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Characterization of water-soluble conjugates of polyacrylic acid and antigenic peptide of FMDV by size exclusion chromatography with quadruple detection

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ABSTRACT

Intermolecular complex by electrostatic interaction and specifically coupled conjugates between polyacrylic acid (PAA) and a synthetic peptide representing 170–188 sequence from the GH loop of VP1 protein of foot-and-mouth disease virus (FMDV) were investigated as an intermolecular model system due to their importance in biotechnology and immunology. In this study, polyacrylic acid (PAA) with a synthetic peptide representing 170–188 sequence from the GH loop of VP1 protein of foot-and-mouth disease virus at a wide range of mixing ratios of components ($C_{Peptide}/C_{PAA}$ 0.1, 0.25, 0.5, 0.75 and 1.0, respectively) were characterized by size-exclusion high performance liquid chromatography with on-line refractive index, UV, light scattering and viscometer detectors. The results revealed that two molecules are both negatively charged as a result of repulsive forces preventing complex formation at neutral pH. Therefore, these molecules bound covalently to each other by using water-soluble carbodiimide when pH levels are higher than the pI of the peptide. High performance liquid chromatography analysis showed that the amount of protein-polymer complex increased and free peptide amount decreased with the increase in molar ratio of the peptide. Also, this paper presents that number of the bound peptide molecules with one PAA molecule was expressed by a Langmuir-type equation as a function of the amount of excess synthetic peptide existing free in the solution.

1. Introduction

The main reasons why synthetic polyelectrolytes are used as adjuvants to stimulate immune system against an antigen underlie the limitations of Pasteur method of vaccination, such as the genetic dependency of immune system or undesirable side effects caused by the multi-component complex systems of the whole or partly destroyed microbes. In addition, synthetic polyelectrolytes are inexpensive and can be modified or designed for desired functionality [1,2].

There are a number of problems associated with vaccine design and development, such as instability, hypersensitivity and the toxicity of suitable carriers and adjuvants for delivery to the immune system. Some of these problems have been overcome with the current technological advances. Furthermore, significant advances in the areas of biotechnology and peptide synthesis have led to the availability of large quantities of pure, potent and highly specific peptides that can be used to generate the appropriate immune responses necessary to combat many human diseases [3].

Synthetic antigens used to generate site-specific antibodies to proteins are of interest in the vaccine development. Synthetic peptides need to be conjugated to a carrier protein or polymer for optimal immunogenicity [4].

Foot-and-mouth disease virus (FMDV) terminally afflicts domestic livestock and has had destructive economic effects on the agricultural industry [5,6]. This disease is characterized by the formation of vesicles on the mouth, tongue, nose and feet [19]. FMD is caused by a single stranded RNA virus bearing the same name (FMDV) which belongs to the family *Picornaviridae* and the genus *Aphthovirus*. It is a small, icosahedral, non-enveloped virus with a single stranded positive sense RNA genome of approximately 8400 nucleotides [5,7,8]. FMDV consists of four protein subunits, VP1-4, of which only VP1 (1D) showed immunogenic activity when the individual proteins were used [9,10]. There are seven serotypes (A, O, SAT1, ST2, SAT3, Asia 1) and more than 65 subtypes. These antigenic variations have implications for the development and use of vaccine strains, those that elicit antibodies capable of neutralizing a broad range of field viruses being preferred to viruses with a narrow antigenic spectrum [11]. Various kinds of vaccines against the virus have been studied [1–4, 12–15] and it is still the subject of many researches.

Polyacrylic acid (PAA) is a strong adjuvant for primary and secondary responses and has been widely used in several studies of polymer-subunit immunogenic systems for better understanding the mechanism of immunostimulating activity of synthetic polyelectrolytes [1,2].

Polymeric immunogens and vaccines, based on conjugates or complexes of synthetic polyelectrolytes with antigenic molecules such as proteins and peptides, have become an extensively studied

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topic in the last decades [1,2]. Although the interaction mechanism of polyelectrolyte complexes/conjugates with cell membrane is known in details [12,13, 16–18], it is important to characterize the physicochemical parameters of polyelectrolyte complexes/conjugates for better understanding the structure-function relationship. Many methods have been use to analyze these systems, such as analytical ultracentrifugation [14,15,19], fluorescence spectrometry [20], circular dichroism spectrometry [21], viscometry [19] and light scattering [22]. Each of these methods needs to be performed together with other techniques to provide comprehensive information about the structure.

The most fundamental parameters for characterizing macromolecules such as polymers, peptides, proteins, polysaccharides, oligonucleotides, and antibodies are their molecular weight and/or molecular weight distribution [23]. Size exclusion chromatography (SEC) with quadruple detection system (Refractive Index, UV, Light Scattering and Viscometer) gives detailed information about molecular weight and/or molecular weight distribution in a fast and reliable way, after calibration with single standard polymer solution [24,25]. A combination of two or more detectors online with chromatographic systems has been used for determination of molecular weight, polydispersity index, hydrodynamic radius, intrinsic viscosity, constants of Mark-Houwink equation and composition of compounds by single analysis of conjugate/complex of polyelectrolyte with antigenic peptide or protein.

Objective of our research is to develop a synthetic polyelectrolyte-peptide based vaccine system against FMDV as a model system, which has longer shelf life, less side effect and price. Characterization of the polyelectrolyte-peptide systems is the first step to achieve obtaining information about the structure-function relationship. In the present study, we attempted to investigate the molecular structure and composition of covalent conjugates of PAA with partially water-soluble chemically synthesized antigenic peptide epitope of VP1 protein of FMDV in the various ratios of components using size exclusion chromatography with refractive index (RI), ultraviolet absorption (UV), light scattering (LS) and viscometry (VIS) detectors.

2. Experimental

2.1. Materials

PAA was purchased from Sigma-Aldrich and weight-average molecular weight (Mw) of this polymer was 100 kDa. Synthetic peptide representing 170–188 from the GH loop of VP1 protein of foot-and-mouth disease virus (Trp-Ala-Thr-Asp-Ile-Ser-Glu-Leu-Leu-Val-Arg-Met-Lys-Arg-Ala-Glu-Leu-Tyr-Cys-Pro) [26–27] was synthesized by using the continuous solid-phase method by Sigma Gynosys. Ttryptophan was added to the N-terminus of the peptide sequence to analyze the peptides with fluorescence spectrometry in further studies. Molecular weight of the peptide sequence is 2395 Da. Isoelectric point (pI) of the peptide is 6.17. Since it is partially soluble in aqueous medium, peptide was dissolved in 10% (v/v) DMSO-water mixture. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was used for the activation of carboxylate groups of PAA and obtained from the Sigma Chemical, St. Louis, MO. NaH_2PO_4, $Na_2HPO_4.7H_2O$, NaCl, NaOH were obtained from Fluka and NaN_3 was from Applichem. Ultra pure water used in preparation of the solutions and chromatographic analysis was obtained from Millipore MilliQ Gradient system.

2.2. Polymer–peptide mixtures

For understanding if there is any interaction between PAA and peptide molecules, parallel solutions between PAA and peptide mixtures were prepared. PAA and peptide solutions were prepared using phosphate buffer (pH 7.0) containing 0,15 M NaCl at 25 °C. Increasing molar ratio of the peptide solutions were added to PAA solution to prepare polymer-peptide mixtures. The weight concentration of PAA was kept constant and equal to 0,05 g/dl. $C_{Peptide}/C_{PAA}$ ratios were 0.1, 0.25, 0.5, 0.75 and 1.0, respectively.

2.3. Polymer–peptide covalent conjugates

Conjugates of PAA and peptide were synthesized by covalent binding using a procedure involving water-soluble carbodiimide [28]. Activation of polymer acid groups was carried out in water (pH 5.0) in a molar ratio of 4:1 (EDC:AA). 15 mg of PAA were dissolved in 1,5 ml of water and the pH of the solution was adjusted to 4.0. Then, EDC was added to PAA solution and it was stirred for 1 hour at room temperature. Increasing molar ratio of the peptide solutions ($C_{Peptide}$ = 0.05, 0.125, 0.25, 0.375 and 0.5 mg/ml) prepared in DMSO-water (10% v/v) mixture was added to the activated PAA and stirred for 12 h at 4 °C and the pH was adjusted with 1 M NaOH to 7.0. After removal of O-acylisourea intermediate by dialysis, the sample was lyophilized. The lyophilized conjugates were dissolved in 50 mM phosphate-buffered saline (PBS) at 4 °C for the chromatographic analysis.

The Viscotek with Quadruple Detector system and OmniSEC 4.1 software programme were used for analysis of molecular weight of PAA and PAA-Peptide conjugates in the solution.

2.4. Size exclusion chromatography with quadruple detection

Viscotek TDA 302 detector system with refractive index (660 nm), right angle light scattering (670 nm) and four-capillary differential viscometer detectors was used for on-line SEC signal detection. A separate UV detector obtained from Viscotek was connected to this detector system and the detectors were in the following order: UV–LS–RI–VIS. 0.2 μm nylon pre-filter was used between the column and detectors. HPLC pump, degasser and autosampler with 100 μl injection loop were built-in Viscotek GPCmax VE 2001 pump system, which is connected to the detectors. OmniSEC 4.1 software programme was used for the acquisition and analysis of SEC data.

Viscotek quadruple detector arrays were calibrated with BSA monomer peak in a mobile phase of PBS at 1.0 ml/min flow rate. 0.185 [24,29] and 0.66 [25,30] were used as dn/dc value and extinction coefficient of BSA, respectively. Also, the column is calibrated using standard polymers of known molecular weight and size. All size exclusion chromatography experiments were repeated three times, and chromatograms are depicted using the mean values of replicate analyses. A Shim-Pack Diol 300 column (500 mm length, 7.9 mm inlet diameter) was used for separation of the peptide, PAA and PAA-Peptide mixture/conjugate. Elution was isocratic and at flow rate of 1.0 ml/min. Phosphate-buffered saline (PBS) was prepared using ultra pure water and consisted of 50 mM phosphate and 150 mM sodium chloride, pH 7.0. 0.05% NaN_3 was added to the mobile phase solutions to prevent biological degradation of the columns. Buffer solutions were filtered through 0.45 μm Millipore cellulose nitrate filter and were degassed before use.

2.5. Theoretical

Classical light scattering theory describes how the molecular weight is related to intensities of incident light. Light scattering caused by particles in solution is proportional molecular weight and concentration of the protein [31]. The light scattering intensity of polymer molecules in solution is proportional to molecular weight and concentration of sample [9]:

$$\frac{KC}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2C \quad (1)$$