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High stability of plant-expressed virus-like particles of an insect virus in artificial gastric and intestinal fluids

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ABSTRACT

The harsh conditions of the gastro-intestinal (GI) milieu pose a major barrier to the oral delivery of protein nanocages. Here we studied the stability of *Nudaurelia capensis* omega virus (NωV) virus-like particles (VLPs) in simulated GI fluids. NωV VLPs capsids and procapsids were transiently expressed in plants, the VLPs were incubated in various simulated GI fluids and their stability was determined by gel electrophoresis, density gradient ultracentrifugation and transmission electron microscopy (TEM). The results showed that the capsids were highly resistant to simulated gastric fluids at pH ≥ 3. Even under the harshest conditions, which consisted of a pepsin solution at pH 1.2, NωV capsids remained assembled as VLPs, though some digestion of the coat protein occurred. Moreover, 80.8% (± 10.2%) stability was measured for NωV capsids upon 4 h incubation in simulated intestinal fluids. The high resistance of this protein cage to digestion and denaturation can be attributed to its distinctively compact structure. The more porous form of the VLPs, the procapsid, was less stable under all conditions. Our results suggest that NωV VLPs capsids are likely to endure transit through the GI tract, designating them as promising candidate protein nanocages for oral drug delivery.

1. Introduction

Viral nanoparticles (VNPs) are a new class of protein cage nanoparticles that are being extensively studied for various pharmaceutical and biomedical applications [1]. One type of VNPs are virus-like particles (VLPs): VLPs are essentially copies of their parent viruses, but they do not contain the genetic material required for the viral replication, and are therefore not infectious. VLPs have several characteristics which makes them ideal nanocarriers for drug and vaccines delivery: first of all, they are biocompatible and biodegradable. Second, they are homogenous and monodispersed [1]. Third, their hollow nature enables the loading of drugs and other molecules in the core [2,3]. Fourth, VLPs are amenable to functionalisation by either genetical or chemical modification [4].

The oral route is the main means of delivery of medicines, with over 70% of all medicines being administered this way [5]. Similarly, bioactive nutrients are generally delivered through the oral route. Nevertheless, during their transit through the gastro-intestinal (GI) tract, drug delivery systems encounter, and must overcome, a series of physiological barriers, including the low gastric pH and the digestive enzymes [6–8]. The harsh GI environment poses a particularly great challenge to protein cage nanoparticles. For instance, ferritin, which is the mostly studied protein cage vehicle for delivery of drugs and bioactive nutrients, has shown limited stability in simulated GI conditions. Thus, several attempts are being made to design ferritin-based drug delivery systems more resistant to degradation (reviewed in [9]).

Nudaurelia capensis omega virus (NωV) is a virus that infects insects, such as moths [10] and NωV VLPs have been produced in insect cells using recombinant technologies [11]. NωV VLPs exist mainly as two forms, i.e. either as procapsid or as capsids: at pH 7.6, NωV initially assembles as an immature porous procapsid of approximately 48 nm diameter, which is made of 240 copies of the 70 KDa coat protein (α-peptide). At pH 5.0, the NωV VLPs rapidly reorganise into a more compact and less porous 42 nm capsid [12]. The irreversible structural transition of procapsid into capsid also triggers a slower auto-proteolysis of the coat protein into a 62 KDa β-peptide and an 8 KDa γ-peptide. This VLP form, containing the cleaved coat protein, resembles the mature virion [12]. Notably, mature VLP capsids are fairly resistant to proteolysis by trypsin [13].

Given their compact structure, their ability to mature at low pH and their resistance to trypsin digestion, we reasoned that NωV VLP capsids might withstand the harsh GI environment. Thus, we have examined

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the stability of NωV VLPs in simulated GI conditions. If our hypothesis, that NωV VLPs could be stable in GI conditions, was confirmed, they could then serve as GI-resistant protein nanocages for translation in oral drug delivery applications.

We produced NωV VLPs using a transient gene expression system in a plant host [14]. VLPs procapsids and capsids forms were prepared by extraction at pH 7.6 and 5.0, respectively. Thus, two formulations were studied, namely, wild-type procapsids at pH 7.6 (wt7.6) and wild-type mature capsids at pH 5.0 (wt5). After extraction and purification of the VLPs, we studied their stability in simulated gastric fluids (SGF) containing the digestive enzyme pepsin at various pHs and in simulated intestinal fluids (SIF) containing pancreatin. The stability of the VLPs was investigated in terms of: 1) chemical stability, i.e. analysing the primary structure of the coat protein by SDS-PAGE; and 2) physical stability of the protein cage assembly, i.e. examining the VLPs' integrity by transmission electron microscopy (TEM) and by sucrose density gradient ultracentrifugation. Here, we show that mature capsids of NωV VLPs – but not procapsids- are highly stable in simulated gastric and intestinal fluids, which is unusual for protein cages.

2. Materials and method

2.1. Materials

pEAQ-*HT*-NωV-WT is a bacterial plasmid designed to transiently express full-length NωV wild-type capsid protein in plants [14]. Complete® Protease inhibitor tablets were purchased from Roche (UK). NuPAGE MOPS buffer, NuPAGE LDS Sample Buffer and NuPAGE polyacrylamide bis–tris gels were bought from Invitrogen (UK). InstantBlue stain was purchased from Expedon (UK). Micro Float-A-Lyzer dialysis tube (100 kDa MWCO) and Amicon centrifugal filters (100 kDa MWCO) were obtained from Spectrum Laboratories and Millipore, respectively. BCA Protein Assay Kit and Bovine Serum Albumin (BSA, 2 mg/mL standard) were purchased from Thermo Fisher Scientific (USA). Pepsin (≥400 units per mg protein) from porcine gastric mucosa and pancreatin (≥3 × USP specifications) from porcine pancreas were bought from Sigma-Aldrich (UK). The composition of simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) is similar to that described previously [15,16] and it is presented in Table 1. Wang et al. have shown that those media can effectively mimic the stability of peptide biopharmaceuticals in real human GI fluids [17]. Thus, SGF and SIF can be used in early stage drug development as a surrogate of human GI fluids, that are difficult to be sourced due to ethical limitations [17].

2.2. Methods

2.2.1. VLPs expression, extraction and purification

Three weeks old *Nicotiana benthamiana* leaves were pressure-infiltrated with a suspension of *Agrobacterium tumefaciens* (strain LBA 4404) containing pEAQ-*HT*-NωV-WT using a syringe [18]. Leaves were harvested 3–4 days post-infiltration.

Extraction and purification of NωV formulations at pH 7.6 and pH 5.0 should yield procapsids and capsids, respectively [19]. Briefly, harvested leaves were homogenised at 4 °C in 2.5 vol of either 50 mM Tris-HCl pH 7.6, 250 mM NaCl (abbreviated as 50 mM Tris) or 50 mM sodium acetate pH 5, 250 mM NaCl (abbreviated as 50 mM NaAc). Both buffers were supplemented with Complete® Protease inhibitor tablets. Extracts were subsequently passed through a double layer of Miracloth and then clarified by centrifugation at 12,000*g* for 20 min at 11 °C. VLPs were then pelleted through a 3.8 mL 30% w/v sucrose cushion, prepared in either 50 mM Tris or 50 mM NaAc, at 30,000 rpm for 3 h at 11 °C on a Surespin 630 rotor. After resuspension of the pellets, the VLPs dispersions were clarified by centrifugation at 12000*g* for 30 mins and then layered on the top of a 10–50% w/v sucrose step gradient prepared in either 50 mM Tris or 50 mM NaAc. VLPs were sedimented for 1.25 h at 40,000 rpm at 11 °C in a TH-641 rotor [19]. Gradients' fractions were manually collected and analysed by SDS-PAGE. The two NwV formulations, i.e. wild type procapsid (wt7.6) and wild type capsid (wt5) were produced using this procedure.

Fractions rich in NωV were pooled together and further purified and concentrated through Amicon centrifugal filters, using 10 mM Tris-HCl pH 7.6, 250 mM NaCl (abbreviated as 10 mM Tris) or 10 mM sodium acetate pH 5.0, 250 mM NaCl (abbreviated as 10 mM NaAc) to exchange the buffer of procapsids and capsid preparations, respectively. The concentration of VLPs in the purified sample was measured using the BCA protein assay. All samples stocks had concentrations ranging between 4.5 and 7.5 mg/mL and were stored in the fridge prior to use.

Table 1
Composition of the simulated gastric and intestinal media.

Media	Abbreviation	Composition
Simulated gastric fluid	SGF pH 1.2	63 mM HCl + 3.2 mg/mL pepsin
	SGF pH 2	10 mM HCl + 3.2 mg/mL pepsin
	SGF pH 3	1 mM HCl + 3.2 mg/mL pepsin
	SGF pH 4	0.1 mM HCl + 3.2 mg/mL pepsin
Simulated intestinal fluid	SIF	50 mM KH_2PO_4 (+NaOH to pH 6.8) + 10 mg/mL pancreatin

2.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One volume of a solution of LDS loading buffer and β-mercaptoethanol (in ratio 3:1) was added to two volumes of the preparation to analyse, before heating for 5 min at 98 °C. Then 10–20 μL of the resulting samples were separated on 12% (w/v) NuPAGE polyacrylamide gels, using MOPS running buffer, at 200 V for 50 min. Gels were stained with Instant Blue [15,20].

2.2.3. Chemical stability of the VLPs in simulated gastric and intestinal fluids

6 μg of VLPs were incubated at 37 °C in 14 μL of each of the simulated gastric fluids (SGF) and intestinal fluids (SIF) (Table 1) for periods of time ranging from 15 min to 4 h. As a control for digestion, bovine serum albumin (BSA) was also incubated in the same media. Samples were then analysed by Commassie-stained SDS-PAGE (described in Section 2.2.2). Images of the gel were taken using a G:BOX EF2 image analysis system. The density of the band corresponding to the coat protein in each line was quantified by densitometry, using ImageJ. Results are expressed as percentage of densitometry unit (DU) compared to a control of untreated VLPs loaded in the same gel [21]. Experiments were performed in triplicates.

2.2.4. Physical stability of the VLPs in simulated gastric fluids

The integrity of the quaternary structure of the VLPs, i.e. the assembly of the protein cage, was assessed by sucrose density gradient ultracentrifugation and by direct visualisation of the VLPs at the TEM [15,16].

For the sucrose density gradient ultracentrifugation, 150 μg of VLPs from the stocks were incubated at 37 °C in 1 mL SGF or SIF (Table 1), for periods of time ranging from 1 h to 4 h. After the incubation in SGF or SIF, samples were diluted in 2 mL of either 50 mM NaAc or 50 mM KH_2PO_4 - NaOH pH 6.8 buffer, respectively, and loaded on the top of a 20–70% sucrose step gradient (in 50 mM NaAc for SGF and 50 mM Tris for SIF). VLPs were sedimented by centrifugation on an AH-650 rotor for 1.25 h at 49300 rpm. Fractions were then collected and analysed by SDS-PAGE.

For TEM, 6 μg of VLPs from the stocks was diluted in 50 μL of SGF pH 1.2, SGF pH 2 or SGF pH 3 (Table 1) and incubated at 37 °C. After 1 or 2 h, pepsin was inactivated by pH neutralisation [22], adding 20 μL of 1 M Tris- HCl at pH 8 (for SGF pH 1.2) or 5 μL of 1 M Tris- HCl at pH

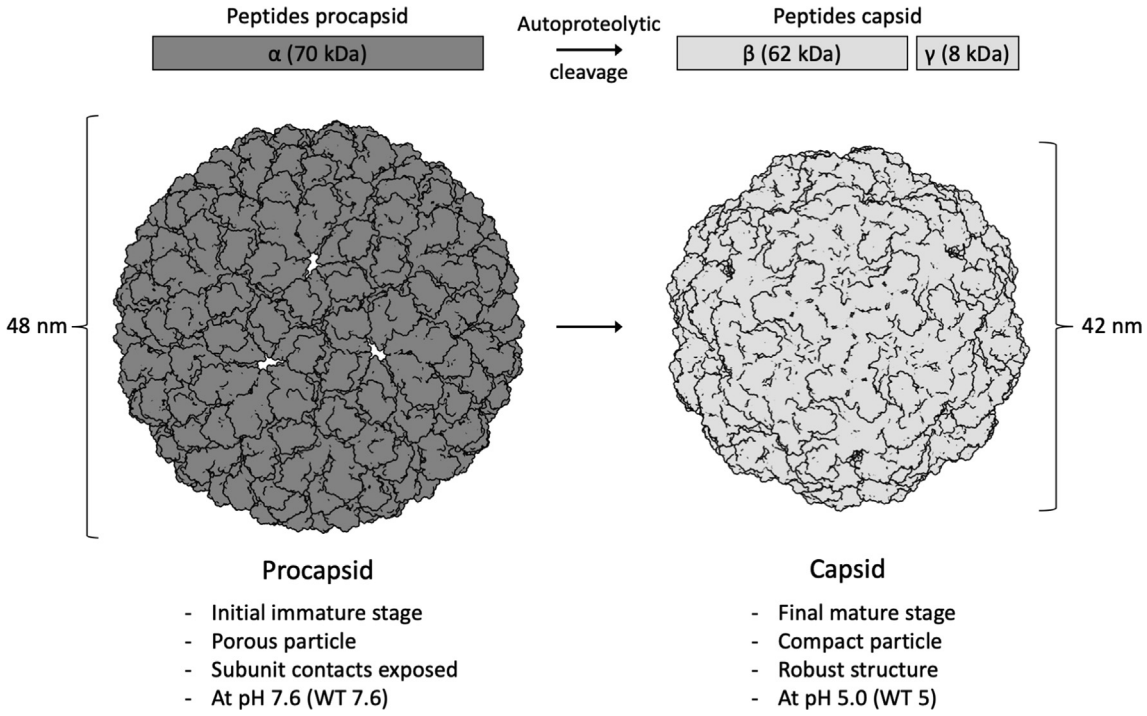

Fig. 1. Schematic representation of the two NωV VLP forms (bottom) and the corresponding coat protein state (top). Immature procapsids are porous and expanded particles prepared by extraction at pH 7.6. Mature capsids are smaller and more compact particles prepared by extraction at pH 5.

8 (for SGF pH 2 and pH 3). Samples were then clarified by centrifugation at 10000*g* for 5 min. The supernatant was collected and adsorbed onto hexagonal, plastic and carbon-coated copper grids. The grids were negatively stained with 2% (w/v) uranyl acetate. A FEI Talos F200C TEM with a Gatan OneView 4 k × 4 k CMOS camera was used for taking the images. Positive controls, prepared by diluting 6 μg of VLPs to a concentration of ≈1.2 μg/mL, were also analysed at the TEM.

VLP formulations incubated in SIF could not be directly imaged at the TEM, due the large amount of insoluble material present in the media. For this reason, after incubation of the VLPs in SIF for 4 h at 37 °C, samples were purified by sucrose gradient ultracentrifugation (described above). Gradient fractions rich in coat protein were pooled together and dialysed against 10 mM Tris to remove the sucrose. After dialysis samples were imaged at the TEM.

3. Results and discussion

3.1. NωV VLPs characterisation

Fig. 1 is a cartoon of the two different NωV VLP forms prepared in this study. The panel on the left shows a wild-type procapsid prepared by extraction at pH 7.6 (wt7.6): this porous procapsid can be converted to capsid by acidification to pH 5.0. The panel on the right contains a wild-type capsid prepared by extraction at pH 5.0 (wt5): the capsid is irreversible locked in its compact conformation [19].

After extraction of the recombinant NωV protein from leaves, VLPs were pelleted and then purified by sedimentation through sucrose gradients. Fig. S1 shows the SDS-PAGE analysis of the collected sucrose fractions. Fractions rich in NωV coat protein were pooled together and further purified and concentrated through centrifugal filters. Fig. 2A shows the protein-stained SDS-PAGE of the purified NωV VLPs stocks. As expected, wt7.6 had the coat protein in the uncleaved α state (70 KDa) (Fig. 2A), while wt5 is characterised by the coat protein cleaved in the β state (62 KDa). It should be noted that wt7.6 does not consist entirely of α peptide; a faint 62 KDa band can also be seen. Thus, readers must consider that wt7.6 contains mainly procapsids VLPs (made of α peptide subunits), but also a small portion of capsids (made of β peptide subunits), as also confirmed by the TEM images (Fig. 2B – discussed later). It should also be noted that over the course of the study, more α protein in the wt7.6 sample converted to the β form over storage, as visible from subsequent gels. To limit this time-dependent conversion of procapsids into capsid, wt7.6 stocks were tested within 4 weeks from extraction in all further experiments described in this work.

Fig. 2B presents TEM images of the VLPs. Particles of 40–50 nm can be seen in all samples. In the case of the wt7.6, a large background of broken particles is present. This instability has been previously described [19] and it is possibly due to the harsh conditions of the TEM analysis: the high concentration of uranyl acetate at a pH near 4 and the high ionic strength might have induced the procapsids to collapse. It has been previously reported that procapsids are unstable at ionic strength > 0.5 [13]. Capsids in wt5 are clearly distinguishable from procapsids, as they remained intact and not penetrated by the stain, in agreement with previous findings [19]. Some unstained particles are also visible in wt7.6, confirming that wt7.6 contains a majority of procapsids, but also a small population of capsids. The heterogeneity of the particles in the sample suggests that wt7.6 might contain also some intermediates between capsids and procapsids.

3.2. Stability in simulated gastric fluids

Human gastric fluids have median pH values between 1 and 2 in fasting conditions. Right after a meal the pH can rise up to around 6–7, to then slowly return back to fasting state values within the following one to four hours [23]. The acidic pH has the ability to denature proteins and thus disassemble the physical structure of protein cages. Besides the low pH, the protease pepsin is another barrier to the gastric stability of VLPs [15,16,21].

3.2.1. Chemical stability

We first studied the extent of digestion of NωV VLPs upon exposure to SGF containing pepsin at pH 1.2, 2, 3 and 4 (Table 1) for 2 h, in order to simulate the variable condition of pH present in the stomach. Fig. 3A indicates that BSA was fully digested at all pHs, after incubation in SGF.

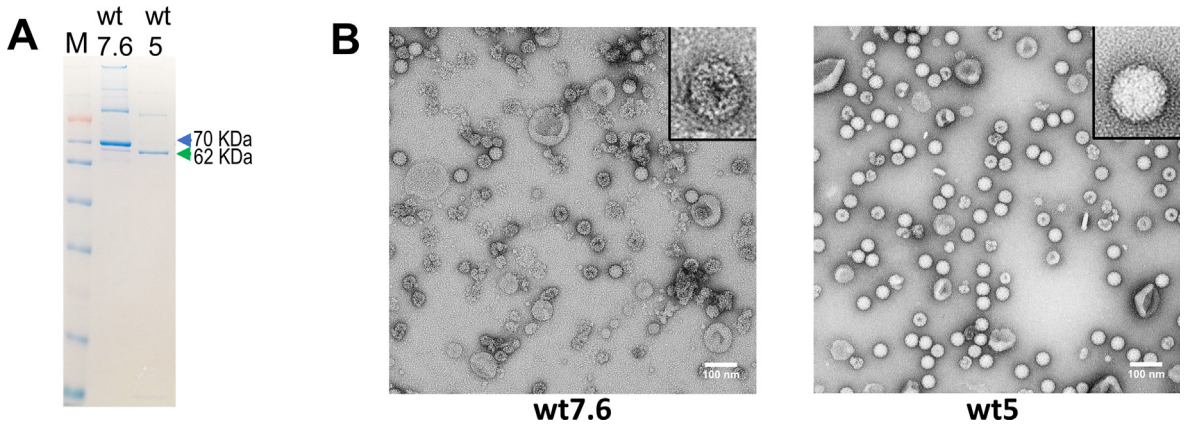

Fig. 2. VLPs characterisation. A: Protein-stained SDS-PAGE of the two formulations of purified VLPs. The blue and green arrows indicated the α and β coat proteins, respectively. B: TEM images. The panels in the top-right of each image show a single procapsid and capsid particle (zoom 4x) in wt7.6 and wt5, respectively.

On the contrary, NωV VLPs capsids (wt5) showed only minimal digestion, which was a function of the pH: the lower the pH, the more was the digestion. As seen in Fig. 3B, the procapsids (wt7.6) were subject to a greater extent of proteolysis compared to the capsids. Moreover, the 70 KDa α coat protein (visible in the control) was totally unstable in SGF at all pHs, unlike the 62 KDa β coat protein. It has been previously reported that the 8KDa γ peptide at the *N*-terminus of the 70 KDa α coat protein is accessible to proteolysis [13]. Thus, it seems that in our experiment the γ peptide at the *N*-terminus had been readily cleaved by the pepsin in SGF.

We then measured the relative density of the gel band of the digested coat protein compared to the control of untreated NωV (Fig. 3C).

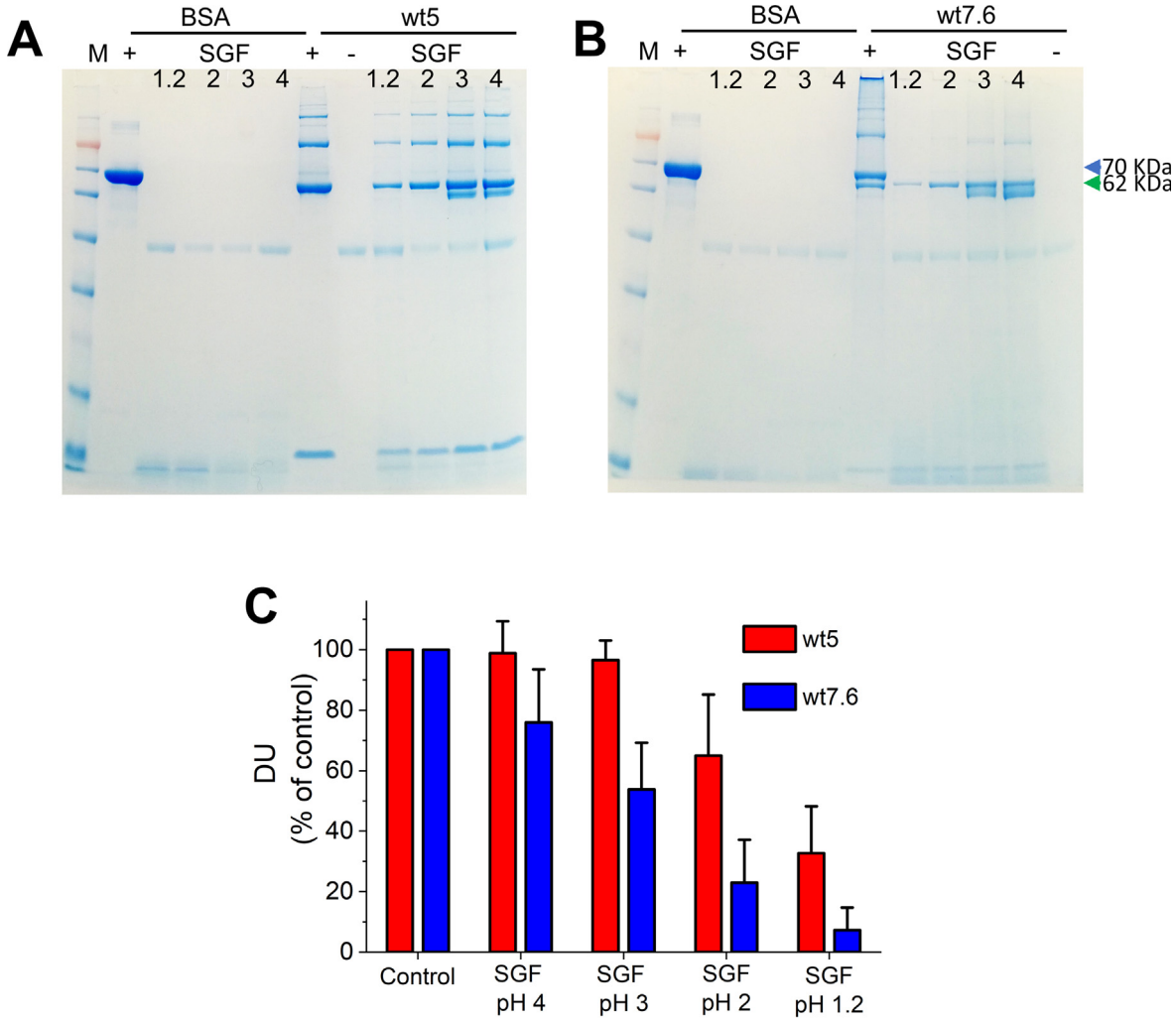

Fig. 3. Chemical stability of NωV wt coat protein upon exposure to SGF at different pH for 2 h. A and B: protein-stained SDS-PAGE of wt5 (A) and wt7.6 (B) exposed to SGF at pH 1.2, 2, 3 and 4. BSA was used as control for digestion. Untreated NωV particles and SGF pH 1.2 were use as positive and negative controls, respectively. C: analysis of the relative density (mean ± SD; n = 3) of the coat protein band in the gels, as a percentage of the positive control.

Results confirmed that VLP capsids resisted digestion in SGF at pH 3 and 4, but they were increasingly less stable in more acidic conditions. At each pH, VLPs procapsids (wt7.6) were always more extensively degraded than capsids (wt5). This can possibly be explained: during the acidification in SGF, procapsids should have rapidly matured into the more stable capsids [24], hindering digestion. However, it seems that before complete maturation, pepsin enzyme could have digested the procapsids. Hence the greater degradation of wt7.6 compared to wt5. It must be noted that the stability of wt7.6 in SGF might even be slightly overestimated compared to the stability of pure procapsids, because this formulation inherently contains a portion of capsids, as previously discussed.

The greater proteolysis at lower pH (Fig. 3) cannot be attributed to an intrinsically higher activity of the pepsin at low pH. Indeed, pepsin activity at pH 1.2 is reported to be > 3-fold lower than at pH 2 [22], while on the contrary, in our work, NωV digestion was greater at pH 1.2. It can be proposed that the lower pH induced conformational changes of NωV, which rendered the protein more exposed to enzymatic attack.

The VLPs were much more stable than BSA, that was totally digested under all conditions. This is probably due to the greater shielding of the NωV proteins which are assembled into nano-shells, compared to the soluble monomers of BSA that are readily exposed to enzymes. The remarkable stability of VLPs has been previously described also for Hepatitis B core antigen (HBcAg) VLPs [16], Cowpea mosaic virus (CPMV) and CPMV VLPs [15]. The highly compact mature NωV capsids are even more resistant to pepsin than the previously studied CPMV and HBcAg VLPs, both of which were completely degraded by pepsin in SGF pH 1.2 [15,16].

The 2 h exposure to SGF pH 1.2 is an extreme condition, possibly never encountered *in vivo* by a delivery system potentially administered in the form of an immediate release solid dosage form or liquid. In the fed state average pH values would be between 2.7 and 6.4 [23]. In the fasted state, values as low as pH 1.2 could be reached, yet the emptying of water, non-nutritional liquids and disintegrated immediate release tablets would be very rapid: Mudie et al. described that upon ingestion of 240 mL of water, half of the gastric volume is emptied in 13 min and the gastric volume returns to baseline in just 45 min in average [25]. Also immediate release disintegrating tablets given in fasting condition are likely to be emptied from the stomach much more rapidly than in 2 h [26]. Thus, we decided to study the digestion of our VLPs in SGF pH 1.2 for periods of 15, 30 and 60 min, which could better reflect gastric residence times of nanoparticles in fasting conditions. Fig. 4A and B shows that the stability of NωV VLPs capsids and procapsids in SGF 1.2 dropped rapidly within the first 15 min and it declined only minimally, thereafter. In other words, the time of exposure to the enzymes did not have a considerable influence on degradation. Also, the digestion of wt7.6 was always greater than that of wt5.

3.2.2. Physical stability

The studies described this far, only evaluated the enzymatic digestion of the NωV coat protein. However, beside the chemical degradation, the low gastric pH could challenge the physical stability, leading to denaturation of the particulate structure. Thus, we studied the physical integrity of capsids and procapsids in SGF. Fig. 5A shows that after incubation of wt5 and wt7.6 formulations in SGF pH 3 for 2 h, NωV was still sedimented in the lower fractions of the sucrose gradient, upon ultracentrifugation. This is typical of assembled VLPs [19]. On the contrary, denatured protein monomers, being smaller and less dense would have not been able to penetrate the dense sucrose fractions upon ultracentrifugation, and would have remained in the supernatant. TEM images, in Fig. 5B, confirmed that NωV VLPs capsids had remained assembled after incubation in SGF pH 3. Even in the case of wt7.6, the VLPs are in the capsid form, as the procapsids were converted into the more stable capsids in acid media. Alternatively, the procapsids might have been degraded and the visible capsids in wt7.6 were those already present in the formulation, as discussed before.

To further challenge the integrity of the VLPs and to simulate extreme fasting gastric pH and exposure time, we incubated NωV VLPs in SGF pH 1.2 for 1 h. Surprisingly, capsids could still penetrate through the gradients (Fig. 6A). We could also visualise intact particles at the TEM (Fig. 6B). In previous studies, we found that CPMV and HBcAg VLPs were remarkably resistant to SGF, yet particles dissembled or aggregated at pH 1.2 [15,16]. Thus, NωV VLP, being able to survive SGF pH 1.2, is a clear winner for its ability to withstand extremely harsh simulated gastric conditions. Also, all studied VLPs [15,16] are far more stable in simulated gastric conditions compared to the much more studied ferritin protein cage [9].

In view of the potential clinical translation of NωV as nanocages for oral drug delivery, the obtained results would suggest that NωV VLPs capsids (wt5) would be completely stable in the fed stomach, where the pH is ≥ 3. Even in the harsher fasting state, though partially digestion

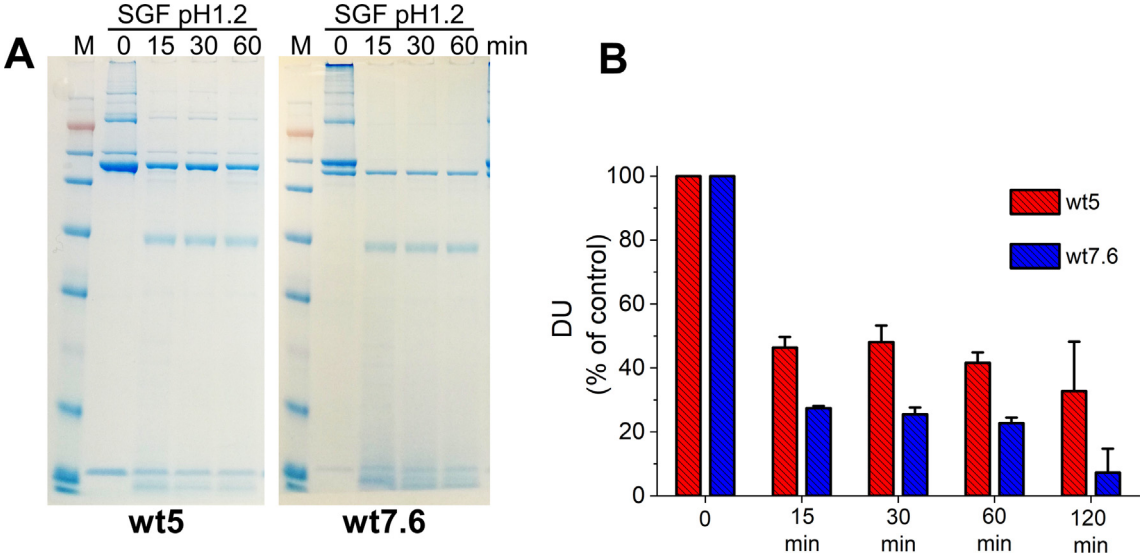

Fig. 4. Effect of time on the chemical stability of NωV wt coat protein upon exposure to SGF pH 1.2. A and B: protein-stained SDS-PAGE of wt5 (A) and wt7.6 (B) exposed to SGF pH 1.2 for 15, 30 and 60 min. Untreated NωV particles were used as positive controls (time 0). C: analysis of the relative density (mean ± SD; n = 3) of the coat protein band in the gels, as a percentage of the positive control. Values relative to the 120 min time point were taken from the previous Fig. 3.

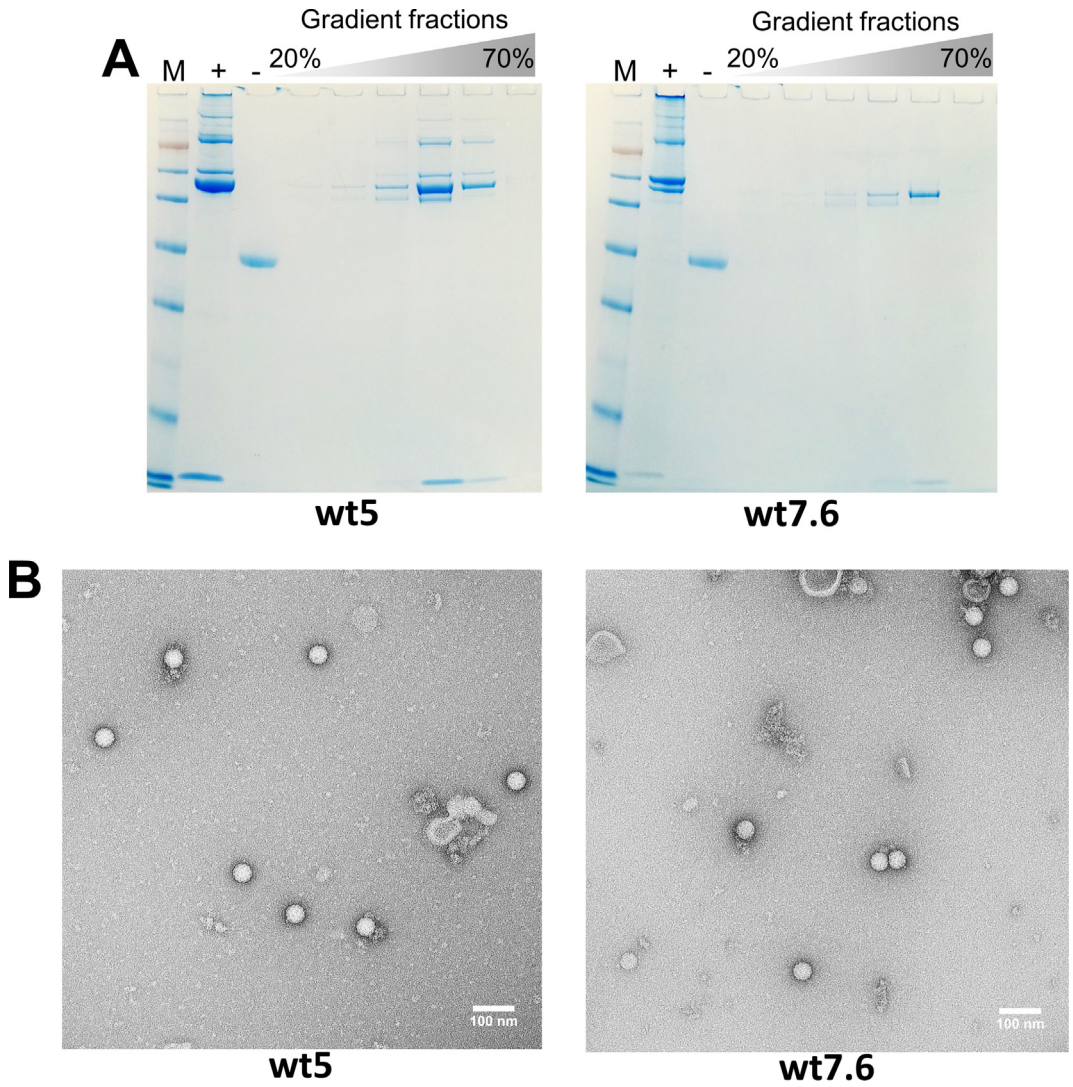

Fig. 5. Physical stability of wt NωV VLPs exposed to SGF pH 3 for 2 h. A: sedimentation profile of NωV in a 20–70% sucrose step gradient upon ultracentrifugation. B: TEM images.

could occur, a considerable portion of VLPs would likely reach the intestine intact.

3.3. Stability in simulated intestinal fluids

After the stomach, orally delivered nanoparticles should traverse the intestinal milieu. Here, the environment is nearly neutral - generally quoted to be between pH 6 and 8 [27] – but intestinal enzymes could have a detrimental effect on the stability of protein-based materials.

To study the effect of intestinal proteases on chemical digestion, we incubated NωV VLPs in SIF (Table 1) for 4 h, considering that the small intestinal transit time of pharmaceuticals is generally modelled to be 3–4 h [28]. SIF contained pancreatin, which is a mixture of enzymes, including the proteases trypsin, chymotrypsin, elastase, and carboxypeptidases [29]. Fig. 7A shows the SDS-PAGE relative to the digestion of NωV VLPs in SIF. Care must be taken in the interpretation of this gel, because SIF, as seen in the negative control, contains a circa 50 KDa protein, right below the NωV coat protein bands, which could confuse the visualisation of the results. In order from the left to right, wt7.6 had the 70 KDa α-coat protein digested by proteases, while the portion of coat protein present as β-form (62 KDa) remained intact. The 62 KDa β-coat protein of wt5 had also resisted degradation. The BSA control was fully digested.

Gels were analysed by densitometry, in order to measure the band density of the coat protein (Fig. 7B). 80.8% (± 10.2%) wild type capsids (wt5) remained stable upon incubation in SIF; the coat protein in wt7.6 was more heavily digested. Bothner et al. had previously demonstrated that the β-coat protein of NωV VLP capsids produced in insect cells were more resistant to trypsin digestion than the α-coat protein of the procapsids [13]. We found the same trend here in our study, though we did not use only trypsin but the whole pool of pancreatic enzymes. Also, once again, it should be noted that the stability of wt7.6 might be overestimated as wt7.6 does not contain only α-coat protein (procapsids), but also the much more stable β-coat protein (capsids).

To ensure that the partial digestion in SIF did not interfere with the particles' assembly, we then studied the physical stability of the VLPs in SIF. Fig. 8A shows that capsids could still sediment through the sucrose gradient. The gradient fractions richest in NωV coat protein were then dialysed to remove sucrose, followed by imaging at the TEM. Fig. 8B confirmed that particles had remained intact in SIF. Even in wt7.6 the surviving VLPs are capsids, and not procapsids. This agrees with Figs. 7 and 8A, where the only residual protein detected after incubation of wt7.6 in SIF was indeed the β-form, i.e. capsids.

In conclusion, NωV VLPs capsids could resist degradation in SIF. In previous studies, HBcAg VLPs, CPMV and CPMV VLPs were also found

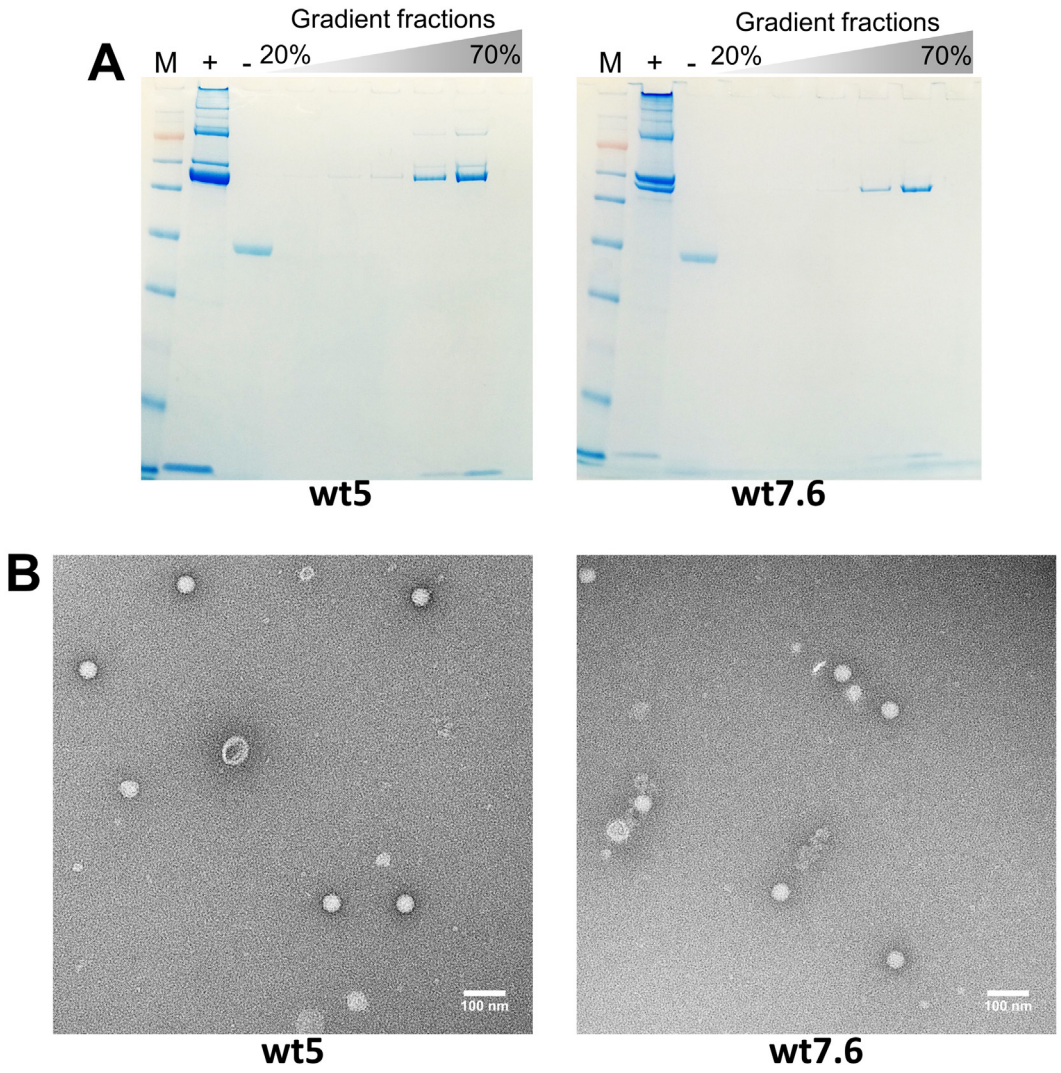

Fig. 6. Physical stability of wt NωV VLPs exposed to SGF pH 1.2 for 1 h. A: sedimentation profile of NωV in a 20–70% sucrose step gradient upon ultracentrifugation. B: TEM images.

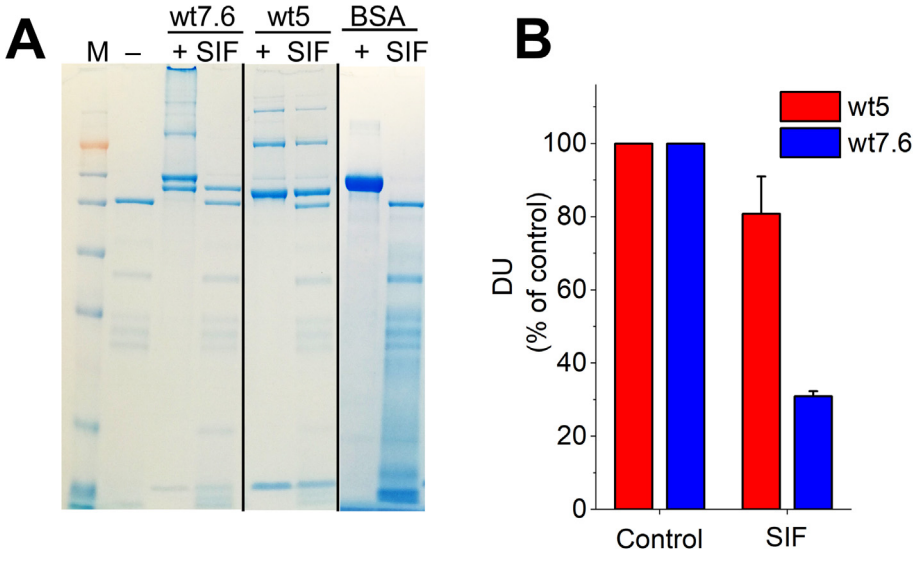

Fig. 7. Chemical stability of NωV coat protein upon exposure to SIF for 4 h. A: protein-stained SDS-PAGE of the two formulations in SIF. BSA was used as control for digestion. Untreated NωV VLPs and SIF were use as positive and negative controls, respectively. B: analysis of the relative density (mean ± SD; n = 3) of the coat protein band in the gels, as a percentage of the positive control.

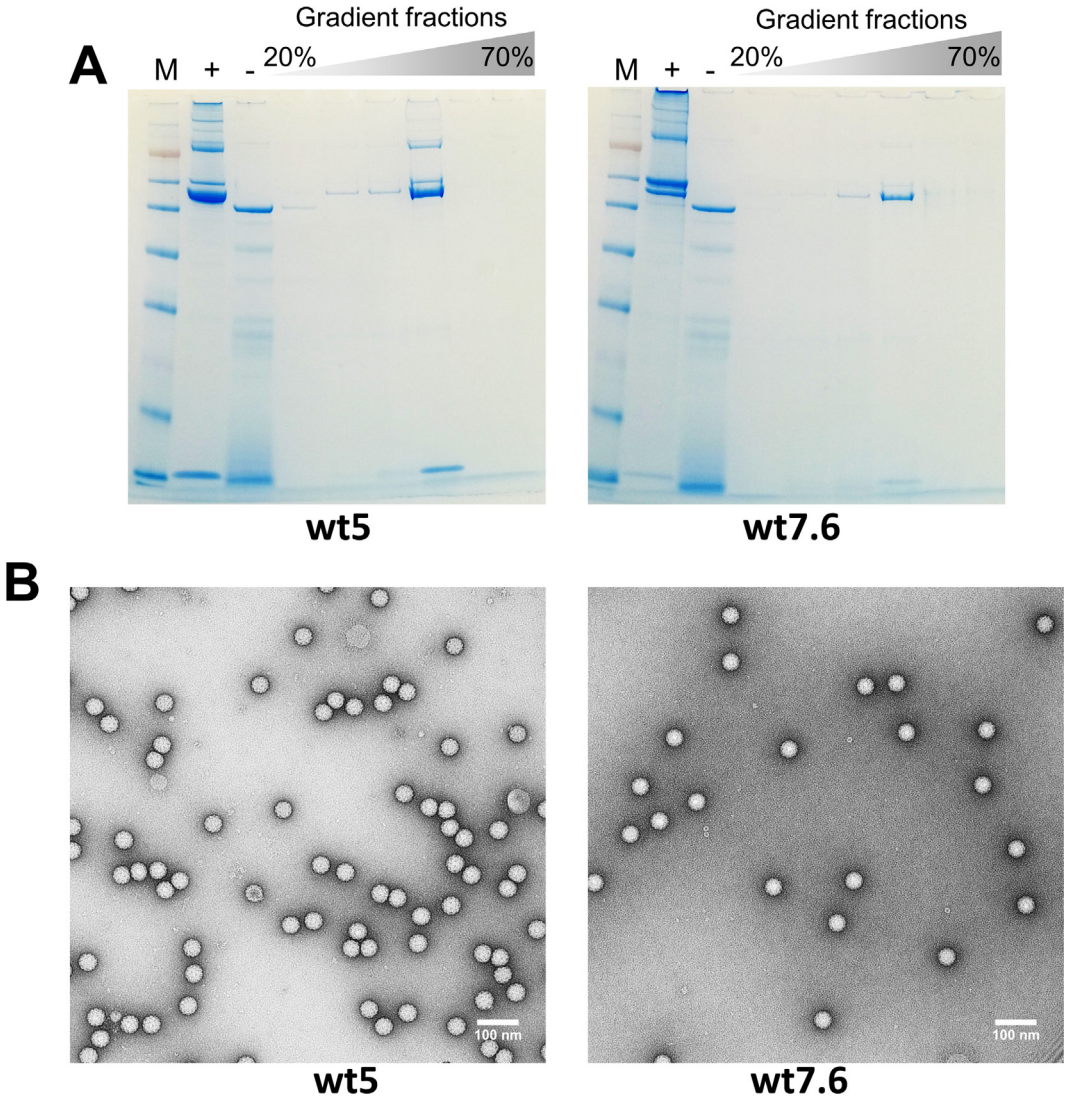

Fig. 8. Physical stability of wt NωV VLPs exposed to SIF. A: sedimentation profile of NωV in a 20–70% sucrose step gradient upon ultracentrifugation. B: TEM images of the dialysed gradient fractions rich in NωV protein.

to be stable in SIF [15,16,30]. All these results taken together indicate that VLPs can be remarkably resistant to digestion from intestinal enzymes, possibly owing to their compact and rigid structure. This is consistent with a study of Wang et al. where cyclic peptides were found to be more stable in SIF, as compared to linear peptides, possibly due to their cyclic structure which increased rigidity and reduced flexibility, ultimately impeding enzymatic digestion [17].

4. Conclusion

We have shown in this study that plant-expressed NωV VLPs capsids remained generally stable under most conditions simulating GI fluids. Even in SGF at pH 1.2, though some proteolysis occurred, the residual VLPs remained intact and assembled. NωV VLP procapsids were much less stable.

The results of this study indicate that NωV VLPs capsids should be able to traverse the harsh GI environment without being considerably degraded. This is remarkable, considering that VLPs are protein-based cages. We proposed then that NωV VLPs could be utilised as a protein-based nanoplatform for the oral delivery of drugs and bioactive nutrients. To move forward clinical translation, future studies should look into the incorporation of therapeutics and/or micronutrients into the NωV nanocarriers. In this regard, the pH-controlled transition of NωV VLPs from an open procapsid to a closed capsid could be exploited for drug encapsulation: the cargo could be loaded by diffusion through the porous procapsid; then the VLPs could be irreversibly locked by the pH-trigger, ultimately enabling the drug retention with the carrier.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: G.P.L. declares that he is a named inventor on granted patent WO 29,087,391 A1 that describes the CPMV-HT system used for transient expression in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2020.08.012.

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