



Norovirus assembly and stability

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Noroviruses are rapidly evolving RNA viruses and are generally known as the main cause of viral gastroenteritis worldwide. Particle stability is of special interest as transmission occurs via the faecal-oral route and virions are able to persist in the environment. Studies on norovirus capsid assembly and disassembly rely mainly on norovirus-like particles. Notably, stability of several human, murine and bovine variants has been investigated revealing distinct patterns of stability and also distinct assembly mechanisms and intermediates. Gathering information on these differences and common features may deepen our understanding of norovirus emergence and can potentially be used to distinguish variants. However, more systematic studies and standardized approaches are required.

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Introduction

Approximately one-fifth of all acute gastroenteritis outbreaks are caused by human noroviruses (NoV) [1]. Acute gastroenteritis involves fever, vomiting, cramping and diarrhea [2]. This illness usually persists for several days only but can take longer in children, the elderly and immunocompromised [3]. Although the prototypical Norwalk virus was discovered over four decades ago, so far no vaccines are available [2].

Outbreak causing noroviruses emerge frequently and are seasonal especially in temperate regions [4]. The dynamics of this diversity are poorly understood. Contributing factors like population immunity, virus evolution and transmission are mainly followed by epidemiological observations as a robust infection model is still missing. Since the virus is mostly foodborne and has to persist on surfaces and in the environment, particle stability is of

interest. Here we summarize current knowledge on norovirus stability and capsid assembly. Notably, there are indications for isolate-dependence of these features revealing important gaps in understanding norovirus emergence. Data on human noroviruses are limited and are complemented with findings on murine, feline and bovine noroviruses. Clearly, directed systematic biophysical studies on different norovirus variants are required to understand why some genotypes are more successfully transmitted than others.

Genetic diversity

The genus *Norovirus* belongs to the *Caliciviridae* family, which is divided into seven genogroups (GI–GVII). Noroviruses infecting humans can be found in genogroup I, II and IV, while murine noroviruses (MNV) are GV and bovine GIII noroviruses [5]. Genogroups are subdivided into genotypes based on VP1 sequence, differing by at least 43% [6]. However, recombination and mutations in both the viral polymerase and the capsid protein VP1 happen frequently. To include both genome regions as nomenclature parameters, P indicates polymerase genotypes (e.g. GII.P4 and GII.4). Thus, recombinant forms can be captured [7–10].

Noroviruses of distinct prevalence emerge frequently and surveillance networks like NoroNet help to capture trends of respective genogroups and genotypes [11]. While GI, which also includes the prototype Norwalk virus, is less frequently found nowadays, GII.4 isolates have been known as the most common cause of clinical gastroenteritis cases [12]. Surveillance studies also imply that GII.17 variants increase in prevalence [13]. Interestingly, genogroup and transmission route correlate: while GII.4 strain transmission was often found to be person-to-person dependent; non GII.4 viruses like several GI isolates, GII.6 and GII.12 were mostly foodborne; and other GI isolates waterborne (reviewed in [8]). These dependencies may be due in part to specific stability patterns of particular isolates.

Norovirus virion stability

Many NoV mediated gastroenteritis outbreaks originate in the consumption of fecal-contaminated food like mussels, oysters, berries and vegetables [14]. Hence, these enteric pathogenic agents have to persist on various surfaces and in harsh environments. Studies on intact human noroviruses are hampered as no robust cell culture system is available. Just recently, cultivation in enterocytes has been established [15^{*}]. However, for infectivity assays on virions reliable cultivation systems are inevitable and thus studies focus on norovirus surrogates.

Proposed norovirus surrogates are amongst others feline calicivirus (FCV), which belongs to the genus *Vesivirus*. Due to its transmission by the respiratory route and its general pH instability, FCV is considered less suitable [16,17]. MNV is often used as a surrogate because it shares many genetic and biochemical features with human norovirus and can infect cells in culture [18] as well as replicate in the gastrointestinal tract of its host. However, human and murine noroviruses have different cell specificities [19]. MNV is more stable than FCV regarding thermal resistance and acid tolerance [16,20]. Nevertheless, these norovirus surrogates are considered suboptimal due to structural variations within human noroviruses [21].

To include human norovirus into comparison studies, stability or infectivity is followed by genome content detection and quantification by reverse transcription-quantitative PCR (RT-qPCR) and plaque assays (reviewed in [22]). Data from systematic evaluation of 12 independent RT-qPCR studies comparing persistence and survival of human NoV and NoV surrogates in foods and the environment suggests that human NoVs are often more resistant to food and environmental control measures (e.g. thermal inactivation and chlorine treatment) than the respective surrogates [22]. Despite various attempts for correction, correlation of viral infectivity to detection of genome content remains difficult as data are often inconsistent [17,23]. In conclusion, RT-qPCR can give valuable indications on stability profiles as long as a proper propagation system for human NoVs is missing.

Studying norovirus stability using virus-like particles

To overcome the limitations of a lacking cultivation system, human NoV studies focus on virus-like particles (VLPs). The Calicivirus capsid is icosahedral and comprises 180 copies of the capsid protein VP1. Expression of VP1 in insect cells leads to self-assembly of VLPs of approximately 36–42 nm in diameter that are morphologically and antigenically comparable to native virions [24]. X-ray crystallography of GI.1 Norwalk $T = 3$ VLPs revealed the division of the capsid protein into a protruding (P) domain and a shell (S) domain (Figure 1). The P domain is further divided into P1 and P2 subdomains. Subdomain P2 is highly variable and, as it is exposed to the surface, involved in determination of antigenicity and cell attachment [25^{**},26,27].

The capsid protein adopts three quasi-equivalent structures, namely A, B and C. The S domain of A/B and C/C dimers is arranged bent and flat, respectively. Switching between A/B and C/C dimers of the S domain is necessary to facilitate curvature whereas P domains build the capsid protrusions. Icosahedral contacts between VP1 dimers are modulated by the S domain, while intra-dimer contacts are modulated by the P domain [26]. The deletion of

amino acids in the P domain that are thought to be involved in these contacts leads to heterogeneous particles with increased size [31^{**}]. Moreover, particles can be formed by assembly of the S domain only showing that the S domain interactions are sufficient for capsid formation [31^{**},32]. Deletion of the P domain results in smaller, smooth particles ($T = 3$, 29 nm) with reduced stiffness compared to wildtype particles indicating that the P domain stabilizes the viral particles [25^{**}].

Limitations of VP1 VLP studies

Recombinant norovirus particles are assembled in absence of genome and therefore lack putative RNA-capsid interactions, which could alter capsid stability. These interactions have been shown to play important roles in assembly and stability of other RNA viruses [33,34]. Another factor likely contributing to capsid stability and assembly behavior is the presence of the minor capsid protein VP2 [35]. Although the function of VP2 could not be deciphered completely, comparison of a GII NoV VP1 only and VP1/VP2 particles indicate a slight stabilizing influence under alkaline conditions [36]. Additionally, VP1/VP2 particles showed decreased protease degradation [32]. The amount of incorporated VP2 molecules is still unknown. The protein putatively interacts with the VP1 S domain and is located in the capsid interior [37].

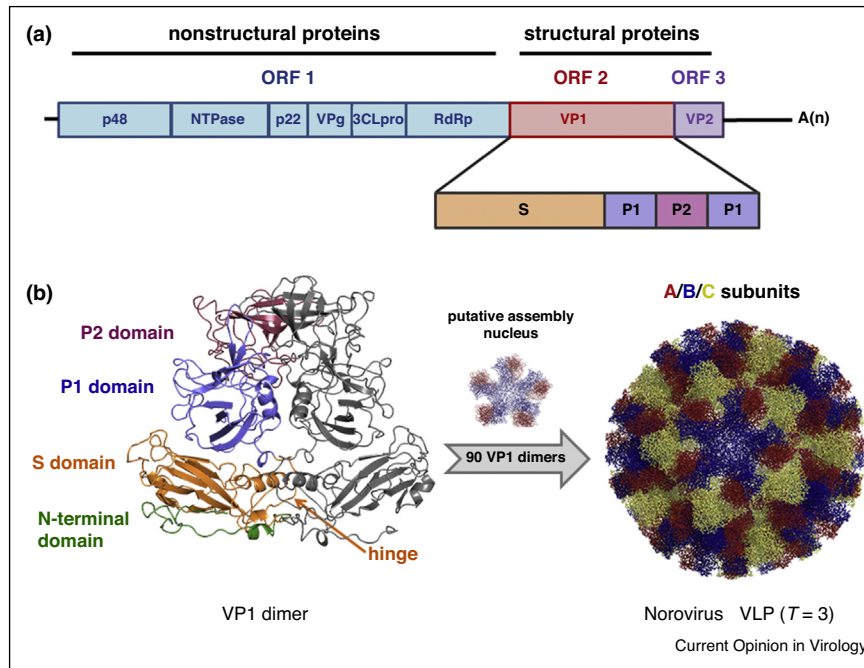
Norwalk stability and capsid assembly

Several studies focus on the effect of pH, temperature and solution additives on capsid stability using various biophysical techniques. GI.1 Norwalk capsids were stable at neutral and acidic pH, whereas disassembly was observed above pH 8 [38,39]. Particles remain intact up to 55 °C. Above this temperature VLPs start to form aggregates or disintegrate into soluble VP1 oligomers in a pH dependent manner [38]. Notably, Norwalk VLP stability was monitored in buffer containing citrate, which can alter capsid morphology as shown for a GII.10 variant [40]. It would be of interest to disentangle the structural and pH effects of citrate in more detail. Aggregation was prevented by adding various common vaccine-excipients [41]. Nanobodies targeting the P domain have also been shown to influence particle stability and promote disassembly [42].

Norwalk capsid disassembly was also followed in detail using native mass spectrometry (MS) [43,44^{**}]. In line with other studies, complete $T = 3$ capsids (VP1₁₈₀) were present at physiological pH [38]. At alkaline pH disassembly into different intermediates could be observed with VP1₆₀ and VP1₈₀ being the most abundant species at high ionic strength (Figure 2).

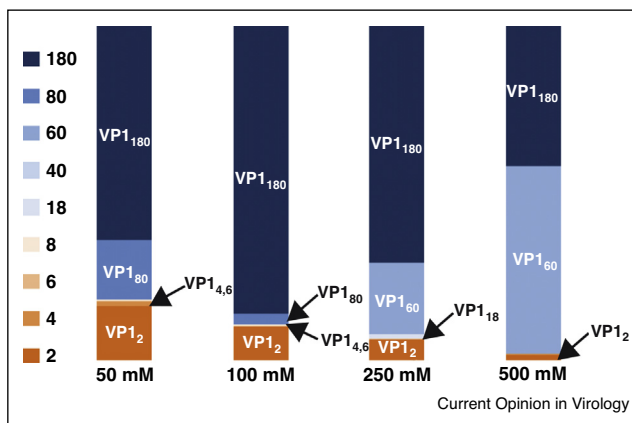
Atomic force microscopy (AFM) images confirmed the presence and spherical morphology of the $T = 1$ VP1₆₀ [44^{**}]. Furthermore, the morphology of VP1₈₀ was probed

Figure 1



Genomic organisation and structure of human noroviruses. **(a)** Noroviruses are non-enveloped RNA viruses with a positive sense single-stranded genome with three open reading frames (ORFs). A polyprotein, which includes the nonstructural proteins such as the RNA dependent polymerase (RdRp), is encoded by ORF1. Two structural proteins, the major and minor capsid protein (VP1 and VP2), are encoded by ORF2 and ORF3, respectively [28,29]. The VP1 protein is divided into shell (S) and protruding (P) domains. **(b)** 90 dimers of the major capsid protein VP1 assemble into icosahedral $T = 3$ norovirus VLPs. The S domains of the VP1 monomers build a shell that surrounds the viral RNA in form of a scaffold. The more flexible P domain is subdivided into P1 and P2 and connected to S via a hinge [25,26,27,30]. The domains are highlighted in the VP1 dimer structure **(left)** and the three quasi-equivalent subunits (A/B/C) forming the capsids are shown in the VLP structure **(right)**. PDB accession number 1IHM [26].

Figure 2



Effect of ionic strength on assembly of VP1 oligomers at pH 8. Assembly was monitored by native MS at pH 8 and a range of ammonium acetate concentrations (50–500 mM). This figure was adapted from the originally published in *Molecular & Cellular Proteomics* [44] © the American Society for Biochemistry and Molecular Biology or © the Author(s).

using native ion mobility MS (IMMS). As the determined collisional cross sections of VP1₁₈₀, VP1₈₀ and VP1₆₀ increased linearly with mass, a spherical shape was also proposed for VP1₈₀. The observed oligomers could also be reassembled into the $T = 3$ native capsids. Further IMMS studies in alkaline, assembly and non-assembly conditions revealed a sheet-like structure of smaller oligomers [45]. The VP1₆ structure was in line with a partial pentameric vertex. Hence, an assembly pathway starting with a decameric nucleus as predicted in [26] comprising a fivefold symmetry axis and proceeding via dimer addition was proposed [45]. An *in silico* AFM study confirmed this finding, as it could be shown that the interface building a quasi-sixfold symmetry axis (B/C of S domains) is the most unstable one [46]. Another study on bovine GIII norovirus reported a similar disassembly but distinct assembly mechanism, which indicates that capsid assembly and stability could be genotype specific [47,48].

Strain-dependent stability profiles

Unlike Norwalk VLPs, native MS revealed high stability of another variant [49]. Interestingly, this GI.17 variant

Table 1

Stability studies of norovirus VLPs. Norovirus VLP stability has been targeted using many different methods and experimental approaches. Here, we compare results of stability studies under various pHs, temperature, ionic strength and other conditions.

genogroup	isolate	reference	methods*	particle size	pH dependency (stable - disassembled - not tested)										temperature, additives, ionic strength and other factors
					3	4	5	6	7	8	9	10			
GI	GI.1 Norwalk	Ausar (2006)	EM, fluorescence, UV absorption, CD, DLS, DSC	45 nm at pH 7; 28 nm at pH 8	3	4	5	6	7	8	9	10	stable up to 55 °C, citrate-phosphate buffer		
		Baclayon (2011)	IMMS, AFM	39 nm; 29 nm S-particles	3	4	5	6	7	8	9	10	role of P domain in capsid stability, increased stiffness under prestress		
		Bertolotti-Ciarlet (2002)	EM, CryoEM, ELISA, Gradient centrifugation	38 nm; 45 nm (P domain mutant); 30 nm (S-particles)	3	4	5	6	7	8	9	10	effect of mutants and deletions on particle size and stability		
		Boyd (2015)	<i>in silico</i> nanoindentation	-	3	4	5	6	7	8	9	10	the interface building a quasi-sixfold symmetry axis (B/C of S domains) is the most instable one		
		da Silva (2011)	DLS, Electrophoretic mobility, QCM-D Adsorption Studies	35 nm	3	4	5	6	7	8	9	10	different attachment efficiencies depending on buffer and ionic strength		
		Kissmann (2008)	CD, fluorescence, DSC	-	3	4	5	6	7	8	9	10	influence of vaccine excipients (sucrose, trehalose, sorbitol, mannitol, lactose, glycerol, dextrose, chitosan); chitosan, glutamate, sucrose, and trehalose enhance stabilization and inhibit aggregation (> 60 °C)		
		Shoemaker (2010)	native MS, IMMS, AFM	various depending on condition	3	4	5	6	7	8	9	10	higher ionic strength reduces alkaline sensitivity		
		Utrecht (2011)	native MS, IMMS	various depending on condition	3	4	5	6	7	8	9	10	shape of small assemblies		
	GI.1 West Chester	White (1997)	EM, sucrose gradient centrifugation	23 nm; 38 nm	3	4	5	6	7	8	9	10	investigation of different capsid sizes		
		Pogan (2018)	native MS, EM	40 nm at pH 6 and 7; also 20 nm pH 6-8	3	4	5	6	7	8	9	10	higher ionic strength reduces alkaline sensitivity		
GI.4 Chiba	Someya (2011)	EM	23 nm; 38 nm	3	4	5	6	7	8	9	10	investigation of different capsid sizes			
		CD, Near-UV intrinsic fluorescence	-	3	4	5	6	7	8	9	10	slight losses of ordered secondary structure at higher temperature and ionic strength			
GII	GII.4 Guangzhou	Yao (2014)	CD, EM	38 nm (VP1 only and VP1/VP2)	3	4	5	6	7	8	9	10	VP2 might have a stabilizing effect on VLPs		
		da Silva (2011)	DLS, Electrophoretic mobility, QCM-D Adsorption Studies	40 nm	3	4	5	6	7	8	9	10	different attachment efficiencies depending on buffer and ionic strength		
	GII.4	Huo (2015)	EM	38 nm; 21 nm	3	4	5	6	7	8	9	10	investigation of different capsid sizes		
	GII.4	Samandoulgou (2015)	CD, Near-UV intrinsic fluorescence	-	3	4	5	6	7	8	9	10	stable at different ionic strengths; small structure changes at higher temperature		
	GII.7	Cuellar (2010)	AFM	28-47 nm	3	4	5	6	7	8	9	10	tested range pH 2 – 10, size increase at basic pH		
	GII.10 Vietnam	Koromyslova (2015)	EM, ELISA, X-Ray crystallography	43-48 nm depending on citrate concentration	3	4	5	6	7	8	9	10	additional ring-like structure after treatment with citrate buffer		
	GII.17 Kawasaki 308	Pogan (2018)	native MS, EM	-	3	4	5	6	7	8	9	10	high and low ionic strength tested		
GIII	GIII.2 Bo/ Newbury2	Tresset (2013)	EM, DLS, SAXS, SANS	-	3	4	5	6	7	8	9	10	low ionic strength promotes disassembly and vice versa		

*Method abbreviations: AFM (atomic force microscopy), CD (circular dichroism), DLS (dynamic light scattering), DSC (differential scanning calorimetry), ELISA (Enzyme-linked Immunosorbent Assay), EM (electron microscopy), IMMS (ion mobility mass spectrometry), MS (mass spectrometry), QCM-D (Quartz Crystal Microbalance with Dissipation), SANS (small-angle neutron scattering), SAXS (small-angle X-ray scattering), UV (ultraviolet) absorption.

was causing gastroenteritis outbreaks in Asia and showed increased prevalence in the last years [50]. Both native $T = 3$ and smaller $T = 1$ particles were observed from pH 6 to pH 10 [49**]. Additionally, a close relative to the GI.1 Norwalk, GI.1 West Chester, showed a Norwalk-like ionic strength dependent disassembly pattern. However, native size capsids showed increased sensitivity to alkaline treatment and VP1₆₀ was already observed at neutral pH. Smaller particles of $T = 1$ formation were also found in Norwalk VLP preparations, although in low abundance [49**,51]. Further studies on GII variants, also forming these smaller particles, proposed VP1 truncation and variable purification conditions as putative reasons [52,53]. However, significant VP1 truncations were not observed in MS and the ability of GI.1 West Chester to reassemble normal $T = 3$ particles from small oligomers indicates that altered balance between $T = 1$ and $T = 3$ capsids is an intrinsic protein property and in agreement with reversible assembly of Norwalk VLPs. This suggests that a few substitutions can shift the balance between capsid sizes.

Stability differences of NoVs from different genogroups were also tested with other biophysical techniques. For example, human NoV variants of GI.1 and GII.4, and FCV were compared using CD and intrinsic UV fluorescence. Less temperature and pH induced structural changes were observed for GII.4 than for the other particles [54]. When solution chemistry properties of GI.1 Norwalk and GII.4 Houston VLPs were compared, Norwalk VLPs aggregated at pH 9 with increasing hydrodynamic radii. The GII.4 variant was stable up to pH 8, however pH 9 was not examined. Furthermore, the two variants showed different attachment efficiencies [39]. Sensitivity to alkaline treatment was also observed for a GII.7 isolate via AFM nanoindentation. From pH 8.5 to pH 10, the observed spring constant dropped indicating decreased capsid stability. Notably, capsids of increased size could still be detected at pH 10 [49**,55]. Comparing these findings to AFM on Norwalk VLPs would be of interest.

Concluding remarks

Norovirus VLPs have been used to study core features of capsid assembly and stability. Biophysical methods like CD spectroscopy are extremely helpful to characterize VLPs. However, they often fail to describe structural changes thoroughly as smaller oligomers are poorly resolved. In AFM nanoindentation, biophysical properties of capsids like size and stiffness can be probed and techniques like IM and native MS are a great tool to monitor stability and decipher smaller capsid assembly states. Insights to capsid disassembly mechanisms could improve the design of capsids for nanotechnological applications and therefore would also be beneficial for norovirus vaccine development. Especially, the latter is extremely hindered by the frequent emergence of

outbreak causing variants. Lately, some worldwide outbreaks were caused by an emerging recombinant polymerase genotype showing mutations in the RNA polymerase gene as well as the VP1 [56]. This indicates that polymerase genotypes can also play a role in emergence, but are usually accompanied by mutations in the capsid protein [57].

Several different NoV isolates were targeted in the studies reviewed (Table 1). On the virion level, stability differences between human NoV and norovirus surrogates were reported. Differences between human NoV isolates were not addressed, although different PCR reduction rates were indicated for tested variants [22,58,59]. In combination with VLP studies, these findings indicate distinguishable stability profiles of different isolates. Notably, a clear comparison was hindered due to variability in tested conditions such as pH range and lacking information on used isolates. On the other hand the broad set of techniques used underpins that many isolates show reduced stability under alkaline conditions.

The infective virus likely exhibits altered stability due to presence of VP2, VPg and RNA. The VPg protein is covalently linked to viral RNA and required for infectivity [60]. Considering these factors, biological relevance of VLP studies is limited. However, until cell culture systems become routine, systematic studies on capsid stability of norovirus isolates of different prevalence are required in order to understand and link their structural properties. This could confirm that genetically close isolates exhibit distinct size and stability profiles and decipher the underlying structural requirements. For adequate comparison, systematic reporting of the exact norovirus isolate is necessary. Nevertheless, available data suggests that isolates could be distinguished by stability. Following this feature by determination of mass and mechanical properties in patient samples could have potential as a diagnostic tool.

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