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Thomas Hahne,[1] Muthukutty Palaniyandi,[2] Tatsuya Kato,[2] Peter Fleischmann,[3] Hermann Wätzig,[1] and Enoch Y. Park,[2,*]

Institute of Medicinal and Pharmaceutical chemistry, Technische Universität Braunschweig, Beethovenstr. 55, 38106, Braunschweig, Germany,[1] Laboratory of Biotechnology, Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, 422-8529 Japan,[2] Institute of Food Chemistry, Technische Universität Braunschweig, Schleinitzstr. 20, 38106 Braunschweig, Germany[3]

[*] Corresponding author. Tel.: +81 54 238 4887; fax: +81 54 238 4887. *E-mail address:* acypark@ipc.shizuoka.ac.jp (E.Y. Park)

Abstract

Human papillomavirus 6b L1 virus-like particles (VLPs) were successfully expressed using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid expression system and rapidly purified using size exclusion chromatography (SEC) after ultracentrifugation procedure and characterized by capillary zone electrophoresis (CZE). The average capillary electrophoresis (CE) migration time was 11 min with the relative standard deviation (RSD) of 0.3% of HPV 6b L1 VLPs. After this threefold fractionation, the CZE samples were still further investigated by dynamic light scattering (DLS) and immuno blotting. The versatile technique, CZE not only proved to be a valuable tool for VLP characterization, but was also found to be reliable and precise. Thus CZE will also be an important option for the quality control of VLPs in pharmaceutical research level.

[*Keywords:* Human papillomavirus 6b L1; Virus-like particles; Silkworm; Capillary zone electrophoresis; Fractionation; Size exclusion chromatography]

Introduction

Subunit vaccines like virus-like particles (VLPs) are potent candidates for immunization, since these VLPs are devoid of genomes but still mimics the shape of the whole virus and they are efficacious to elicit immune response (1). Taking the advantage of capsid protein, their autonomous folding to form structural conformation and potential immune protection has drawn a lot of focuses on studies to improve their ability in various applications like vaccines, drug delivery and protein trafficking analysis (2). Several VLPs has been successfully developed using various expression systems and analyzed by several analytical techniques to prove their conformation and authenticity. Different expression systems like *E. coli*, yeast, transgenic plants, mammalian cell line and insect cell lines has successively produced various VLPs and several recombinant VLPs have been commercialized (3).

One of the contagious sexually transmitted diseases causing from genital warts to cervical cancer is human papillomavirus (HPV), which is a non-enveloped DNA virus with more than 100 different genotypes that infect humans. Ninety percent of HPV infections causing genital infections are from HPV 6 and 11 (4). HPV are icosahedral shaped viruses with 72 pentamers forming the capsid L1 proteins which function is to adhere and enter into the squamous epithelial cells in the genital region forming warts

(5–6). Protection against HPV infection using vaccine has been successful to some extent, commercially available vaccines like Cervarix® (Glaxosmithkline) and Gardasil® (Merck), but these vaccines are not cost-effective to third world countries. A simple and high-throughput production system from upstream and downstream processing of the VLPs will be effective to reduce the cost burden of vaccines (7).

Baculovirus based expression system using silkworm has been competitively able to express and produce recombinant proteins and VLPs. Much advancement has been achieved in this system; especially, *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid-based expression is fast, inexpensive and non-laborious using silkworm larva (8). Recently, we have expressed HPV 6b L1 capsid protein in silkworm expression system, which successfully formed VLPs and manipulation of their epitopes (9–10). However it is relatively difficult to purify VLPs from silkworm for pharmaceutical research purpose, which is the key hindering step in downstream processing. The method of its elimination should not pose a risk to the structure and integrity of the target protein. Traditionally, protein isolation methods use combinations of filtrations and chromatography-based purifications. Purifying and analyzing those recombinant VLPs involves multiple steps, which in turn decreases the recovery ratio.

Though gel based electrophoresis is the widely used technique in laboratories

around the world to analyze proteins, the most common approach is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recently capillary electrophoresis (CE) replaces traditional gel electrophoresis, because this technique has been standardized to evaluate organic drug compounds, pharmaceutically important proteins and peptides (11). In the CE, electrophoretic separations of analytes are performed in a fused silica capillary tube of particular length. It is a very efficient separation technique, also available for quantifying several viruses and VLPs (12). CE replaces traditional gel electrophoresis step by step, as it is particularly non-laborious and provides excellent separation capabilities for proteins. This flexibility makes CE a very effectual alternative over the 1D-SDS-PAGE (13). Various proteins, peptides and viruses were analyzed using CE as efficient electrophoretic technique (14, 21-23). Improvements of capillary zone electrophoresis (CZE) as an analytical tool, are made based by the prevention of denaturing or binding of analytes to the fused silica capillaries using detergents and/or suitable buffers.

In this study, we tried to purify VLPs employing size exclusion chromatography (SEC) and characterizing them by CZE, which could be a corroborated method in future for high-throughput analysis of VLPs.

MATERIALS AND METHODS

Reagents and materials Sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, acetanilide and Thesit® were obtained from Sigma-Aldrich (Steinheim, Germany) sodium borate was purchased from Merck (Darmstadt, Germany). All samples and buffers were prepared using double distilled water. Fifth-instar silkworm larva (Ehime Sansyu Co. Ltd., Yahatahama, Ehime, Japan) was used for the expression of HPV L1VLPs. Fused silica capillaries of 50-µm internal diameter were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Preparation of samples and buffers Phosphate buffered saline (PBS, pH 7.4) was used as lysis and elution buffer. This buffer solution was prepared by dissolving 137 mM of NaCl, 2.7 mM of KCl, 10 mM of NaH_2PO_4, 2 mM of KH_2PO_4 in 1000 ml doubly distilled water. The background electrolyte (BGE) used for separation is 100 mM sodium borate which was adjusted to pH 8.3 with HCl, later 10 mM Thesit® was added (15). Acetanilide as electro-osmotic flow marker was added into the SEC fractions containing VLPs to a final concentration of 0.5 µg/ml. All buffers and samples were filtered through 0.45 µm filter before use.

Expression and purification of HPV 6b L1 VLPs The VLPs were produced from silkworm larvae using BmNPV bacmid-based expression system (9). In brief, using standard molecular techniques the HPV 6b L1 gene was transformed into BmNPV bacmid and the DNA of recombinant BmNPV bacmid was injected into silkworm. Post-injection time of 7 days the fat body of silkworm larvae was collected and sonicated.

Sucrose gradient separation and size exclusion chromatography Five hundred microliters of fat body lysates were applied on to the 25–60% sucrose gradient prepared in PBS (pH 7.4). Separation of contaminants was achieved by centrifugation at 120,000 × g for 3 hrs at 4°C. Centrifuged sample were aliquoted into 500-µl fractions. Fractions containing HPV 6b L1 VLPs were filtered through 0.45-µm filters. Primary purified HPV 6b L1 VLP fraction was applied to size exclusion chromatography (SEC). Proteema 300 A column (PSS, Mainz, Germany) packed with 5 µm particle size of porosity 300 Å (300 × 8 mm), was equilibrated with 10 column volume of PBS (pH 7.4). One hundred microliters of purified HPV L1 fractions of sucrose gradient separation were injected and separated. Elution was carried out with PBS (eluent A) and PBS containing 1 M sodium chloride (eluent B) using a linear gradient over 30 minutes

(0–100% B in 30 min) starting the gradient with the beginning of the separation. The used flow rate during separation was 0.5 ml/min (16). The collected 1 ml size exclusion fractions including the target protein were confirmed using western blot. The HPV 6b L1 VLPs were further analyzed using CZE.

Capillary zone electrophoresis Agilent 7100 capillary electrophoresis system with inbuilt diode array detector was used for the analysis (Agilent Technologies, Waldbronn, Germany). Peaks were detected at 214 nm with a reference wavelength of 360 nm. Integration was done using in-house integration software corrected integration software system (CISS, Würzburg, Germany) and data processing using Microsoft EXCEL™. Before use, conditioning of fused capillaries was done by flushing at 1000 mbar of 1 M sodium hydroxide and subsequent with background electrolyte (BGE) for 15 min each. Preconditioning before each run was done using BGE for 2 min and post-conditioning carried out using 1 M sodium hydroxide solution and rinsing with double distilled water for 2 min. During preconditioning as well as post-conditioning a pressure of 1.0 up to 2.0 bar was applied. The size exclusion fractions were injected hydrodynamically with 100 mbar for 25 seconds into the capillary. Separation of analytes was carried out at 25 kV and a mean current of 23 μA using BGE in the inlet

and PBS in the outlet. Temperature was maintained at 25°C throughout the run. Samples and running electrolytes were filtered and sonicated for 3 min before analyzing the sample in CE system. The volumes of fifteen consecutive injections were collected in one vial during the separations. During preconditioning and post-conditioning other vials were used, which leads to separation from other components of the SEC fractions which migrates slower than the VLP's

Characterization of VLPs Dynamic light scattering (DLS) and immuno-analysis were carried out using Zetasizer nano ZS DLS analyzer fitted with 532 nm laser lamps (Malvern Instruments, UK), to prove that CE based separation and analysis were authentic VLPs. Fractions (1 ml) of SEC and CZE were subjected to DLS analysis at 25°C with 10 consecutive measurements. Western blot were carried out for both SEC and CZE fractions, to confirm the presence of VLPs, the fractions were subjected for SDS-PAGE and transferred to PVDF membrane using trans-blot® semi electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). Blots were developed by immuno staining with H6.J54 HPV L1 primary antibody (a kind gift from Neil Christensen, Pennsylvania State University College of Medicine, USA) and anti-mouse secondary antibody conjugated with HRP (GE Healthcare, Tokyo, Japan)

were used against VLPs. ECL plus detection reagents were used to detect HPV 6b L1 protein using Versa Doc Imager and analyzed using Quantity-One software (Bio-Rad).

RESULTS AND DISCUSSION

Sample preparation To achieve partial purity of the VLPs from cell lysates sucrose gradient ultracentrifugation and SEC were performed. Sucrose gradient centrifugation has been chosen for preliminary purification to obtain formed VLPs from lysates. Fractionated sucrose gradient separated samples were re-clarified using SEC. The separation was achieved by 1M NaCl, and the elution was approximately when 60–80% of the NaCl gradient were performed (data not shown), which was later confirmed by DLS and immunoblot detection.

Characterization of VLPs using CZE In order to perform an efficient CE analysis the rinsing was initially done with the 1 M sodium hydroxide in combination with the anionic surfactant SDS (17). However, even though the protein adsorption to the capillary walls was reduced, the obtained relative standard deviation (RSD) remained unsatisfactory. Weiss et al. employed non-ionic surfactants such as Thesit® (15) which neutralize the anionic silanolate group on the inner capillary surface, minimizing the protein adsorption to the capillary wall. Pressures from 1.0 bar up to 2.0

bar and further rinses with water and BGE containing Thesit® were employed (2 mins each), which subsequently reduced the drift of the migration times to an RSD of less than 1% (17–18). Note that the addition of non-ionic detergent pose a serious stability problem with enveloped viruses which have lipid bilayer. However, HPV L1 is a non-enveloped virus, which allows for analyzing and prevention of aggregation of VLPs under the running conditions.

Analyzing the electropherogram of HPV 6b L1 VLPs using CISS, the migration times of acetanilide and the HPV L1 VLPs were 2.5 and 11.0 min, n=10, with RSD of 0.3 and 0.6%, respectively (Fig. 1A), which was better than the generally accepted level of 2%, proving the employed CZE method is precise. The corresponding RSD of the peak areas was 6.2% (Fig. 1B). This remaining variability could be due to a still persisting adsorption at the inner capillary surface. The signal to noise ratio (S/N) of HPV L1 VLPs was calculated as 147 (mean of five consecutive runs) using the Agilent chemstation software. This value is greater than 100, which is accepted as sufficient for optimal precision of CE systems (19). If a scale up analysis to commercial scale of CZE fractionation was desirable, the methods described here could be transferred to free flow zone electrophoresis (FFZE), which provides very similar selectivity with much higher

sample loading capabilities.

Hydrodynamic radius of HPV 6b L1 VLPs and immune analysis

Hydrodynamic radius of VLPs in series purification of SEC and CZE was measured (Fig. 2). In both samples, a peak at around 7 nm was mainly detected. This size corresponds to HPV L1 capsomeres (11-12 nm) (20), indicating these peaks have HPV 6b L1 capsomeres and most of the purified L1 protein formed these ones. HPV 6b L1 proteins expressed in silkworm larvae formed various sizes of L1 VLPs (10 – over 100 nm) (9). In this study, samples of SEC contained mainly capsomeres and also VLPs whose size ranged between 100 and 300 nm. Samples of CZE fractionation also mainly show capsomeres and several sizes of VLPs, with additional species with sizes of approximately 20, 50 and 700 nm. HPV 16 L1 protein purified from yeasts can be disassembled into the L1 capsomeres under the low ionic strength and high pH in the presence of a reducing agent (24). In addition, they formed in complete HPV VLPs and uniform VLPs were obtained by dis- and re-assembling (25). This suggests that HPV 6b L1 protein from silkworm formed or disassembled into some particles (the sizes of 20 and 50 nm) under the CZE condition. Particles with the size of 700 nm might be aggregated forms because its size was too large compared to the HPV L1 particles.

L1 proteins purified by SEC and CZE were also detected by western blot (Fig. 3). In both samples, two bands appeared. Based on the molecular weight of L1 protein, the upper and lower bands correspond to full-length of L1 protein and partially degraded L1 protein, respectively, indicating that the main peak in CZE contains purified HPV L1 protein. This HPV L1 protein is prone to be degraded in the fat body of silkworm larvae (9). Sometimes recombinant HPV 6b L1 proteins are detected in the fat body of silkworm larvae by western blotting as several bands. Full-length of L1 protein can not be separated from degraded L1 protein because full-length of L1 protein might form into capsomeres and VLPs with degraded L1 protein (26).

CZE can also be applied to the investigation of the quality of purified recombinant protein due to its reproducibility and reliability. However, individual CZE condition has to be investigated in detail. For example, degraded L1 protein was not completely separated from full-length L1 protein by CZE in this study, because fraction collection was the main goal in the present work. It seems that several peaks in DLS results might be caused by partial degradation of L1 proteins.

In this work we demonstrated the feasibility of CZE to analyze and quantify VLPs, including fraction collection. CZE analysis fulfills the requirement of fewer amounts of sample and high throughput. The achieved precision of CZE analysis is

essential, since VLPs e.g. for vaccines intended for human use require a high standard for their quality and quantity. Even without a sophisticated protocol, analysis of CZE fractions allows for further characterization by DLS and SDS-PAGE and subsequent western blotting with sufficiently high sensitivity. A more elaborated fraction collection protocol could further improve sensitivity and selectivity of this approach. Modifying and even scaling up of this approach using other free zone and capillary electromigration techniques will pave the way for new quality control standards of pharmaceutical bioproducts.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Rustenbeck for making it possible to use the ultracentrifuge at the institute of pharmacology, toxicology and clinical pharmacy in Technische Universität Braunschweig. We also thank Prof. Neil Christensen for generously the providing antibodies. Furthermore the authors thank Polymicro Technologies for providing the capillaries used in this work. This work was supported by a grant from the German academic exchange services (DAAD).

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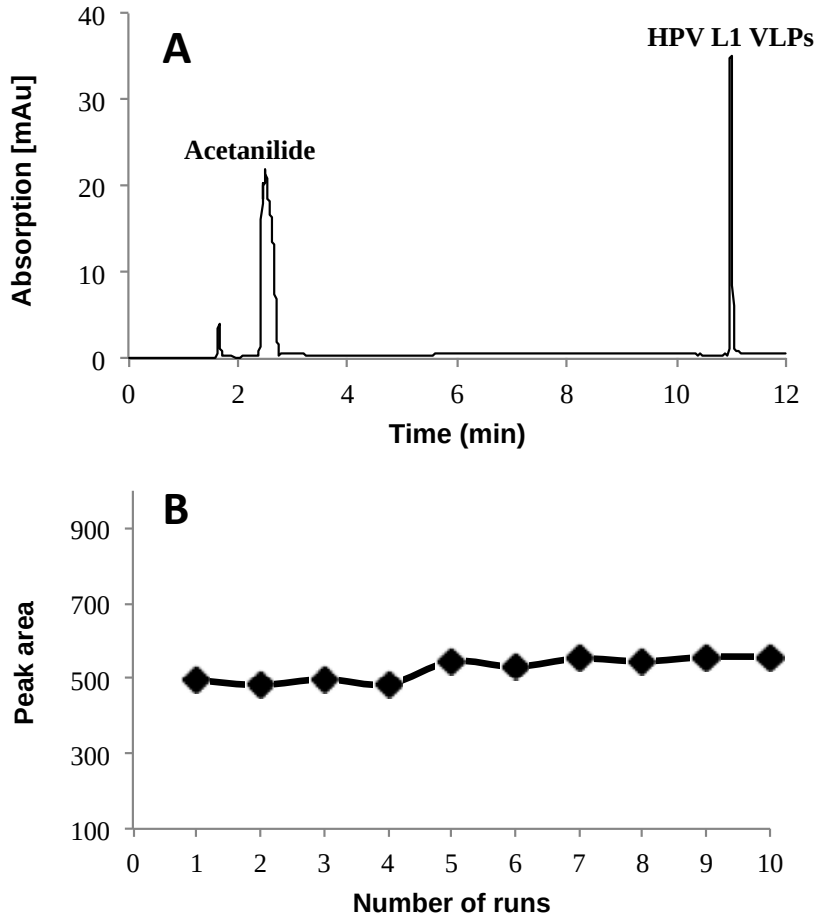
Figure legends

FIG. 1. Electropherogram of the HPV 6b L1 VLPs analyzed by CZE using acetanilide as electroosmotic flow marker (A) and its stability during 10 CE runs (B).

FIG. 2. Dynamic light scattering analysis of HPV L1 VLPs. (A) Partially purified VLPs after SEC. (B) Injected SEC fraction, further fractionated by CZE of HPV L1 VLPs. Fraction (1 ml) was analyzed at 25°C.

FIG. 3. Immuno blot of HPV L1 VLPs. Lane M: Molecular weight marker, Lane 1: Partially purified fraction after SEC, Lane 2: Fraction of capillary zone electrophoretic analysis of HPV L1 VLPs. HPV L1 VLPs were detected against HPV L1 primary antibody. Arrow indicates full-length of HPV L1 protein.


A
HPV L1 VLPs
Acetanilide
Absorption [mAu]
40
30
20
10
0
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2
4
6
8
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12
Time (min)
B
Peak area
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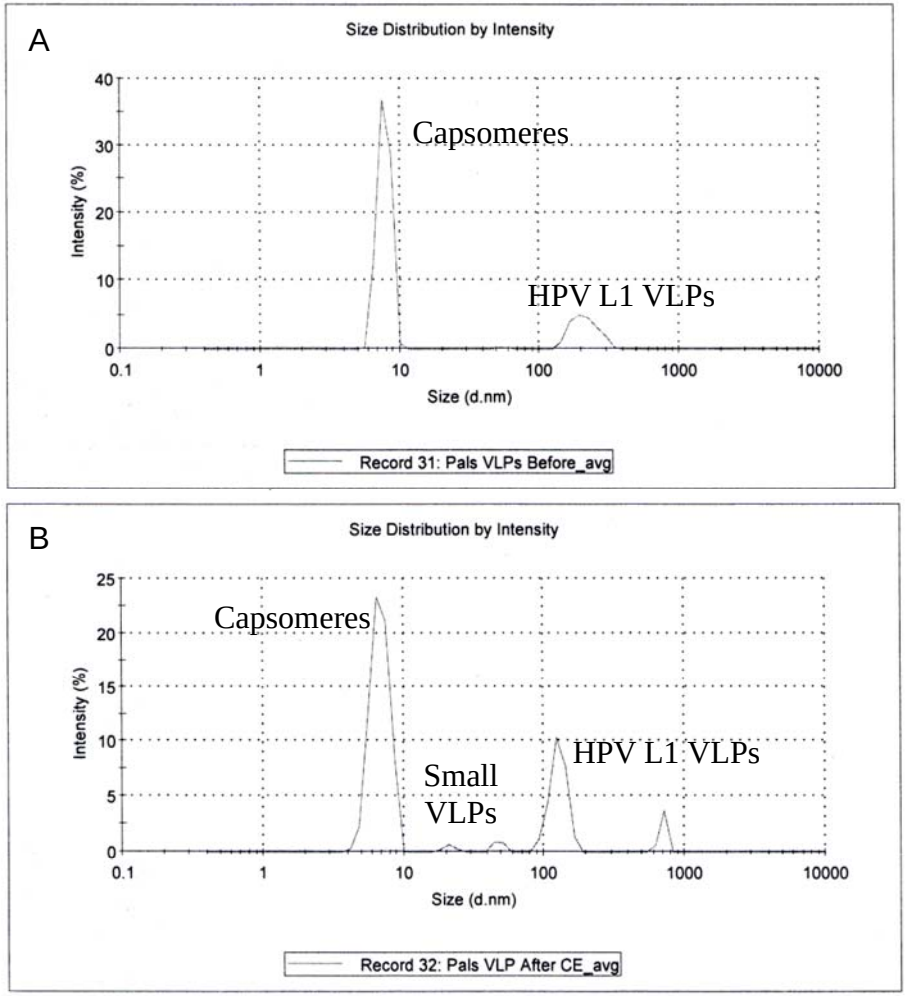
Number of runs


A
Size Distribution by Intensity
Capsomeres
HPV L1 VLPs
Intensity (%)
Size (d.nm)
Record 31: Pals VLPs Before_avg
B
Size Distribution by Intensity
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Small
VLPs
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Intensity (%)
Size (d.nm)
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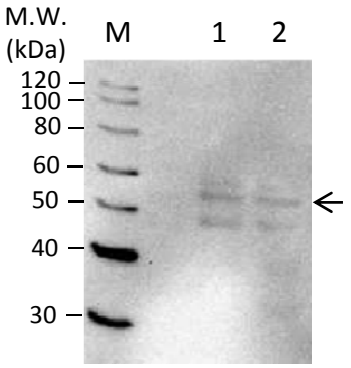
M.W.
(kDa)
M
1
2
120
100
80
60
50
40
30