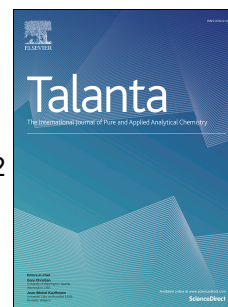


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Interaction studies between human papillomavirus virus-like particles and laminin 332 by affinity capillary electrophoresis assisted by bio-layer interferometry

Aurore Boclinville, Marylène Vandevenne, Ernesto Ambroggio, Nicolas Thelen, Marc Thiry, Nathalie Jacobs, Alain Brans, Marianne Fillet, Anne-Catherine Servais



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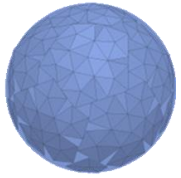
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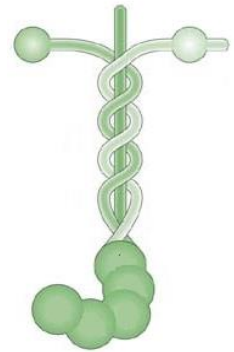
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HPV16-VLP



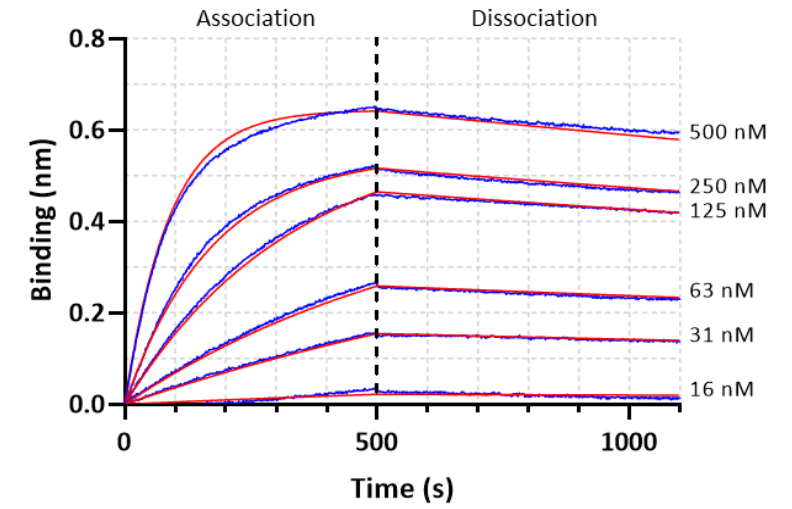
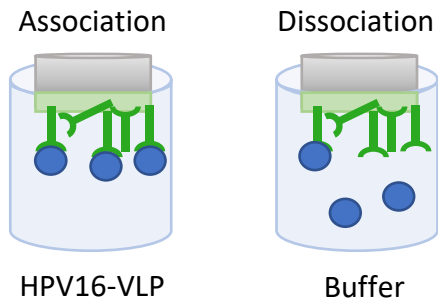
BLI

BINDING STUDY

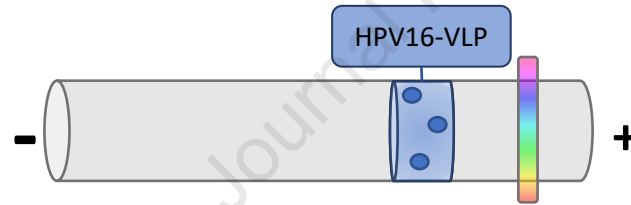
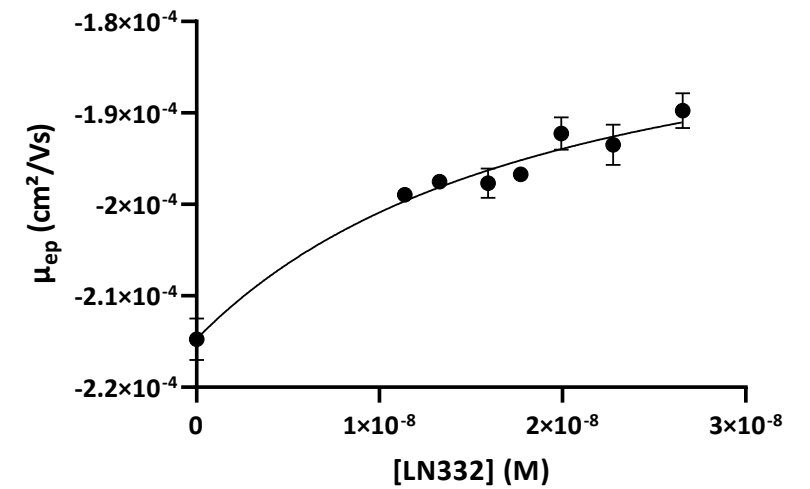
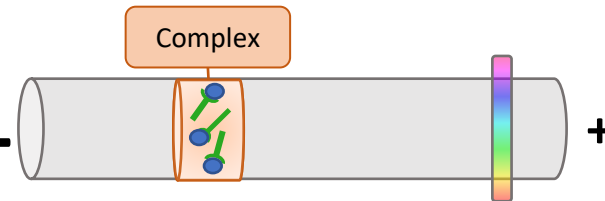


LN332

ACE



HPV16-VLP

HPV16-VLP
preincubated
with LN332

Interaction studies between human papillomavirus virus-like particles and laminin 332 by affinity capillary electrophoresis assisted by bio-layer interferometry

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Abbreviations

ACE, affinity capillary electrophoresis; APS, aminopropylsilane; BGE, background electrolyte; BLI, bio-layer interferometry; ECM, extracellular matrix; HPV, human papillomavirus; HSPG, heparan sulfate proteoglycan; K_D , equilibrium dissociation constant; k_{off} , dissociation rate constant; k_{on} , association rate constant; LN, laminin; M, internal marker; VLP, virus-like particle.

Abstract

Human papillomavirus (HPV) interacts, *in vitro*, with laminin 332 (LN332), a key component of the extracellular matrix. In this study, we performed bio-layer interferometry (BLI) and affinity capillary electrophoresis (ACE) to investigate the binding properties of this interaction. Virus-like particles (VLPs), composed of the HPV16 L1 major capsid protein, were used as HPV model and LN332 as the VLPs binding partner. Using BLI, we quantitatively determined the kinetics of the interaction, via the measurement of VLP binding and release from LN332 immobilized onto the surface of aminopropylsilane biosensors. We found an averaged k_{on} of $1.74 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and an averaged k_{off} of $1.50 \times 10^{-4} \text{ s}^{-1}$. Furthermore, an ACE method was developed to study the interaction under physiological conditions, where the interactants are moving freely in solution, without any fluorescence labeling. Specifically, a constant amount of HPV16-VLPs was preincubated with increasing LN332 concentrations and then the samples were injected in the capillary electrophoresis instrument. A shift in the migration time of the HPV16-VLP / LN332 complexes, carrying an increasing number of LN332 molecules bound per VLP, was observed. The mobility of the complexes was found to decrease with increasing LN332 concentrations in the sample. It was used to quantify stability constant. From BLI and ACE approaches, we reported an apparent equilibrium dissociation constant in the nanomolar range (8.89 nM and 17.7 nM, respectively) for the complex between HPV16-VLPs and LN332.

Keywords

Human papillomavirus; Virus-like particles; Laminin 332; Dissociation constant; Affinity capillary electrophoresis; Bio-layer interferometry

1. Introduction

Human papillomavirus (HPV) infection is a necessary cause of cervical cancer, which is the fourth leading cause of cancer death, with 604,000 new cases and 342,000 deaths worldwide in 2020 [1]. Papillomaviruses are non-enveloped, circular, double-stranded DNA viruses of approximately 55 nm in diameter. Their capsid is composed of two proteins, the major capsid protein L1 (~ 55 kDa) and the minor capsid protein L2 (~ 50 kDa). It contains 360 molecules of L1 monomer arranged into 72 pentamers forming a $T = 7$ icosahedral lattice. The number of copies of the minor capsid protein L2 is undetermined but it is estimated to be up to 72 molecules per capsid [2]. More than 200 different HPV types have been identified [3]. The most common HPV types detected in biopsy samples are HPV16 and HPV18. Together they are responsible for approximately 70 % of cervical cancers [4, 5].

HPV requires the presence of a microwound that allows the virion to infect the mitotically active basal cells in the lowest layers of the epithelium. The virus depends on the differentiation of the epithelium to produce its capsid proteins, L1 and L2. The receptor(s) implicated in HPV binding and entry into the host cells has (have) not been fully elucidated, even though several possible candidates have been identified [6, 7]. It has been shown that HPV16 requires the binding to heparan sulfate, located on heparan sulfate proteoglycans (HSPGs) either expressed on the surface of keratinocytes or residing in the basement membrane [8]. With cell culture, it was demonstrated that HPV preferentially binds to components of the extracellular matrix (ECM) by immunocytochemistry [9, 10]. It is worth noting that, in cell culture models, the ECM is used to mimic the basement membrane. It has also been shown by immunohistochemistry that HPV16 might initially interact with laminin 332 (LN332, formerly laminin 5), a key ECM component secreted by basal keratinocytes [11]. Since HSPGs are ubiquitously expressed through all layers of the epithelium, this might contribute to the anatomical-site specificity [12]. The interaction between HPV16-VLPs and LN332 was confirmed by ELISA [10]. Nevertheless, the equilibrium dissociation constant (K_D) value relative to the binding with the individual component LN332 was not yet reported. Laminins are major basement membrane proteins being multidomain heterotrimers composed of alpha (α), beta (β) and gamma (γ) polypeptide chains. The name of laminins is linked to their composition in α , β and γ chains. This means that LN332 is composed of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains [13].

In vitro HPV production requires a very complex cell culture system in order to mimic the epithelium as the viral cycle is keratinocyte differentiation-dependent. Moreover, it does not

allow to obtain large quantities of virus. For these reasons, most studies on papillomavirus use virus-like particles (VLPs) as a model. HPV-VLPs, isolated from recombinant protein expression system, result from the self-assembly of only L1 protein or L1 and L2 proteins. These particles are morphologically and immunologically like native virions [14].

Traditional binding assays between viruses and ligands, including enzyme-linked immunosorbent assay (ELISA), isothermal titration calorimetry and flow cytometry, have drawbacks such as long analysis times, large quantities of purified binding partners, low throughput and some of them require additional fluorescent labeling steps [15]. In addition, labeling may change the conformation of the biomolecule or alter the binding interface due to steric hindrances and therefore may interfere with the biological activity of the molecule.

Affinity capillary electrophoresis (ACE) is particularly interesting to study biomolecular interactions because it relies on the modification of the charge-to-size ratio of an analyte upon its interaction with the ligand. Moreover, ACE allows the analysis of interactions in solution and does not require any attachment of the entities on a solid support, contrary to immunoassays, such as ELISA. Therefore, each available binding site is accessible. In ACE, several configurations are available, depending on the interaction to be studied (reviewed in [16]). Nevertheless, they can be divided into two major groups. In brief, if the analyte and the ligand have a high affinity, equilibrium-mixture analysis can be employed. In this case, the analyte is preincubated with increasing concentrations of the ligand until the equilibrium is achieved and then a part of the sample is injected. On the contrary, if the affinity between the analyte and the ligand is low, mobility-shift analysis is employed. In this second case, the ligand is added to the background electrolyte (BGE) at different concentrations and the analyte is injected. The K_D can be determined by plotting the analyte migration time as a function of the ligand concentration in the BGE [17]. ACE is a widely used technique for investigating complex formation [18]. However, there are few publications concerning the study of interactions with viruses in ACE [17, 19–21]. The studied viruses were human rhinovirus and poliovirus. To the best of our knowledge, the interaction of VLPs with a receptor using ACE has never been investigated.

Bio-layer interferometry (BLI) is another label-free method widely used to determine the dynamics of macromolecular interactions. Briefly, BLI is an optical analytical technique that measures the change of a biolayer thickness at the tip of a biosensor. It monitors in real-time the interaction between two different components: the ligand, which is immobilized onto the

surface of the biosensor; and the analyte, the binding partner that is free in solution [22]. In addition to the K_D , BLI also allows determining the binding kinetics of the studied interaction (the values of the association and dissociation rate constants, k_{on} and k_{off} , respectively). Recently, the interaction of HPV11-VLPs or HPV18-VLPs with RNA was studied using BLI [23].

The present study aims to quantitatively determine the binding properties of HPV16-VLPs to LN332. Figure 1 presents an overview of the size and shape of the HPV16-VLPs and LN332. As shown in this Figure, LN332 is two times longer than HPV16-VLPs. The kinetics and binding affinity of the interaction were first studied by BLI. An ACE methodology was then developed to study the interaction under physiological conditions without the need of immobilization for one of the binding partners and without fluorescence labeling. To overcome sensitivity issues in CE which usually require the derivatization of one interacting species for the determination of dissociation constants in the nanomolar range, a method proposed by S. Kiessig et al. was used [24]. In this method, the analyte is preincubated with various concentrations of the ligand before the injection and variations in the electrophoretic mobility of the HPV16-VLP / LN332 complexes are followed. The outcome of our study is the determination of the binding constant and kinetics of the HPV16-VLP / LN332 complex.

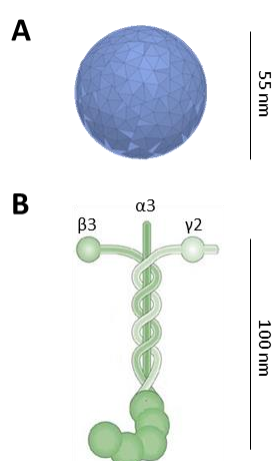


Figure 1. Schematic representation of HPV16-VLP (A) and LN332 (B).

2. Materials and methods

2.1. Chemicals and reagents

Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), poly(ethylene oxide) (PEO) (MW 200,000 g mol⁻¹), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane-sulfonic acid sodium salt (HEPES-Na), sodium citrate, phthalic acid, polysorbate 80 (Tween[®] 80) 10 % solution, *N,N*-dimethylformamide (DMF) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (Saint-Louis, MO, USA). Hydrochloric acid fuming 37 % (HCl), ortho-phosphoric acid 85 % (H₃PO₄), sodium chloride (NaCl), polyethylene glycol (PEG) 6000 and glycine were provided by Merck (Darmstadt, Germany). Sodium hydroxide (NaOH), disodium hydrogen phosphate, sulfuric acid 95-98 % and polysorbate 20 (Tween[®] 20) were purchased from VWR (Leuven, Belgium). Sodium dodecyl sulfate (SDS) was obtained from Fisher Scientific (Loughborough, United Kingdom). Ethylenediaminetetraacetic acid (EDTA) was acquired from Acros Organics (New Jersey, USA). Laminin 332 (LN332) 0.1 mg mL⁻¹ and laminin 211 (LN211) 0.1 mg mL⁻¹ were sourced by Bio-Connect (Huissen, The Netherlands). Phosphate buffered saline (PBS) was provided by Lonza (Bâle, Switzerland). 96-well plates Nunc Maxisorp were obtained from Thermo Fisher Scientific (Waltham, USA). Ultra-pure water was supplied by a Milli-Q equipment (Millipore, Bedford, MA, USA) and Chromafil[®] Xtra PVDF syringe filters (0.20 µm) were from Macherey-Nagel (Düren, Germany). Rabbit anti-mouse HRP antibody was obtained from Dako (Glostrup, Denmark). HPV16 L1 conformational antibody supernatant was kindly provided by Prof. M. Müller (German Cancer Research Center (DKFZ), Heidelberg, Germany). HPV-VLP based vaccine, Gardasil[®]9 from Merck & Co. (Lyon, France), was also used.

2.2. HPV-VLP samples

HPV16-VLPs were produced using the baculovirus-insect cell expression system. SF21 insect cells (Thermo Fischer Scientific) were infected by a recombinant baculovirus carrying the L1 gene of HPV16. These cells were then harvested and HPV16-VLPs were purified on a cesium chloride gradient following the procedure described in [14]. HPV16-VLPs were finally resuspended in 150 mM NaCl and 0.01 % Tween[®] 80. L1 protein was quantified using a micro-BCA protein assay kit obtained from Thermo Fisher Scientific (Waltham, USA). HPV16-VLP samples were characterized using different techniques. HPV16-VLP conformation was checked by transmission electron microscope (cf. Supplementary Figure S1). For this purpose, HPV16-VLPs were applied on a carbon film grid (Laborimpex, Brussel, Belgium) for 2 min and negatively stained with 1.5 % ethanolic uranyl acetate for 2 min. To confirm HPV16-VLP

conformation, an ELISA was performed with a conformational antibody following the protocol described by Bettonville et al. [25] (cf. Supplementary Figure S2).

2.3. Bio-layer interferometry

BLI experiments were performed using an Octet HTX instrument (Sartorius) available at the Robotein facility which is part of the BE- Instruct ERIC Centre. All assays were carried out in a 384-well microplate (Greiner Bio-One), with a final volume of 80 μ L per well. All the binding assays were performed at 30 °C. For all experiments, aminopropylsilane (APS) biosensors (Sartorius) were used for the immobilization of LN332 via non-specific interactions (weak hydrophobic and electrostatic interactions). The APS biosensors were hydrated in PBS for at least 10 min before use. Prior to all measurements, the biosensors were cleaned by deeping the sensors into a 10 mM glycine buffer (pH 1.5) for 30 s and then into PBS for 30 s. This “regeneration” cycle was performed 5 times. A baseline step of 90 s was then performed in PBS. Next, purified LN332 was loaded onto the biosensors for 90 or 180 s using different LN332 concentrations from 1.25 to 2.5 μ g mL⁻¹. A baseline was then monitored for 120 s in PBS buffer. The biosensors were then saturated using an HBSt-BSA solution buffer (10 mM HEPES-Na pH 7.6, 150 mM NaCl, 3 mM EDTA, 0.005 % Tween[®] 20, 0.5 % BSA) for 120 s to prevent non-specific binding. The binding against the HPV16 L1 protein-based viral particles was recorded for 240 to 500 s using different HPV16 L1 protein concentrations ranging from 16 nM to 1500 nM (diluted in HBSt-BSA). The dissociation was recorded in HBSt-BSA solution buffer for 600 s which corresponds to a period of time sufficient to dissociate 5% of formed complex (based on the determined k_{off} value). Longer dissociation periods were also applied (up to 1800 s) but it did not lead to significant changes in the k_{off} value.

Recorded data were corrected by subtracting the signals from a reference sensor immobilized with LN332 but without any analyte (HBSt-BSA instead of HPV16 L1 proteins). All the data were analyzed using the Octet Data Analysis HT software version 12.0, considering the analyte concentration as the HPV16 L1 protein concentration and fitted using a 1:1 interaction model and a global fit. Therefore, a single set of kinetic parameters was obtained for all tested concentrations by nonlinear least-squares fitting.

2.4. Affinity capillary electrophoresis

2.4.1. Instrumentation

All capillary electrophoresis (CE) experiments were performed on a Hewlett Packard ^{3D} Capillary Electrophoresis system (Agilent Technologies Waldbronn, Germany) operated by

Agilent OpenLab CDS C.01.07 software. The instrument is equipped with an autosampler and a temperature control system ($15 - 60\text{ }^{\circ}\text{C} \pm 0.1\text{ }^{\circ}\text{C}$). Bare fused-silica capillaries with an internal diameter of $50\text{ }\mu\text{m}$ were purchased from Polymicro Technologies (Phoenix, AZ, USA). The total and the effective lengths of the capillary were 48.5 cm and 8.5 cm , respectively. The capillary was dynamically coated with PEO according to the procedure described below.

2.4.2. PEO coating procedure

The PEO coating procedure used was previously reported in [26, 27]. Each new capillary was first conditioned by successive rinses with H_2O , 1 M NaOH , 0.1 M NaOH and H_2O (5 min each). The capillary was coated by flushing with H_2O (5 min), 1 M NaOH (5 min), 1 M HCl (10 min), H_2O (10 min), $0.2\text{ }\%$ PEO (5 min) and H_2O (5 min). Each day, the dynamic coating was regenerated by successive flushes with H_2O (5 min), 1 M HCl (5 min), $0.2\text{ }\%$ PEO (5 min). Moreover, PEO coating was regenerated before each run by flushes with H_2O (3 min), 1 M HCl (3 min), $0.2\text{ }\%$ PEO (5 min) and BGE (2 min). Flushes were performed at a pressure of approximately 1 bar at the inlet vial. The $0.2\text{ }\%$ PEO solution was prepared in water containing 0.1 M HCl .

2.4.3. Electrophoretic conditions

Experiments were performed using the outlet injection mode. Samples were injected hydrodynamically by applying a pressure of -50 mbar during 15 s . The separation was achieved using a voltage of 10 kV (normal polarity mode) and the detection was carried out using an on-column diode-array detector set at 280 nm . During the analysis, the capillary cassette was thermostated at a temperature of $15\text{ }^{\circ}\text{C}$ by high-velocity air stream.

2.4.4. Preparation of buffer and samples

The BGE was composed of 0.01 M Tris HCl , 0.01 M HEPES-Na , 0.1 M NaCl and $0.1\text{ }\%$ PEG 6000, $\text{pH } 7.4$ (adjusted with $0.1\text{ M H}_3\text{PO}_4$) containing 1.5 mM SDS .

For all experiences, all mixtures were prepared with a constant amount of HPV16-VLP (2.19 nM) and different concentrations of laminin. Before the experiment, HPV16-VLP production was diluted with 150 mM NaCl and $0.01\text{ }\%$ Tween[®] 80 solution. LN332 and LN211 were diluted with PBS to the required concentration. Phthalic acid was added to each mixture at a final concentration of 0.1 mg mL^{-1} as internal marker (M). The mixtures were prepared in the following proportions: $2\text{ }\mu\text{L}$ phthalic acid solution, $8\text{ }\mu\text{L}$ HPV16-VLP solution and $10\text{ }\mu\text{L}$ laminin solution. All mixtures were incubated 1 hour at room temperature before CE analysis, except for the time course of complex formation where the mixtures were incubated from 15 to

240 min. An HPV16-VLP standard was prepared with 10 μ L PBS instead of laminin solution in the same conditions.

For the LN332 matrix effect experiment, an HPV16-VLP matrix sample was performed by replacing the laminin solution by 10 μ L of a 10 % glycerol and 0.02 % sodium azide solution and a blank replacing the HPV16-VLP solution by 8 μ L of a 150 mM NaCl and 0.01 % Tween[®] 80 solution. As negative control, mixture was prepared with a 12-fold molar excess of LN211 instead of LN332. LN211 standard was prepared by replacing the HPV16-VLP by 8 μ L of 150 mM NaCl and 0.01 % Tween[®] 80 solution. LN332 standards were prepared by replacing the VLP solution by 8 μ L of 150 mM NaCl and 0.01 % Tween[®] 80 solution. To study the complex formation, HPV16-VLP solution was incubated with a 5- to 12-fold molar excess of LN332. Each incubated sample was injected three times.

2.4.5. Determination of K_D

The K_D of HPV16-VLPs / LN332 complex was determined according to Equation (1) reported by Kiessig et al [24] :

$$S_{obs} = S_0 + \frac{(S_{max}-S_0)}{[A]_0} \cdot \left\{ \frac{([A]_0+[B]_0+K_D)}{2} - \sqrt{\frac{([A]_0+[B]_0+K_D)^2}{4} - ([A]_0 \cdot [B]_0)} \right\} \quad (\text{Eq. 1})$$

where S_{obs} is the response (i.e. the electrophoretic mobility of the complex in this study) caused by a certain concentration of B in the sample. The other reactant A was used at a fixed concentration. S_{max} is the maximum electrophoretic mobility achievable by the addition of B to the incubation mixture. The values for K_D and S_{max} are determined simultaneously by nonlinear regression using GraphPad Prism[®] version 8 software (GraphPad Software Inc., La Jolla, CA).

The electrophoretic mobility (μ_{ep}) of the complex was calculated considering the electroosmotic flow following the Equation (2):

$$\mu_{ep} = - \left\{ \frac{L \cdot l}{V} \cdot \left(\frac{1}{M_t} + \frac{1}{M_{teo}} \right) \right\} \quad (\text{Eq. 2})$$

Where L is the capillary total length, l the capillary effective length, M_t the migration time of the complex and M_{teo} the migration time of the electroosmotic flow. M_{teo} was determined by reversing the polarity (i.e. – 10 kV) and injecting a sample of 0.05 % DMF solution. The sample of DMF was injected at the beginning and at the end of the sequence relative to the analysis of the different incubated mixtures for K_D determination. M_{teo} was the averaged migration time of the DMF peak.

3. Results and discussion

3.1. HPV16-VLPs / LN332 interaction study by BLI

Binding assays were carried out using BLI. Ligand (LN332) immobilization was performed on APS biosensors via noncovalent hydrophobic and/or electrostatic interactions (Figure 2A).

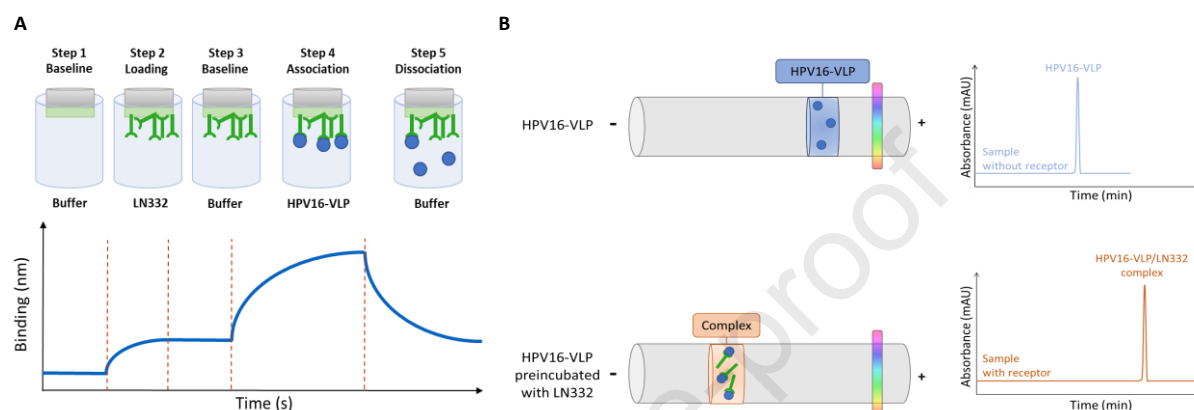


Figure 2. Setup of the bio-layer interferometry binding experiments (A). APS sensors were washed (step 1, baseline) in PBS buffer, incubated with LN332 (step 2, loading), washed again (step 3, baseline) in PBS and HBSt-BSA buffer, incubated with HPV16-VLPs (step 4, association), followed by a wash (step 5, dissociation) in HBSt-BSA buffer. Set-up of the ACE method (B). HPV16-VLPs and LN332 are preincubated. The mixture is then analyzed by CE. Electrophoretic mobility shift is then used to quantify the interaction.

Figure 3 shows the sensorgrams obtained for the determination of the binding kinetics and affinity parameters. These parameters were determined using binding curves obtained with six HPV16 L1 concentrations (Figure 3, blue lines). The data were fitted assuming a 1:1 model (Figure 3, red lines) and a global fit mode allowing to calculate the binding kinetics (k_{on} and k_{off}) and binding affinity (K_D).

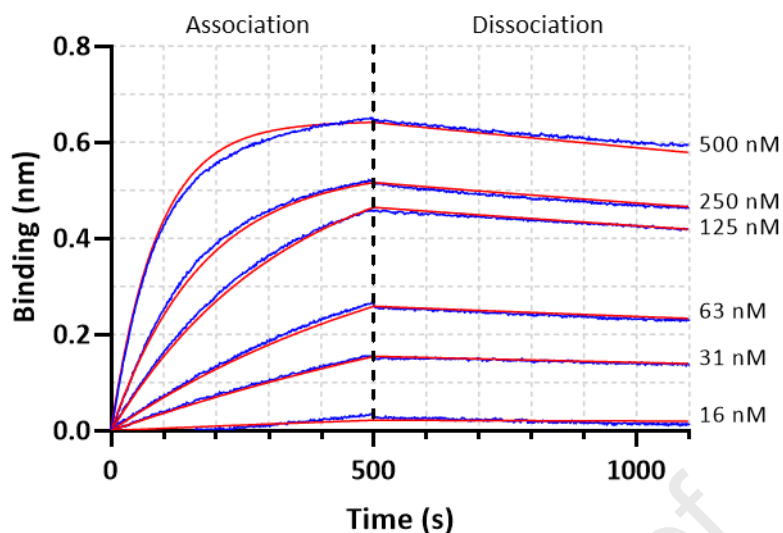


Figure 3. Binding kinetics of HPV16-VLPs to LN332. Association and dissociation of the complex was monitored from 0 to 500 s and from 500 to 1100 s, respectively. The HPV16 L1 concentration window used for this assay was comprised between 16 nM and 500 nM. Blue lines represent experimental data and red lines correspond to the fitted model used to calculate the binding parameters. A 1:1 model ($R^2 > 0.99$) provided a K_D value of $7.51 (\pm 0.03)$ nM.

It should be noted that the experiments were performed four times, varying the LN332 concentration and loading time as well as the HPV16 L1 concentration and association time (See Supplementary Figure S3 for the sensorgrams obtained for these experiments). As shown in Table 1, the results of all assays are consistent. The binding between HPV16-VLPs and LN332 was found to have an averaged k_{on} of $1.74 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and an averaged k_{off} of $1.50 \times 10^{-4} \text{ s}^{-1}$. The binding affinity, K_D value, was found to be in the nanomolar range (averaged K_D based on the four experiments is of 8.89 nM).

The K_D was also calculated non-kinetically with BLI using the extrapolated equilibrium response values. In this case, the K_D value obtained was 23.5 nM (for the data set shown in Figure 3), which is significantly higher compared to the one measured using the kinetic data (7.51 nM). Furthermore, we can also observe an apparent low association rate when the analyte concentration is 16 nM (Figure 3, which is a concentration equivalent to twice the K_D considering the kinetic data). At this concentration, if the K_D is 7.51 nM, we should observe a much faster association kinetic. This indicates that, in the context of this work, BLI overestimates our binding affinity when using the kinetic data. It is very likely that this overestimation comes from the fact that we use a multivalent analyte: the HPV-VLPs which can interact with neighboring LN332 molecules that are immobilized onto the biosensors and can

297 also re-associate during the dissociation phase. Indeed, this is a well-documented phenomenon
298 that occurs with any surface-based technique (like BLI and surface plasmon resonance). These
299 methods are vulnerable when using multivalent binders because it artefactually measures slow
300 off-rates (k_{off}), due to re-binding events that can occur in a way that depends on the
301 immobilisation density [28]. Therefore, the underestimation of the k_{off} leads to an
302 underestimation of the K_D ($K_D = k_{off}/k_{on}$).

Table 1. BLI results for HPV-VLPs/ LN332 interaction.

Loading time	[LN332]	[HPV16 L1]	k_{on}^*	k_{off}^*	$K_D \pm SE$	R^2
(s)	($\mu\text{g mL}^{-1}$)	(nM)	($10^4 \text{ M}^{-1}\text{s}^{-1}$)	(10^{-4} s^{-1})	(nM)	
180	2.5	[125 – 1500]	1.14	1.22	10.72 ± 0.03	0.9996
90	2.5	[31 – 750]	1.45	1.29	8.95 ± 0.06	0.9973
90	1.25	[16 – 500]	2.09	1.76	8.40 ± 0.03	0.9988
90	1.25	[16 – 500]	2.29	1.72	7.51 ± 0.03	0.9987

* All standard errors (SEs) < 0.01

3.2. HPV16-VLPs / LN332 interaction study by ACE

3.2.1. Set-up of the ACE method

The separation in ACE is based on the modification of the electrophoretic mobility due to the specific interaction of an analyte with a ligand. Equilibrium-mixture analysis is generally used for the investigation of high-affinity interactions ($K_D = 10^{-12} - 10^{-9}$ M), while mobility-shift assay is performed to study weak to moderate interactions ($K_D = 10^{-8} - 10^{-4}$ M) [29]. Based on the K_D determined by BLI, the interaction can be considered as high. To study interactions with dissociation constants below the micromolar range, LIF detection is generally used for sensitivity reasons [30, 31]. In the present study, it would require the derivatization of LN332, with the risk of modifying the binding affinity between HPV16-VLPs and LN332. Therefore, to study the interaction under physiological conditions, the method proposed by S. Kiessig et al. based on the usual set-up of equilibrium-mixture was applied [24]. This approach was also used in several other studies [32–35]. A constant amount of HPV16-VLPs was preincubated with increasing LN332 concentrations before the CE analysis of the mixtures. Once injected, the electrophoretic mobility of the complexes was followed, as illustrated in Figure 2B.

Mixtures of HPV16-VLP and LN332 were analyzed in CE using the method previously developed by V. Bettonville et al. for the study of intact HPV-VLPs [27]. Briefly, this method is based on the use of a PEO-coated capillary and a low SDS concentration in the BGE (1.5 mM SDS) to avoid particle adsorption onto the capillary wall and aggregation providing a reproducible HPV-VLP analysis. The short-end injection mode together with a voltage of 10 kV were also used to perform the analysis of the complexes in short analysis time and under a relatively low current (43 μ A), allowing the complex to stay intact during the electrophoresis. Moreover, it was highlighted in a previous study that HPV16-VLP electrophoretic mobility is influenced by their concentration as long as the latter is higher than 50 μ g mL⁻¹ [25]. Therefore, HPV16-VLPs were diluted to reach a final concentration in the mixtures of 43 μ g mL⁻¹ (corresponding to 2.19 nM HPV16-VLPs). Indeed, as variations in the electrophoretic mobility are monitored, it is necessary to work with particle concentration for which the migration time is not concentration dependent.

Under these conditions, LN332 was found to migrate towards the anode, as it is the case for HPV16-VLPs. Figure 4 shows the electropherograms of a sample of HPV16-VLPs (trace A) and a sample of LN332 (trace F). As LN332 was not detectable in the concentration range used for the assay, it was analyzed at a high concentration (trace F) to identify the migration window.

Indeed, the migration window between HPV16-VLPs and LN332 is important to study their interaction. Upon complex formation, changes in size, shape, and charge compared to the free compounds can occur and the expected mobility of the complex is intermediate to the mobilities of the reacting species [29].

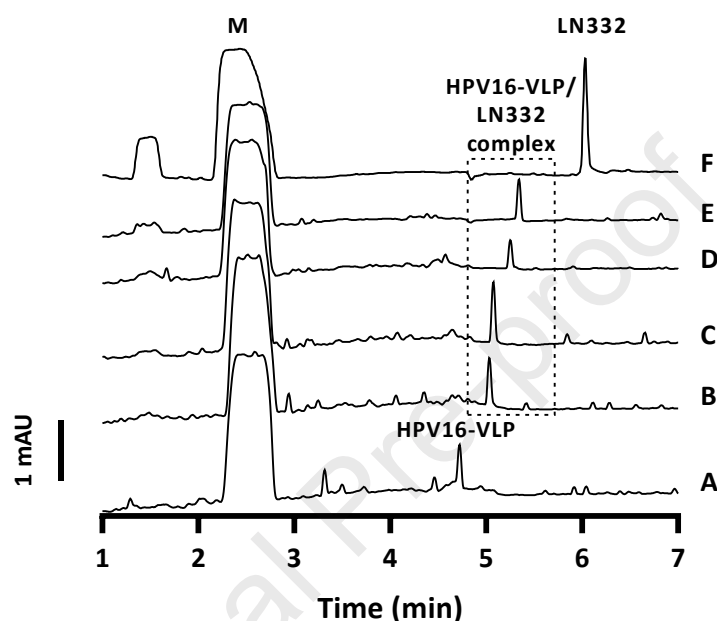


Figure 4. Formation of complexes between HPV16-VLPs and LN332. CE analysis of HPV16-VLPs at 2.19 nM (A) incubated with 11.38 (B); 13.28 (C); 19.92 (D); 22.76 nM (E) of LN332 and CE analysis of LN332 at 153 nM (F). Separation conditions: BGE, 0.01 M Tris HCl, 0.01 M HEPES-Na, 0.1 M NaCl and 0.1 % PEG 6000, pH 7.4 buffer containing 1.5 mM SDS; 48.5 cm (8.5 cm to the detector) x 50 μ m PEO-coated capillary; voltage + 10 kV; detection at 280 nm; pressure injection for 15 s at - 50 mbar; T = 15 °C. Other conditions as described in Section 2.

3.2.2. Kinetics

In equilibrium-mixture analysis, the analyte and the ligand form a complex before the CE analysis. After the injection of the mixture in the capillary filled with the BGE that does not contain any ligand, the dissociation of the complex is avoided if the k_{off} is lower than $[\ln(100/90)]/t$, where t is the time required to separate peaks. In this way, less than 10 % of the bound ligand dissociates during the CE separation [36]. Considering a k_{off} of $1.50 \times 10^{-4} \text{ s}^{-1}$ (obtained from BLI experiments), the separation time must be smaller than 12 min, which is

the case since the migration time of the last migrating compound (i.e. LN332) was about 6 min. Thus, the dissociation of the complex can be considered negligible in our set-up.

3.2.3. LN332 matrix effect

For the interaction study, it is worth noticing that HPV16-VLP stock suspension was diluted in 0.15 M NaCl and 0.01 % Tween[®] 80, while LN332 stock solution was diluted in PBS. To keep the sample matrix constant, all mixtures contained the same volumes of the solution of phthalic acid (used as internal marker), the HPV16-VLP suspension (at a constant concentration of 2.19 nM) and the LN332 solution (concentrations ranging from 11 to 27 nM). The matrix of the LN332 stock solution is made up of PBS, 10 % glycerol and 0.02 % sodium azide. To verify that the LN332 matrix does not influence the migration time of the HPV16-VLPs, an HPV16-VLP standard (containing the same volumes of internal marker, HPV16-VLP suspension and PBS as in the mixtures) and an HPV16-VLP matrix sample (prepared in the same way as for the standard but replacing PBS by LN332 matrix) were analyzed. Both samples showed comparable migration times, demonstrating that the LN332 matrix does not influence the migration time of HPV16-VLPs (See Supplementary Figure S4).

3.2.4. Specificity of the method

T. Culp et al. showed that HPV11-VLPs failed to bind to laminin 211 (LN211, formerly laminin 2) [11]. LN211 has the same general structure as LN332 but has different α , β and γ chains (review in [37]). LN211, which is mainly expressed in muscle and peripheral nerve, was used in this study as an irrelevant receptor. Figure 5 shows the electropherograms of an LN211 standard sample (trace A), of HPV16-VLP standard sample (trace B), and HPV16-VLP sample incubated with LN211 (trace C). As shown in this figure, incubation of HPV16-VLPs with LN211 did not induce changes in the migration time of HPV16-VLP peak. This experiment illustrates the specificity of the developed method.

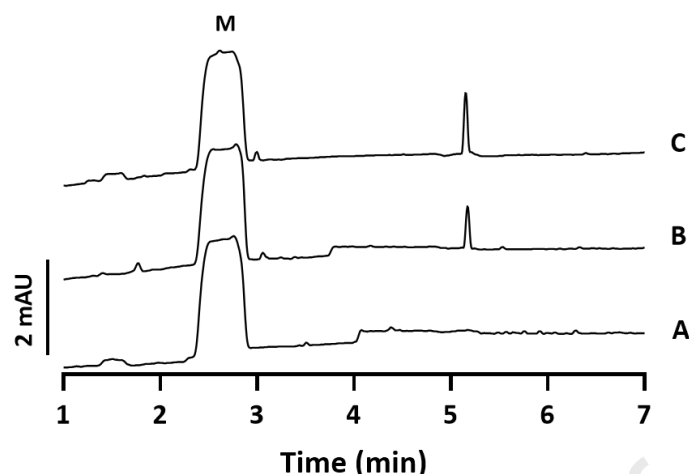


Figure 5. CE analysis of control sample with LN211: LN211 standard at 26.56 nM (A); HPV16-VLP standard (B); HPV16-VLP with 26.56 nM of LN211 (C). Separation conditions: BGE, 0.01 M Tris HCl, 0.01 M HEPES-Na, 0.1 M NaCl and 0.1 % PEG 6000, pH 7.4 buffer containing 1.5 mM SDS; 48.5 cm (8.5 cm to the detector) x 50 μ m PEO-coated capillary; voltage + 10 kV; detection at 280 nm; pressure injection for 15 s at - 50 mbar; T = 15 $^{\circ}$ C. Other conditions as described in Section 2.

3.2.5. Time course of complex formation

To determine whether incubation for 1 hour at room temperature was sufficient to attain equilibrium, HPV16-VLPs were mixed with the highest concentration of LN332 used in all following experiments, namely 26.56 nM. The mixtures, incubated for 15, 30, 60, 90, 120 and 240 min, were then analyzed by CE immediately (See Supplementary Figure S5). The migration time of the peak corresponding to the complex did not change whatever the incubation time. It can be concluded that the equilibrium is reached in the used method.

3.2.6. Determination of K_D

A constant amount of HPV16-VLPs was incubated with increasing concentrations of LN332 (ranging from 11.38 nM to 26.56 nM), corresponding to 5- to 12-fold molar excess of LN332. As shown in Figure 4 (traces B to E), incubation of HPV16-VLPs with LN332 gives rise to one single peak for each molar ratio, corresponding to the HPV16-VLP / LN332 complex. Moreover, the peak corresponding to the complex was shifted to higher migration times with increasing concentrations of LN332 in the sample. It can be assumed that those peaks having different migration times correspond to the presence of populations of complexes with

increasing numbers of LN332 molecules bound per VLP. As the peak of the complex remains sharp whatever the molar ratio, this would suggest the existence of homogenous complexes with all HPV16-VLPs carrying the same number of LN332 molecules at each molar ratio. As mentioned earlier, LN332 was not detectable in the concentration range used for the assay (See Supplementary Figure S6).

The electrophoretic mobility of the complex was calculated from the migration time of the peak of the complex and considering the electroosmotic mobility. It should be noted that phthalic acid was not used in the calculation of the electrophoretic mobility. Phthalic acid was used as internal marker only to confirm that the shift in migration time is due to the formation of the complex and not to a lack of reliability of the method. Since the complex migrated as an anion, its electrophoretic mobility was expressed as a negative value (See Section 2.4.5.). The electrophoretic mobility was plotted as a function of the concentration of LN332 in the sample (the HPV16-VLP concentration being constant), resulting in a binding curve which allowed the quantification of the interaction (cf. Figure 6). As can be seen in this Figure, the electrophoretic mobility of the complex decreased in absolute value with increasing concentrations of LN332. Although peak area repeatability of the complex was not studied, the repeatability of the electrophoretic mobility of the complex was found to be very satisfactory (RSDs lower than 1.1 % for $n = 3$, repetitions of the same incubated sample).

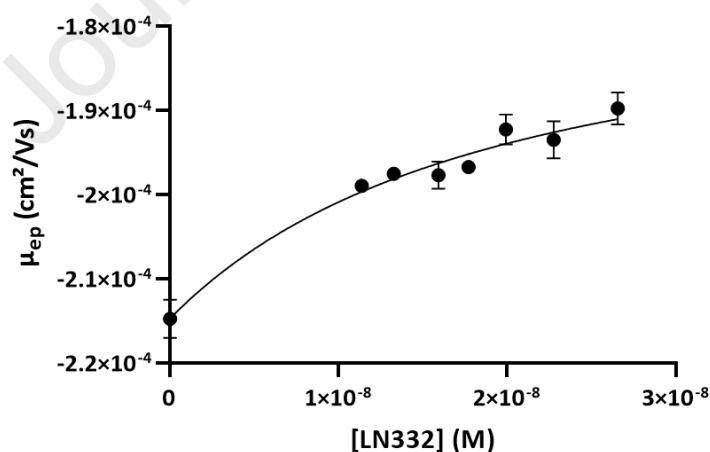


Figure 6. Dependence of the electrophoretic mobility of HPV16-VLPs on LN332 concentration in the sample. Non-linear regression estimates an apparent K_D value for HPV16-VLP/LN332 interaction of 17.7 ± 0.3 nM. The calculation of the K_D resulted from the analysis of 8 incubated samples which were injected 3 times each (total number of points: 24, with RSDs of the electrophoretic mobility lower than 1.1 % for each incubated sample).

For the determination of the K_D an equation derived from the law of mass action was used (see Eq. 1), assuming a 1:1 model. A non-linear regression, on the global set, estimates an apparent K_D for the HPV16-VLP / LN332 binding of 17.7 ± 0.3 nM.

4. Concluding remarks

The goal of this study was to evaluate the attachment properties of HPV16 to its potential physiological receptor LN332. For this purpose, two analytical approaches, ACE and BLI, were used. Both techniques have advantages and disadvantages which are summarized in Table 2. A drawback of BLI is that LN332 is immobilized onto the biosensor, which can affect the interaction interface of the protein and therefore impact the association/dissociation rates and thus the corresponding binding affinity. Indeed, the immobilization, which is not oriented, can lead to conformational changes and/or decrease the accessibility of the binding interface. This is not the case in ACE where the interaction takes place in solution before the analysis. Thus, the binding interface of both binding partners are highly accessible for interaction. Another advantage of ACE is that it is a separative technique. Thus, the samples do not need to be pure, as the impurities are separated from the complex. On the other hand, the analysis in ACE is performed under the application of an electric field, which can influence the interaction between both biomolecules and thus modify their affinity. Moreover, BLI and ACE do not measure the K_D in the same manner. Indeed, in BLI, the K_D is determined from the kinetics of the interaction (k_{on} and k_{off}), while our approach in ACE is based on the analysis of the interaction at equilibrium.

Table 2. Comparison of BLI and ACE techniques and the corresponding K_D obtained for the complex between HPV-VLPs and LN332.

Techniques	Immobilization	Labeling	Electric field	Separation	Real-time kinetics	K_D
BLI	yes	no	no	no	yes	8.89 nM
ACE	no	no	yes	yes	no	17.7 nM

The configurations used in both set-ups are different. In BLI, a constant amount of LN332 is loaded onto the surface of the biosensor and incubated with various HPV16-VLP concentrations while in ACE, a constant amount of HPV16-VLPs is incubated with different LN332

concentrations. Although both configurations are different, the concentrations and implemented ratios were in the same magnitude order. Indeed, the molar ratios between HPV16-VLPs and LN332 in ACE and in BLI ranged from 1:5 to 1:12 and from 1:1 to 1:46, respectively. It should be noted that the investigated ratios between VLPs and LN332 were based on the study highlighting the role of LN332 as transient receptor for HPV where a ratio of 1:10 was used in ELISA [11].

Even if these two techniques are conceptually different and the approaches followed in both cases are not the same, the K_D obtained are similar and are found to be in the nanomolar range (cf. Table 2). These techniques can be used for future studies of the interaction between HPV and host cell receptors and can contribute to a better understanding of HPV entry pathways.

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- Determination of the kinetics of the interaction between HV16-VLPs and LN332 by bio-layer interferometry.
- Analysis of preincubated samples in CE and quantitation using the mobility of the complexes.
- HPV16-VLPs bind to LN332 with K_D in the nanomolar range.
- It is the first application of ACE to study the interaction of VLPs with a receptor.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: