Biotechnology and Bioprocess Engineering 17: 755-763 (2012)
DOI 10.1007/s12257-012-0067-5

RESEARCH PAPER

Effects of Downstream Processing on Structural Integrity and Immunogenicity in the Manufacture of Papillomavirus Type 16 L1 Virus-like Particles

Don Yong Chang, Hyoung Jin Kim, and Hong-Jin Kim

Received: 2 February 2012 / Revised: 11 Marcy 2012 / Accepted: 12 Marcy 2012

Abstract There is increasing demand for virus-like particles (VLPs) as a platform for prophylactic vaccine production. However, little attention has been paid to how downstream processing affects the structure and immunogenicity of the VLPs. In this study, we compared three methods of purifying human papillomavirus type 16 (HPV16) VLPs, each including the same cation-exchange chromatography (CEC) step. Method T-1 uses both ammonium sulfate precipitation (ASP) and a step to remove precipitated contaminating proteins (SRPC) prior to CEC, while T-2 uses only the SRPC step prior to CEC and T-3 includes neither step. We compared the structural integrity and immunogenicity of the HPV16 VLPs resulting from these three methods. All three preparations were highly pure. However, the final yields of the VLPs obtained with T-2 were 1.5 and 2 fold higher than with T-1 and T-3, respectively. With respect to structural integrity, T-1 and T-2 HPV16 VLPs had smaller hydrodynamic diameters and higher reactivity towards monoclonal anti-HPV16 neutralizing antibodies than T-3 VLPs, indicating higher potentials of T-1 and T-2 VLPs for eliciting anti-HPV16 neutralizing antibodies. Moreover, it was confirmed that the T-1 and T-2 HPV16 VLPs elicit anti-HPV16 neutralizing antibodies more efficiently than T-3 HPV16 VLPs do in mice immunizations: the abilities for eliciting neutralizing antibodies were in the order T-2 VLP > T-1 VLP > T-3 VLP. We conclude that the process design for purifying HPV VLPs is a critical determinant of the quality of the final product.

Don Yong Chang, Hyoung Jin Kim, Hong-Jin Kim*
College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea
Tel: +82-2-820-5613; Fax: +82-2-816-7338
E-mail: hongjink@cau.ac.kr

Keywords: downstream process, papillomavirus, virus-like particle, immunogenicity

1. Introduction

Virus-like particles (VLPs) are multimeric protein complexes, the shapes of which are similar to naturally occurring virions [1]. The VLPs produced by recombinant technology range from 22 to 200 nm in size [2]. VLPs have superior immunogenicity because the dense and repetitive display of epitopes on their surface elicits strong immune responses [3]. For these reasons, there is a current market demand for VLPs as prophylactic vaccines [4]. VLP-based prophylactic vaccines against hepatitis B virus (HBV) and human papillomavirus (HPV) have been commercialized [2], and VLP-based prophylactic vaccines against hepatitis E virus (HEV), influenza virus, human immunodeficiency virus (HIV) and Norwalk virus (NV), are in preclinical development or in clinical trials [2].

There are two VLP-based prophylactic HPV vaccines. Gardasil® (Merck) consists of HPV VLPs of types 16, 18, 6, and 11 produced in *Saccharomyces cerevisiae* (*S. cerevisiae*) while Cervarix™ (GlaxoSmithKine) consists of HPV VLPs of types 16 and 18 produced in insect cells [5]. These vaccines are made from recombinant L1 proteins because these can self-assemble into HPV VLPs [6,7]. HPV type 16 (HPV16) is considered the most important type because it is responsible for 50% of cervical cancer cases worldwide. The retail price of both Gardasil® and Cervarix™ is approximately $120 per dose, and $360 for the full series [8]. These are the most expensive vaccines produced so far [5]. The major cause of their high retail price lies in downstream processing, which contributes

80% of the total production costs and is regarded as inefficient, expensive and labor intensive [9,10]. Cervical cancer leads to 250,000 deaths annually and most deaths occur in developing countries [11]. In addition, it is estimated that every five-year delay in introducing a vaccine results in 1.5 ~ 2 million HPV-related deaths [9]. The current HPV vaccines are unaffordable in developing countries. The price is thought to have to fall to below $2 per dose to be accessible by the low-income populations in countries whose per capita gross domestic product is less than $1,000 [9]. Therefore, the efficient and inexpensive downstream processing is the key to the widespread use of HPV vaccines.

Previously, we developed a one-step chromatographic method for purifying HPV16 VLPs produced in *S. cerevisiae* [12]. The yield by this method was high and the L1 protein recovered was over 95% pure. Before the cation-exchange chromatography (CEC), cell lysates are submitted to ammonium sulfate precipitation (ASP) followed by a step that precipitates and removes contaminants (SRPC). 60 ~ 80% of contaminating protein is removed by the ASP and subsequent SRPC [12]. However, this purification method remains inconvenient because the ASP and SRPC require dialysis and centrifugation steps that add to costs. In addition, ASP and SRPC are procedures not traditionally employed in purifying VLPs, and their effects on the structure and immunogenicity of HPV16 VLPs have not been investigated in detail. Better understanding of their effects should permit the design of an efficient downstream process.

A compromise between purity, yield, biological activity and production cost in downstream processing has to be achieved to develop a high efficacy and low cost vaccine. However, most studies of downstream processing for VLPs or virus antigen have focused only on the purity or yield of the target antigen because of the complexity of processing. In addition, the structural integrity and immunogenicity of the antigens are not directly comparable because different expression systems, dosing schedules and formulations for immunization have been used. In the present investigation, we compared three variants of the CEC procedure described below. The complete procedure including the ASP and SRPC is designated T-1. The procedure with the ASP omitted is designated T-2 and the procedure with both the ASP and SRPC omitted is designated T-3. These procedures are summarized in Table 1.

We first evaluated the purities and final yields of L1 protein obtained by the three procedures, and then examined their structural integrity and immunogenicity. Our findings indicate for the first time how downstream processing affects the quality of the HPV VLP recovered.

2. Materials and Methods

2.1. Cell culture

S. cerevisiae Y2805 was transformed with plasmid (YEGα-MCS-HPV16 L1 MO) harboring a codon-optimized HPV16 L1 gene [13]. The transformants were cultured for 144 h in 150 mL of YPDG medium (1% yeast extract, 2% peptone, 7% glucose, and 1% galactose) as described previously [14]. The cells were harvested, washed once with phosphate-buffered saline (PBS) and frozen at -70°C until purification.

2.2. Purification of HPV16 L1 protein using method T-1

T-1 was performed as described [12]. Cells were mixed 1:1 with disruption buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1.7 mM EDTA, 0.01% Tween 80) and disrupted by vortexing with glass beads (Biospec Products, USA). Cell debris was removed by centrifugation at 12,000 × *g* for 15 min. The soluble fraction was precipitated with 45% saturated ammonium sulfate (Duchefa Biochimie, the Netherlands) and precipitated proteins were recovered by centrifugation at 12,000 × *g* for 15 min. The proteins were resuspended in phosphate-buffered saline (PBS) plus 0.01% Tween 80 and dialyzed against buffer for SRPC (10 mM sodium phosphate pH 7.2, 150 mM NaCl + 0.01% Tween 80) for 4 h. After then the concentration of protein was adjusted to 5 mg/mL by addition of the SRPC buffer and held for 12 h at room temperature (RT) to permit precipitation of contaminating proteins, which were removed by centrifugation at 12,000 × *g* for 15 min. The clarified supernatant was dialyzed against the binding buffer for CEC (2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4,

Table 1. Purification procedures of T-1, 2, and 3 methods

Process	Purification method		
	T-1	T-2	T-3
1. Cell disruption	○	○	○
2. Ammonium sulfate precipitation (ASP)	○	Omitted	Omitted
3. Step removing precipitated contaminants (SRPC)	○	○	Omitted
4. Cation-exchange chromatography (CEC)	○	○	○
5. Concentration using membrane filter device	○	○	○

0.5 M NaCl, pH 7.2 + 0.01% Tween 80) for 3 h and loaded onto a 1.8 × 3 cm column packed with P-11 phosphocellulose cation-exchange resin (3 mL of resin, Whatman, UK). The column was washed with binding buffer and bound HPV16 L1 protein was eluted by successive addition of 0.6, 0.7, 0.8, 0.9, and 1 M NaCl. The eluted fractions were collected and concentrated with a centrifugal filter device with a cut-off of 100 kDa (Amicon Ultra, Millipore, USA).

2.3. Purification of HPV16 L1 protein by method T-2

To purify HPV16 L1 protein using method T-2, the cells were disrupted, and cell debris was removed as described for T-1. The clarified cell lysate was dialyzed against the buffer for SRPC for 4 h and left at RT for 12 h without addition of ammonium sulfate (ASP). The suspension was centrifuged at 12,000 × *g* for 15 min, and the subsequent procedures for were as above.

2.4. Purification of HPV16 L1 protein using T-3 method

To purify HPV16 L1 protein by Method T-3 the cell lysate was dialyzed against the buffer for cation-exchange chromatography for 4 h, and the precipitated proteins contained in the dialyzed sample were removed by centrifugation at 12,000 × *g* for 15 min. Subsequent steps for CEC and concentration of L1 proteins were same for T-1 and T-2 methods.

2.5. Determination of protein concentration and SDS-PAGE analysis

Protein concentrations were determined with a Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories, USA) with bovine serum albumin (BSA; Pierce, USA) as standard. SDS-PAGE was performed according to the method of Laemmli [15]. Proteins were fractionated on 12% polyacrylamide gels and visualized with a silver-staining kit (GE Healthcare, USA).

2.6. Transmission electron microscopy (TEM)

Samples of purified HPV16 VLPs (about 0.3 mg/mL) were dialyzed against PBS plus 0.01% Tween 80 (final NaCl conc. 0.13 M), absorbed onto carbon-coated grids and negatively stained with 2% phosphotungstic acid. Electron microscopy was performed on a TEM200CX at a final magnification of 234,000 × [16].

2.7. Dynamic light scattering (DLS)

The size distributions of the HPV16 VLP preparations were analyzed using a DLS-700 system (Otsuka Electronics, Japan). Samples of the HPV16 VLPs were prepared in 25 mM MOPS containing 75 mM NaCl pH 7.2, at a final protein concentration adjusted to 40 μg/mL. Duplicate measurements were made, and a representative result with each VLP preparation is presented.

2.8. Size exclusion chromatography (SEC)

The SEC analysis was performed as described with slight modifications [17]. Each purified HPV16 VLP preparation (0.3 mg) was loaded onto Superose-6 resin (1.5 × 32 cm, GE Healthcare), which was equilibrated with PBS containing 0.325 M NaCl plus 0.01% Tween 80 pH 7.2. The SEC column was used at a flow rate of 0.3 mL/min. The elution profiles of the L1 proteins were acquired using Autochro-2000 software (Young Lin Instrument Co., South Korea). The proportions of assembled and dissociated form of L1 were determined with NIH open source software Image J (http://rsbweb.nih.gov/ij/) [14].

2.9. Enzyme-linked immunosorbent assays (ELISAs) using anti-HPV16 monoclonal antibodies (Mabs)

The ELISAs were performed as described previously with minor modifications [18]. We used the anti-HPV16 neutralizing monoclonal antibodies (Mabs), H16.V5 and H16.E70, to detect neutralizing epitopes on the surface of the purified HPV16 VLPs. 96-well ELISA plates were coated overnight at 4°C with purified HPV16 VLPs (400 ng per well) and blocked with 5% BSA in PBS plus 0.05% Tween 20 (PBS-T). The Mabs were serially diluted, incubated with the coated HPV16 VLPs for 2 h at 37°C and detected with HRP-conjugated polyclonal anti-mouse IgG antibody (Pab, Bethyl, USA). Color reactions were developed using *o*-phenylenediamine (Sigma, USA) and measured at 492 nm. Mabs H16.V5 and H16.E70 were kindly provided by Dr. N. D. Christensen (Pennsylvania State University, College of Medicine, USA).

2.10. Mouse immunization

The mice (Orient Bio Inc., South Korea) were divided into eight groups, each consisting of 6 ~ 10 mice. In this study, two kinds of immunizations were performed. Mice groups were immunized three times at two-week intervals with HPV16 VLPs, in combination with or without adjuvant. For the immunization without adjuvant, the control mice (n = 6) were subcutaneously inoculated with 100 μL of PBS. Mice of the T-1, T-2 and T-3 HPV16 VLP groups (eight mice each) were similarly immunized with 50 ng aliquots of T-1, T-2 and T-3 HPV16 VLPs at two-week intervals, without adjuvant. For the immunization with adjuvant, T-1, T-2, and T-3 HPV16 VLP groups (9 ~ 10 mice each) were subcutaneously immunized with 5 ng of VLPs, in combination with aluminum hydroxide (alum, 200 μg per dose). The control mice (n = 6) were immunized with 100 μL of PBS containing 200 μg of alum. Ten days after the third immunizations, serum samples were obtained from each group and stored at -70°C for subsequent assay.

Table 2. Purification of HPV16 L1s by methods T-1, T-2, and T-3. Cells were cultured in 150 mL medium for 144 h. Data are mean ± SD from four independent experiments

Purification method	Wet weight of cells (g)	Cell lysate protein (mg)	Protein loaded for CEC (mg)	Percent of contaminants removed prior to CEC (%)	Purified HPV16 L1 protein (mg)
T-1	8.0 ± 0.4	150 ± 70	38 ± 25	76 ± 10	0.34 ± 0.1
T-2	8.0 ± 0.4	157 ± 71	111 ± 51	30 ± 10	0.52 ± 0.1
T-3	8.0 ± 0.4	157 ± 77	131 ± 59	16 ± 5	0.24 ± 0.1

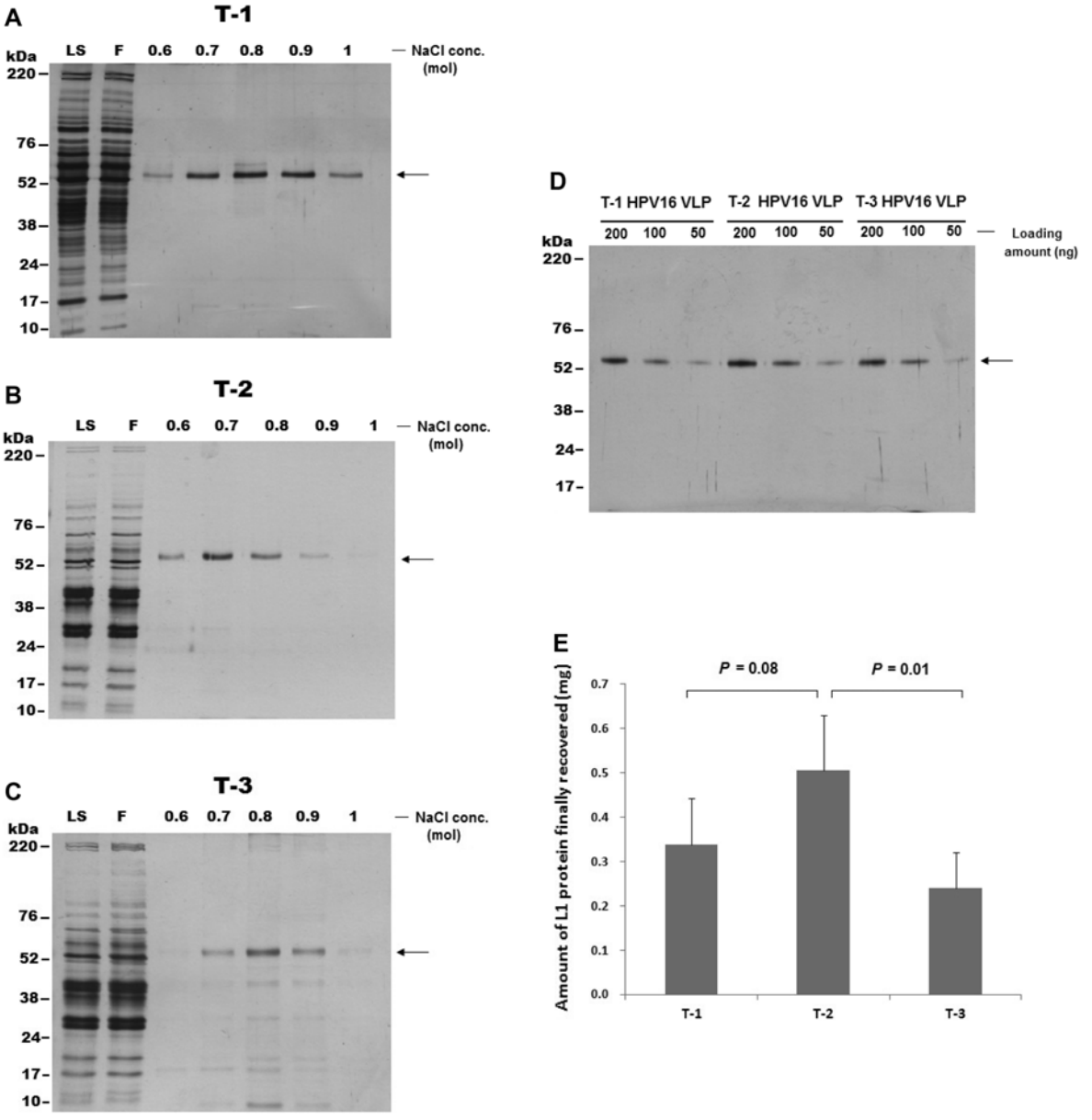

Fig. 1. Results of purifications by methods T-1, 2, and 3. Panels A, B, and C are SDS-PAGE data obtained by the three methods after cation-exchange chromatography. LS and F refer to the loading samples for cation-exchange chromatography and the flow-through, respectively. Numbers in the figure captions indicate molar concentrations of NaCl in the elution buffer, and the arrow indicates the position of L1 protein. Panel D shows the final products purified by the T-1, 2, and 3 methods. The protein concentrations of HPV16 VLP preparations were determined, and 200, 100, and 50 ng of each VLP preparation were loaded in panel D. Panel E shows the amounts of L1 protein finally recovered by the T-1, 2, and 3 methods. Each L1 protein was obtained from 150 mL of culture. Data are mean ± SD of four independent experiments. Details of the purification results are presented in Table 2.

2.11. Determination of anti-HPV16 L1 IgG titers

Anti-HPV16 L1 IgG titers were determined as described previously [19]. 96-well ELISA plates were coated with 100 ng per well of purified HPV16 VLP and blocked with 5% skim milk in PBS-T. Thereafter, serial dilutions of the mice sera were incubated with the coated HPV16 VLPs for 1 h at 37ºC. Binding of anti-HPV16 L1 IgGs to the HPV16 VLPs was detected using HRP-conjugated anti-mouse IgG Pab (Bethyl, USA), and the reactions were developed as described above. The end-point titers of the vaccination group were set at an optical density (OD) of 1.5 times the OD of control mouse serum [20].

2.12. Neutralization assays

The neutralizing activities of mouse sera were determined using a secreted alkaline phosphate (SEAP)-based pseudovirus (PsV) assay as described, with slight modifications [18]. The HPV16 PsVs were produced using the plasmids p16sheLL (containing the HPV16 L1 and L2 genes) and pYSEAP (reporter plasmid). Both plasmids were kindly provided by Dr. J.T. Schiller (NIH, Bethesda, USA). Briefly, the mouse sera were diluted 1:50 and mixed with Optiprep density gradient-purified PsVs for 1 h at 4ºC; these mixtures were then added to 293TT cells seeded at 2×10^4 cells per well in 96-well cell culture plates and the plates were incubated for 72 h. The neutralization activities of the mice sera were determined as follows: neutralization (%) = (value for PsV alone – value for PsV plus mouse serum) / (value for PsV alone – value for blank) × 100.

2.13. Statistical analysis

The statistical significance of differences between groups was determined using two-tailed Student's *t*-tests. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Purification of HPV16 VLPs by methods T-1, 2, and 3

First we evaluated the purity and final yields of L1 protein.

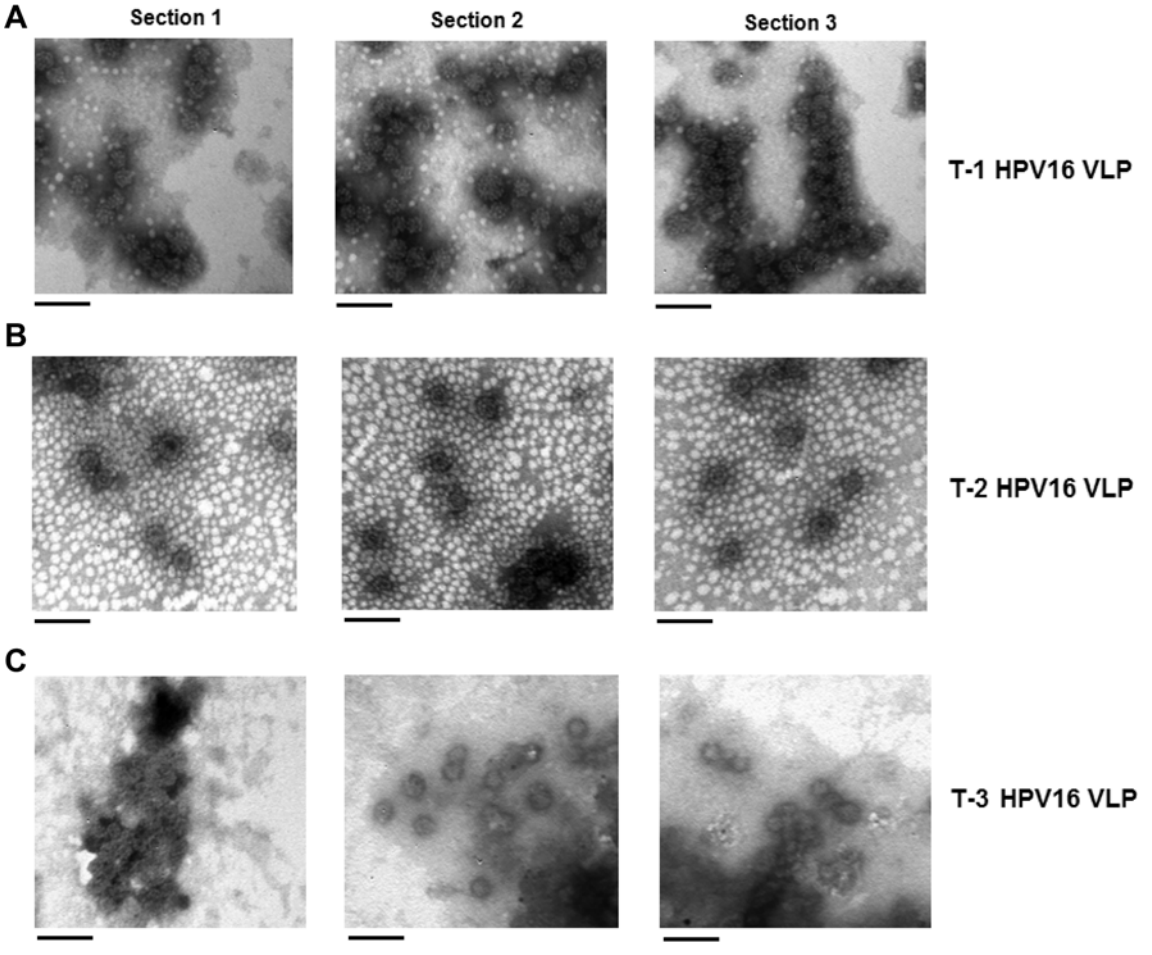

Fig. 2. TEM analysis of T-1, T-2, and T-3 HPV16 VLPs. Panels A, B, and C are TEM images of T-1, T-2, and T-3 HPV16 VLPs. The results for each VLP preparation were obtained from three sections on carbon coated grids. Bars indicate 100 nm. Magnification is 234,000 ×.

As shown in Table 2, 76, 30, and 16% of contaminating protein was removed in methods T-1, 2 and 3, respectively, prior to the CEC. Therefore, T-2 and T-3 removed less protein than T1. However, there was no significant difference between methods T-1, T-2 and T-3 in the purity of the L1 eluted from the CEC (Figs. 1A, 1B, and 1C). Finally we showed that the three L1 products were almost 100% pure after concentrations with a membrane filter device (Fig. 1D).

The yield of L1 protein resulting from method T-2 was 1.5 and 2 fold higher than those from method T-1 and T-3, respectively (Fig. 1E and Table 2). These results indicate that ASP is not essential for obtaining high purity L1 protein with a high yield although it is useful for removing contaminating proteins prior to CEC (Table 2). In summary, these results suggest that the optimal protocol for obtaining high purity of L1 protein with high yield is method T-2.

3.2. Structural integrities of T-1, 2, and 3 HPV16 VLPs

To investigate the structural integrity of the three HPV16 VLP preparations, we performed TEM, DLS and SEC analyses. As shown in Fig. 2, all of the HPV16 VLPs seen by TEM had mean diameters of 40 nm. It has been shown that the hydrodynamic diameter of HPV VLP is inversely correlated with their *in vitro* antigenicity [21]. To compare the antigenic potential of the three preparations, we performed a DLS analysis (Fig. 3). This indicated that the mean diameters of T-1, 2 and 3 HPV16 VLPs were 103, 114 and 183 nm, respectively (Fig. 3). Therefore, the hydrodynamic diameter of T-3 HPV16 VLP was significantly higher than those of the T-1 and T-2 HPV16 VLPs, suggesting that the antigenic potential of the T-3 HPV16 VLP would be lower than those of the T-1 and T-2 HPV16 VLPs.

We also compared the SEC profiles of the three VLP preparations. As shown in Fig. 4, most of T-1 and T-2 HPV16 VLP were eluted between 15 and 35 min as a major peak, indicative of the fully assembled form. On the other hand, T-3 HPV16 VLP eluted as two major peaks of the assembled and dissociated forms. In a DLS analysis, the mean particle size of T-3 HPV16 VLPs was significantly higher than those of T-1 and T-2 HPV16 VLPs (Fig. 3). In addition, we showed that T-3 HPV16 VLPs gave no monomer L1 band in non-reducing Western blot analysis (Sup-I), indicating that there were no dissociated forms prior to the SEC. Therefore it seems that the T-3 VLPs were dissociated during SEC by the hydrostatic pressure, or friction with the SEC resin. In summary, these results mean that the structures of T-1 and T-2 HPV16 VLP s were more superior to that of T-3 HPV16 VLP.

3.3. Comparison of potential for eliciting neutralization antibodies between T-1, 2, and 3 HPV16 VLPs

The neutralizing epitopes on the surfaces of the three HPV16 VLP preparations were detected using anti-HPV16 neutralizing Mabs, H16.V5 and H16.E70. As shown in Fig. 5, the reactivity of these Mabs towards T-3 HPV16 VLPs was significantly lower than that towards T-1 and T-2 HPV16 VLPs, while those towards T-1 and T-2 HPV16 VLPs were similar. Therefore, T-3 HPV16 VLPs possess less neutralizing epitopes than T-1 and T-2 HPV16 VLPs. In other word, these results indicate that the potential for eliciting neutralizing antibody of T-3 HPV16 VLPs should be lower than those of the T-1 and T-2 HPV16 VLPs.

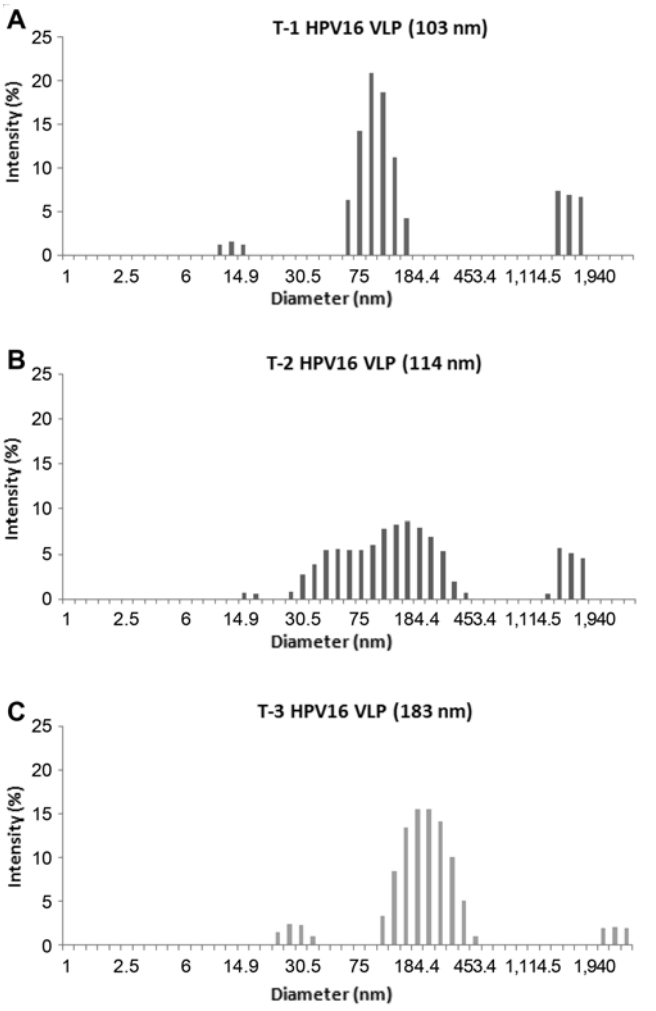

Fig. 3. Particle size distributions of T-1, T-2, and T-3 HPV16 VLPs. Each HPV16 VLP preparation was analyzed by DLS as described in Materials and methods. A, B, and C are the results for T-1, T-2, and T-3 HPV16 VLPs, respectively. Parentheses indicate the mean hydrodynamic diameters of the HPV16 VLPs. Data are representative of two analyses giving similar results.

3.4. Comparison of the immunogenicity of T-1, 2, and 3 HPV16 VLPs

Mice were immunized three times with the HPV16 VLPs with or without adjuvant, to compare their immunogenicity

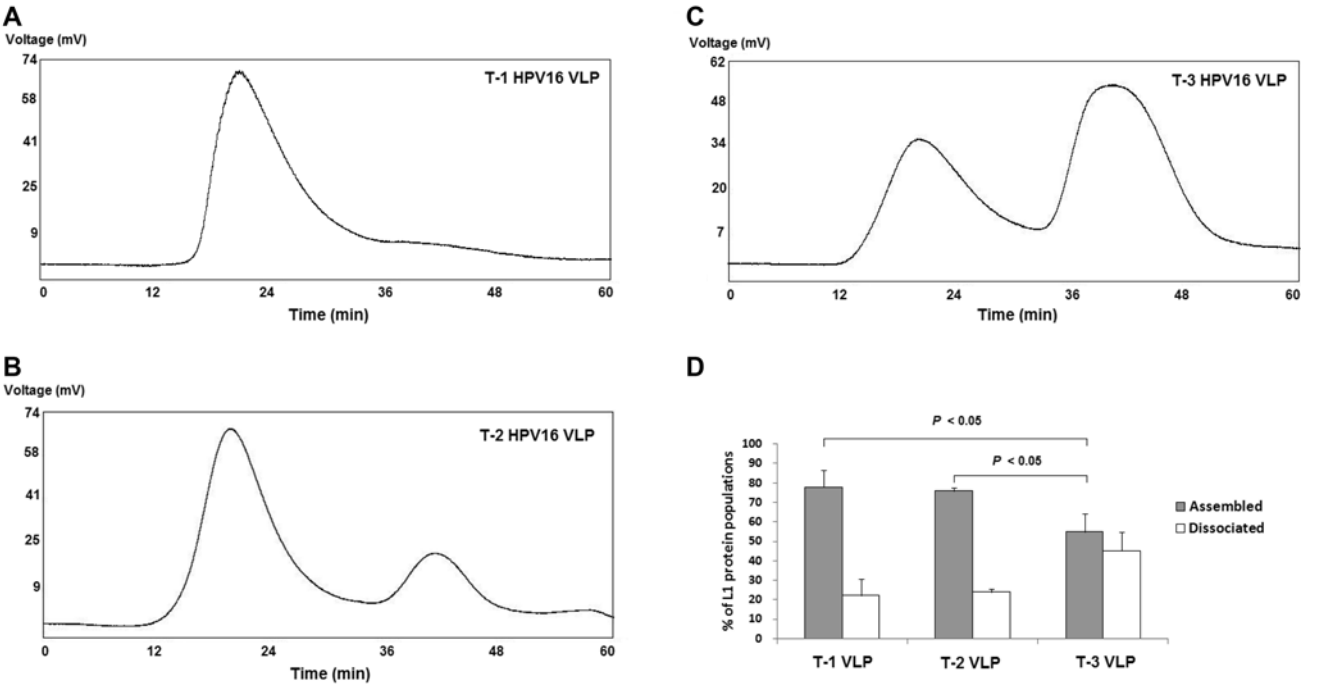

Fig. 4. SEC profiles of T-1, T-2, and T-3 HPV16 VLPs. For each, 0.3 mg of HPV16 VLPs was loaded onto Superpose-6 resin, and the elution profile was acquired with Autochro-2000 software. Panel A, B, and C is representative result of T-1, T-2, and T-3 HPV16 VLPs, respectively. Panel D shows the proportions of assembled and dissociated forms of L1 proteins. Data are mean ± SD of four analyses. The sum of assembled and dissociated forms was set at 100%.

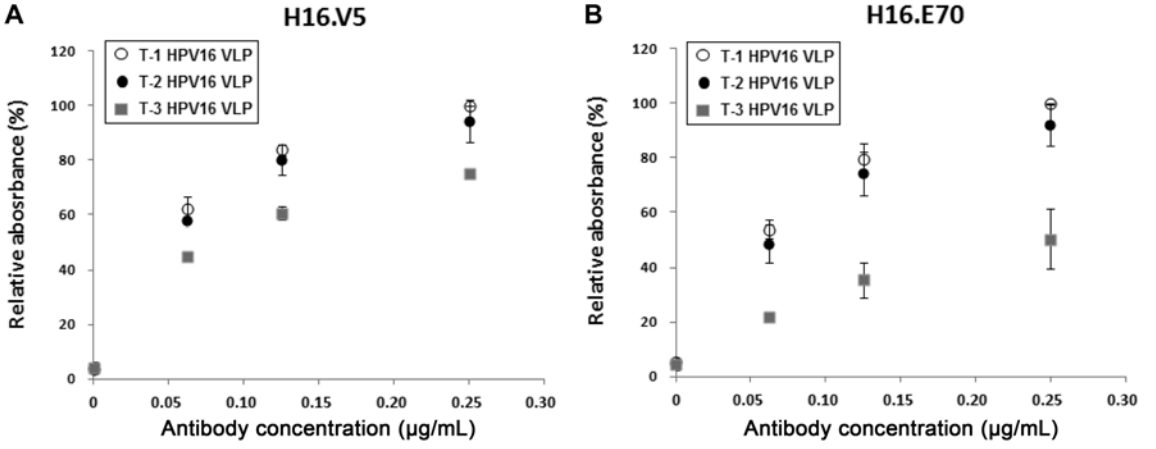

Fig. 5. The reactivity of neutralizing anti-HPV16 Mabs towards T-1, T-2, and T-3 HPV16 VLPs. To investigate the reactivity of Mabs towards the three preparations, 400 ng of VLPs was coated per well, and the coated VLPs were detected using serial dilutions of the Mabs. A and B show the reactivities of H16.V5 and H16.E70 towards the three VLP preparations. Data are mean ± SD of two independent experiments. The optical densities of T-1 HPV16 VLPs reacted with 0.25 μg/mL of Mabs are set at 100% in panels A and B, respectively.

(Fig. 6). The anti-HPV16 L1 IgG titers and neutralizing activities of the mice sera were measured ten days after third immunizations. T-2 HPV16 VLP elicited anti-HPV16 neutralizing antibodies more efficiently than T-1 and T-3 HPV16 VLPs not only in immunization without adjuvant but also in that with adjuvant. On the other hand, T-3 HPV16 VLP less effectively elicited the anti-HPV16 neutralizing antibodies than T-1 and T-2 HPV16 VLP did those (Figs. 6B and 6D). Therefore, it concludes that the method T-2 is most useful to obtain high quality of L1 protein with high yield.

Previous reports have indicated that larger hydrodynamic diameter of HPV VLPs is correlated with lower *in vitro* antigenicity, and that the lower reactivity of H16.V5 and

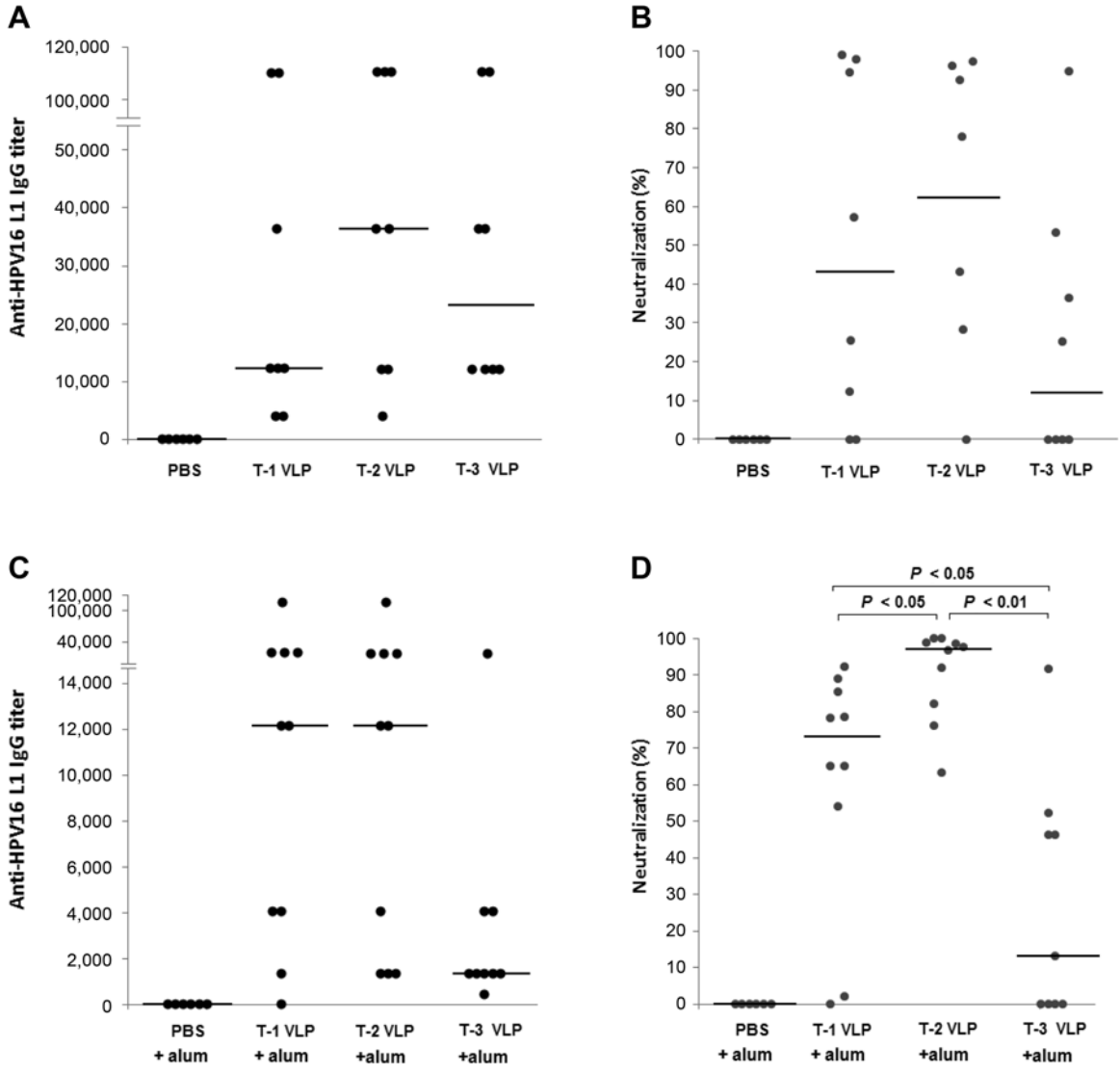

Fig. 6. Humoral immune responses following immunization with T-1, T-2, and T-3 HPV16 VLPs. Anti-HPV16 IgG titers and neutralizing activities were determined by ELISAs and PsV-based neutralization assays after 3rd immunizations, respectively. A and B are the results for anti-HPV16 L1 IgG titers and neutralization activities when the mice were immunized with 50 ng of VLP per dose without adjuvant (PBS, n = 6; T-1 HPV16 VLP, n = 8; T-2 HPV16 VLP, n = 8; T-3 HPV16 VLP, n = 8). C and D are the results for anti-HPV16 L1 IgG titers and neutralization activities when the mice were immunized with 5 ng of VLP per dose, in combination with alum (PBS, n = 6; T-1 HPV16 VLP, n = 10; T-2 HPV16 VLP, n = 10; T-3 HPV16, VLP n = 9). Horizontal solid lines indicate median values, respectively.

H16.E70 towards HPV16 VLP is correlated with reduced ability to elicit anti-HPV16 neutralizing antibodies [21-24]. In this study also, the higher hydrodynamic diameter and lower affinity to H16.V5 and H16.E70 of T-3 HPV16 VLPs were reflected in weaker elicitation of anti-HPV16 neutralization antibodies (Figs. 6B and 6D). Therefore, hydrodynamic diameter and reactivity to Mabs are thought to be useful indicators of the ability of HPV16 VLPs to elicit neutralizing antibodies.

The HPV16 VLP resulting from method T-2 showed the highest immunogenicity and superior structural integrities despite the fact that the method does not include ASP (Figs. 3, 4, 5, and 6). In addition, the final yield of L1 protein resulting from method T-2 was higher than those from methods T-1 and T-3 (Fig. 1E, Table 2). Therefore, we think that T-2 is the best choice not only with respect to the quality of the HPV16 VLPs but also to that of purification efficiency. These results indicate that the structure and immunogenicity of the VLPs are to be superior during the SRPC step. The SRPC step separates the L1 proteins from contaminating proteins and renders samples transparent. On the other hand, the T-3 was less transparent than T-2 prior to CEC. Therefore, it seems that the SRPC preferentially removes contaminants that hinder

the correct assembly of the VLPs. In conclusion, our results suggest that treatments that improve the structure of HPV VLPs, such as SRPC, are important in the manufacture of VLP-based vaccines.

4. Conclusion

In this study, we designed three kinds of downstream processes for HPV16 VLP and compared the structural integrity and immunogenicity of the HPV16 VLPs resulting from these downstream processes. The HPV16 VLPs purified from three kinds of downstream processing showed different yield, structural integrity and immunogenicity. T-2 HPV16 VLP showed superior structural integrity and immunogenicity than T-1 and T-3 HPV16 VLPs, indicating that the step SRPC renders the quality of VLP superior. In summary, we suggest that the process design for purifying HPV VLPs is a critical determinant of structural integrity and immunogenicity, and we anticipate that the optimized downstream processes described above for HPV16 VLP will reduce the delay before an HPV vaccine can be in widespread use.

Acknowledgements

We thank Dr. N. D. Christensen (Pennsylvania State University College of Medicine, USA) for kindly providing the Mabs (H16.V5 and H16.E7). We thank Hye-Lim Kwag and Yingji Jin who supported the animal experiments. This study was supported by grant from the Korean Ministry of Rural Development Administration (Next-Generation BioGreen 21 Programme Nos ADFC2011-PJ008330).

References

1. Morenweiser, R. (2005) Downstream processing of viral vectors and vaccines. *Gene Ther.* 12: 103-110.
2. Vicente, T., A. Roldao, C. Peixoto, M. J. Carrondo, and P. M. Alves (2011) Large-scale production and purification of VLP-based vaccines. *J. Invertebr. Pathol.* 107: 42-48.
3. Bachmann, A. S., G. Corpuz, W. P. Hareld, G. Wang, and B. A. Coller (2004) A simple method for the rapid purification of copia virus-like particles from Drosophila Schneider 2 cells. *J. Virol. Methods* 115: 159-165.
4. Pattenden, L. K., A. P. Middelberg, M. Niebert, and D. I. Lipin (2005) Towards the preparative and large-scale precision manufacture of virus-like particles. *Trends Biotechnol.* 23: 523-529.
5. Madrid-Marina, V., K. Torres-Poveda, G. Lopez-Toledo, and A. Garcia-Carranca (2009) Advantages and disadvantages of current prophylactic vaccines against HPV. *Arch. Med. Res.* 40: 471-477.
6. Conway, M. J. and C. Meyers (2009) Replication and assembly of human papillomaviruses. *J. Dent. Res.* 88: 307-317.
7. Seo, P. S., S. Y. Heo, E. J. Han, J. W. Seo, S. J. Ghim, and C. H. Kim (2009) Bacterial expression and purification of human papillomavirus type 18 L1. *Biotechnol. Bioproc. Eng.* 14: 168-174.
8. National Cancer Institute, Human Papillomavirus (HPV) Vaccines, http://www.cancer.gov/cancertopics/factsheet/prevention/HPV-vaccine.
9. Padmanabhan, S., T. Amin, B. Sampat, R. Cook-Deegan, and S. Chandrasekharan (2010) Intellectual property, technology transfer and manufacture of low-cost HPV vaccines in India. *Nat. Biotechnol.* 28: 671-678.
10. Walsh, G. (2010) Biopharmaceutical benchmarks 2010. *Nat. Biotechnol.* 28: 917-924.
11. National Cancer Institute (2007) Women's Health Report, Fiscal Years 2005-2006. NCI Women's Health Report FY2005-2006.
12. Kim, H. J., S. Y. Kim, S. J. Lim, J. Y. Kim, S. J. Lee, and H. -J. Kim (2010) One-step chromatographic purification of human papillomavirus type 16 L1 protein from *Saccharomyces cerevisiae*. *Protein Expr. Purif.* 70: 68-74.
13. Kim, H. J., S. J. Lee, and H. -J. Kim (2010) Optimizing the secondary structure of human papillomavirus type 16 L1 mRNA enhances L1 protein expression in *Saccharomyces cerevisiae*. *J. Biotechnol.* 150: 31-36.
14. Kim, H. J., H. L. Kwag, Y. Jin, and H. -J. Kim (2011) The composition of the carbon source and the time of cell harvest are critical determinants of the final yield of human papillomavirus type 16 L1 protein produced in *Saccharomyces cerevisiae*. *Protein Expr. Purif.* 80: 52-60.
15. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
16. Kim, S. Y., H. J. Kim, and H. -J. Kim (2011) Simple and convenient chromatography-based methods for purifying the pseudovirus of human papillomavirus type 58. *Protein Expr. Purif.* 76: 103-108.
17. Mukherjee, S., M. V. Thorsteinsson, L. B. Johnston, P. A. DePhillips, and A. Zlotnick (2008) A quantitative description of *in vitro* assembly of human papillomavirus 16 virus-like particles. *J. Mol. Biol.* 381: 229-237.
18. Thones, N., A. Herreiner, L. Schadlich, K. Piuko, and M. Muller (2008) A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than viruslike particles with respect to the induced antibody response. *J. Virol.* 82: 5472-5485.
19. Kim, S. N., H. S. Jeong, S. N. Park, and H. -J. Kim (2007) Purification and immunogenicity study of human papillomavirus type 16 L1 protein in *Saccharomyces cerevisiae*. *J. Virol. Methods* 139: 24-30.
20. Kim, H. J., J. K. Kim, S. B. Seo, H. J. Lee, and H. J. Kim (2007) Intranasal vaccination with peptides and cholera toxin subunit B as adjuvant to enhance mucosal and systemic immunity to respiratory syncytial virus. *Arch. Pharm. Res.* 30: 366-371.
21. Shi, L., G. Sanyal, A. Ni, Z. Luo, S. Doshna, B. Wang, T. L. Graham, N. Wang, and D. B. Volkin (2005) Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. *J. Pharm. Sci.* 94: 1538-1551.
22. Culp, T. D. and N. D. Christensen (2004) Kinetics of *in vitro* adsorption and entry of papillomavirus virions. *Virol.* 319: 152-161.
23. Ryding, J., L. Dahlberg, M. Wallen-Ohman, and J. Dillner (2007) Deletion of a major neutralizing epitope of human papillomavirus type 16 virus-like particles. *J. Gen. Virol.* 88: 792-802.
24. Christensen, N. D., N. M. Cladel, C. A. Reed, L. R. Budgeon, M. E. Embers, D. M. Skulsky, W. L. McClements, S. W. Ludmerer, and K. U. Jansen (2001) Hybrid papillomavirus L1 molecules assemble into virus-like particles that reconstitute conformational epitopes and induce neutralizing antibodies to distinct HPV types. *Virol.* 291: 324-334.