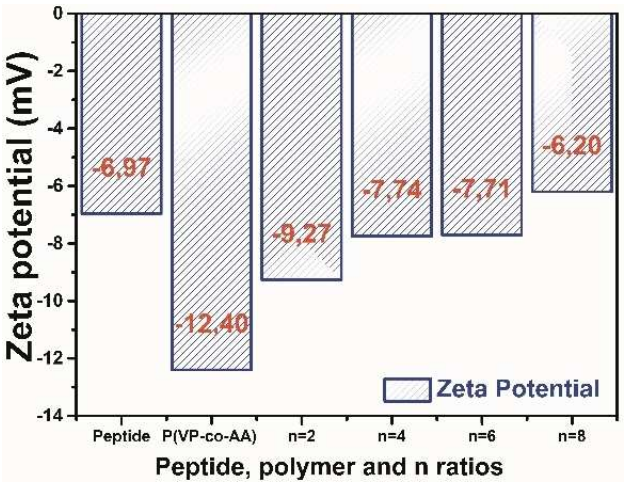

Figure 4. Zeta potential values of peptide, polymer and bioconjugates ($n = 2, 4, 6, 8$)

were found −6.97 mV and −12.40 mV respectively, the zeta potential of bioconjugates ($n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$) were determined −9.27 mV, −7.74 mV, −7.71 mV and −6.20 mV respectively. Peptide, polymer and conjugates appear to be negatively charged.

When the zeta potential values are compared, the potential of $n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]}$ is closer to zero than of the [P(VP-co-AA)]

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polymer. The negative charge transfer arising from forming an amide bond via covalent conjugation reaction between negatively charged carboxyl groups (-COOH) of the polymer and amino groups ($-NH_2$) of the peptide was seen an increase in the zeta potential value. The conjugation reaction is proved to occur for all proportions in the peptide and polymer conjugates carried out in this study. As the ratio of $n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]}$ increases, the zeta potential value approaches zero linearly. The positively increasing of zeta potential by conjugation of the peptide to the polymer might trigger cellular uptake of bioconjugates. In the literature; Chen et al, Liu et al, Motskin et al, and many others have reported that cellular uptake is good enough in less negatively charged particles compared to the peptide or polymer.[17] Thus this synthesized peptide polymer bioconjugates could be a promising strategy for the design of synthetic peptide vaccine against to the rabies disease.

Determination of cell viability

The cell viability graphs for the $n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2, 4, 6, 8$ conjugate ratios comparative with the peptide and the polymer are shown in Figure 5. ECV304 human epithelial cells were used in the study with MTT method at concentrations of 1, 5, 10 and 50 μg/mL peptide, polymer and bioconjugates. The percentage values for each ratio are compared with the control. A decrease in the cell viability of the pure peptide compared to the control at all studied concentrations. The biocompatible polymer [P(VP-co-AA)] can be expressed by looking at the graph, which does not show any toxic effects in all studied concentrations. It can be said that the bioconjugates are not toxic at concentrations of 1, 5, 10 μg/mL. At a concentration of 50 μg/mL, some conjugate ratios had negative effects on the cells.

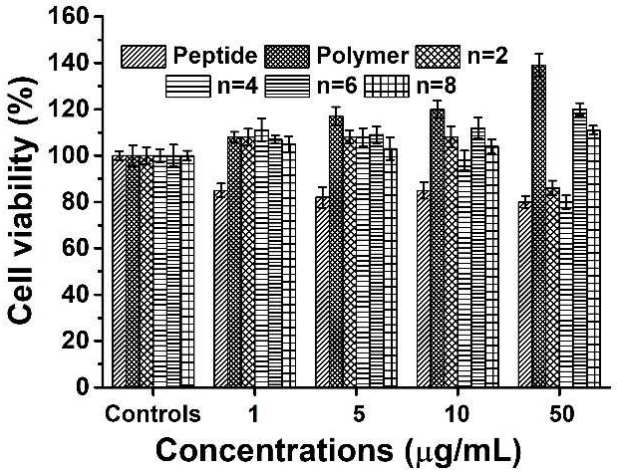

Figure 5. The cytotoxicity values of the bioconjugates ($n_{peptide}/n_{[P(VP\text{-}co\text{-}AA)]} = 2$, 4, 6, 8) synthesized in the presence of EDC compared to the peptide and the polymer

When the cell viability graph is examined, it is seen that pure peptide alone has low toxic properties. When we look at [P(VP-co-AA)] alone, it has been observed that there is no negative effect on cell viability in the 1–50 range, where concentration increases. This result indicates that [P(VP-co-AA)] does not show toxicity to cells. When the conjugates of the peptide and poly (vinyl pyrrolidone-co-acrylic acid) copolymer are considered, the negative effect on the cells generally to increase as the amount of peptide in each ratio increases up to the concentration of 50 μg/mL. It can be said that the poly (vinyl pyrrolidone-co-acrylic acid) copolymer bioconjugates with the peptide significantly reduces the toxicity of the peptide in the bioconjugates. Cell viability studies of conjugates have the ability to establish a preliminary study of the use of our bioconjugates in living organisms.

Conclusions

In the current study, the synthesis of the specific 31D antigenic peptide epitope by the microwave-assisted solid phase peptide synthesis was performed. The peptide characterized using LC–MS and HPLC was obtained in high purity. After the synthesis and characterization of the [P(VP-co-AA)], a biocompatible copolymer, the covalently bound water-soluble conjugates of the peptide and the polymer were synthesized in the presence of the EDC as a crosslinker for the first time. Conjugates synthesized was characterized using GPC and ZetaSizer. In the toxicity study, cell viability was maintained at all concentrations of the polymer, but toxicity was detected at all concentrations of the peptide. As a result of the cytotoxicity study, covalent conjugation of the peptide to the polymer reduces the toxicity of the peptide and plays a role in determining the biocompatible concentrations of the bioconjugates. In the light of all these the data, gained the synthesized water-soluble bioconjugates are seen as a strong candidate for future rabies vaccine studies.

Supporting Information Summary

Supporting information includes experimental section, detailed analysis of Poly(N-vinyl-2-pyrrolidone-co-acrylic acid) and graphic of size and PDI values of conjugates.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Conjugation · Cytotoxicity · Polymeric Adjuvant · Rabies · SPPS

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Submitted: April 17, 2019
Accepted: August 16, 2019

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