MS2 Virus-like Particle as an Efficient Drug Delivery Tool: Optimizing the Synthesis and Purification Protocol

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Abstract

Introducing bacteriophage MS2 virus-like particles (VLPs) as gene and drug delivery tools increases the demand for optimizing their production and purification procedure. PEG precipitation method is used efficiently to purify VLPs, while the effects of pH and different electrolytes on the stability, size, and homogeneity of purified MS2 VLPs, and the encapsulated RNA sequences remained to be elucidated.

In this regard, a vector, capable of producing VLP with an shRNA packed inside was prepared. The resulting VLPs in different buffers/solutions were assessed for their size, polydispersity index, and ability to protect the enclosed shRNA. We report that among Tris, HEPES, and PBS, with or without NaNO₃, and also NaNO₃ alone in different pH and ionic concentrations, the 100mM NaNO₃-Tris buffer with pH:8 can be used as a new and optimal MS2 VLP production buffer, capable of inhibiting the VLPs aggregation. These VLPs show a size range of 27-30nm and suitable homogeneity with minimum 12-month stability at 4°C. Moreover, the resulting MS2 VLPs were highly efficient and stable for at least 48 hours in conditions similar to *in vivo*. These features of MS2 VLPs produced in the newly introduced buffer make them an appropriate candidate for therapeutic agents' delivery.

Introduction

Delivering small organic or inorganic molecules into the biological systems for therapeutic and research purposes has always been a challenge for biologists. In addition to natural barriers challenging the reach of the target organ and further, inside the cells, the stability of the material of interest to be delivered and that of the delivering agent, plus their safety and toxicity levels introduce additional limitations and challenges. In this line, various approaches such as those involving nanoscale platforms have been tested to find optimal delivery agents^{1,2}. Viruses have naturally evolved optimal and efficient targeted cargo delivery and, hence, have successfully been used by biologists for the same purposes. However, nearly all of the naturally occurring viruses that may be most suitable for a specific cargo delivery purpose are also pathogenic and/or posing other threats. Therefore, various modifications are applied to a virus before it could safely be used as a vector. Virus-like particles (VLPs), which are frequently used by biologists for targeted and efficient cargo delivery, are viral particles normally devoid of considerable pathogenicity.^{3,4} The MS2 VLP is a 27nm spherical RNA bacteriophage virus-like particle, consisting of 180 identical 129-amino acid coat protein subunits of ~ 14 kDa that form the icosahedral face for a T = 3 surface lattice⁵⁻⁷. MS2 VLP due to its small size and special shape, as well as its ability to encapsulate different nucleic acids, proteins, and other small molecules, and its endocytic route of entry has been considered a suitable vector for delivering into and tracking cargoes in the targeted cells⁸. The coat proteins of MS2 VLP can be recombinantly expressed and assembled in bacteria and yeasts⁹⁻¹². It is conveniently prepared, packaged, delivered, and has high safety margins. Thus, the MS2 VLP has been used as a potential medicinal carrier¹³ for the treatment of various diseases such as prostate cancer¹⁴, hepatocellular carcinoma^{15,16}, and other diseases^{8,17-20}. It has also been used for various research purposes including the investigation of RNA trafficking in live cells²¹, in platforms of vaccines and peptide epitope identification²²⁻²⁷, and quality control of DNA and RNA virus detection²⁸⁻³⁰.

It is well characterized that nucleic acids and other small molecules can be packaged in the MS2 VLP via their interaction with a short RNA stem-loop structure (19 nucleotides: *pac* site), which in turn binds to coat protein dimers^{5,31-33}. Thus, the interaction with the 5'-terminus of the so-called *pac* site has been used as a preferred strategy to encapsulate different cargoes^{2,8}. In this respect, it is very important to understand the behavior and assembly of virus-like particles and their aggregation and stability in different conditions. To make efficient VLPs, various physicochemical factors such as pH, temperature, and ionic strength of the solution, the ideal size, homogeneity, and stability of produced VLPs, and their capability to protect cargoes from degradation need to be taken into account. Likewise, in the recombinant production of MS2 VLPs, upon the transformation of plasmids expressing MS2 coat protein and a part of maturase into the bacteria, the most appropriate pH and buffers to support the stability and homogeneity of VLPs are sought after^{12,34-36}.

Different studies have shown that the amounts of electrolytes are important factors for the assembly of MS2 bacteriophage and related VLPs^{8,9,35}. On the other side, if we consider the p*H*_c as a critical pH value of the buffer used for the production of particles, the values below and above p*H*_c may lead to repulsive electrostatic interactions and stable suspension of VLPs^{9,37}. It has been shown that MS2 VLP particles remain in the detached form at 100mM NaNO₃ concentration of buffer solution, and the isolated forms of VLPs in low ionic strength (1mM and 10mM) of NaNO₃ buffer were merely seen at a pH value near the isoelectric pH (between 3 and 4)⁹. In this study, we introduce a new and optimized buffer to prepare the most stable condition for VLPs. To simulate the *in vivo* conditions, we assess the stability of VLPs produced in this buffer after incubation in 50% FBS and at 37°C temperature. We report improved buffer conditions that are used for the preparation of VLPs with appropriate size, shape, and stability.

Results

NaNO₃-Tris (100mM, pH:8) buffer provides the best condition for shape, particle size consistency, and dispersity of VLPs.

To confirm the expression of the coat protein of VLPs, ~ 14kDa disassembled protein of purified MS2 VLPs was separated by electrophoresis on 12.5% acrylamide gel and detected using specific antibody and Western blot method. BL21 (DE3) bacterial lysate showed both monomers and dimers of coat protein (Supplementary. Fig. S1).

The influence of different NaNO₃ concentrations (1mM and 100mM, pH:8) and pH values (7 and 8) were assessed in MS2 VLP expression, production, and purification by the PEG precipitation method. The reduction of NaNO₃ concentration could increase the particle size to larger than 100nm ($p < 0.001$, Fig. 1a), probably because of PEG aggregation around the MS2 VLPs or agglomeration of the VLPs. PDI values in the 100mM NaNO₃ solution were lower than the values in the 1mM NaNO₃ solution (Fig. 1b). While TEM results indicated the size and shape variation in the 1mM NaNO₃ solution, the MS2 VLPs in the 100 mM NaNO₃ solution disclosed much more shape uniformity. The 1mM concentration of NaNO₃ induced MS2 VLPs deformation (Fig. 1c).

Decreasing the pH of 100mM NaNO₃ solution from 8 to 7 drastically increased the average size of particles (Fig.1d, $p < 0.0001$). Also, the PDI values were increased in lower pH (Fig.1e). TEM analysis of VLPs at different time points (during 35 days after VLP preparation) indicated a reduction in the size and an increase in the uniformity of VLPs when the 100mM NaNO₃ solution (with pH 7 or 8) was used (Fig.1f, g). Additionally, more negative zeta potential values were registered in 100mM NaNO₃ (pH:7) compared to 100mM NaNO₃ (pH:8) (Supplementary, Fig. S2). To investigate the cause of these changes, the pH of NaNO₃ solution was evaluated through different time points during a 60 days period which showed the reduction of pH value from 8 to 5.5 (Fig.1h). pH values below 6, resulted in instability and dissociation of MS2 particles.

Considering the severe pH fluctuation of NaNO₃ solutions, during MS2 VLP production and purification, different buffers were used to stabilize the pH-dependent changes in particle size and PDI. As shown in Figs. 2a and b, the type of buffer had a significant effect on the particle size and PDI. While particle size and PDI of VLPs produced in HEPES (pH: 8) were higher than 1 μ m (out of range of detection), lowering the pH to 7.4 could reduce the size and expand its PDI variation between 0.05 to 0.33. TEM analysis was performed on samples with particles smaller than 100nm in size. The results showed more homogeneous particles in Tris buffer (pH:8) compared to particles in PBS buffer (pH:7.4) (Fig. 2c, and Supplementary Fig. S3b).

To find a more effective and stable production buffer for PEG precipitation of VLPs, solutions of NaNO₃ (100mM) in Tris (1M, pH: 7.4 and 8), HEPES (pH: 7.4 and 8), and PBS (pH:7.4) were examined. In general, NaNO₃ addition to various buffers boosted the VLP preparation efficiency. Although the particles in NaNO₃-HEPES (pH:7.4) were smaller than particles in NaNO₃-HEPES (pH:8) (extremely large, out of range of detection), yet the size of particles in NaNO₃-HEPES (pH:7.4) was bigger than the size of the native MS2 bacteriophage (27-30nm) (Fig. 2d). The average PDI value was about 0.31 for particles in NaNO₃-HEPES (pH:7.4). In this case, suspensions of VLPs with particle sizes below 100nm were observed by TEM microscopy. Additionally, VLPs produced in NaNO₃-Tris (pH:7.4) were much bigger than particles in NaNO₃-Tris (pH: 8) ($p < 0.0001$). VLPs in NaNO₃-PBS (pH:7.4) displayed a much smaller size than the native MS2 bacteriophage (27-30nm). PDI measurement of VLPs produced in NaNO₃-Tris (pH:7.4) showed a wide size distribution in comparison to VLPs in NaNO₃-Tris (pH: 8), which was also confirmed by TEM analysis (Fig. 2e and f). This discrepancy might be explained by the VLP instability in a narrow pH range, so that only small differences in pH may lead to MS2 VLP aggregation. Zeta potential could not be evaluated because of the buffering nature of HEPES, PBS, and Tris (Supplementary, Fig. S2).

MS2 Virus-like particles produced in the NaNO₃-Tris (pH:8) were more stable than VLPs produced in the NaNO₃ solution (pH:8).

To assess the stability of VLP coat proteins in NaNO₃ (100mM, pH:8) solution and NaNO₃-Tris (100mM, pH:8) buffer, protein concentration was measured by spectrophotometry. Protein concentration and the number of VLPs in NaNO₃ (pH:8) solution decreased over time (1, 2, 3, 4, 7, and 12 months), while it was stable for VLPs in NaNO₃-Tris (pH:8) ($\approx 40\text{mg ml}^{-1}$, $\approx 9 \times 10^{15}$) (Fig. 3a and b). Moreover, particle size assessment of VLPs in two different solutions noticeably indicated the narrow and remarkably homogeneous distribution of VLPs produced in NaNO₃-Tris (pH:8) buffer (Fig. 3c, Supplementary, Fig. S3a).

Considering the RNA sequence packed in MS2 VLPs (Fig. 3e), we performed RT-PCR to evaluate the stability of these RNAs in NaNO₃ (pH:8) and NaNO₃-Tris (pH:8) solutions. Four amplicons (99, 138, 177, and 216 bp) were detected on 2% agarose gel. These amplicons for VLPs generated in NaNO₃-Tris (pH:8) buffer were detectable for 12 months, while for VLPs produced in NaNO₃ solution they were detected for 3 months (Fig. 3d).

VLPs prepared in NaNO₃-Tris buffer remained stable in the presence of serum

To make an environment similar to *in vivo* condition and in favor of further using VLPs in pharmaceutical studies, MS2 VLP suspensions produced in NaNO₃ (100mM, pH: 7 and 8) solutions and NaNO₃-Tris (100mM, pH:8) buffer were incubated with different serum concentrations (10%, 30%, and 50%). While VLPs in NaNO₃ (100mM, pH:7) had a bigger size than VLPs in the same buffer with pH:8, by rising serum concentrations, a decreasing trend of VLP size was observed which was an indicator of serum effects on PEG dispersity. In contrast, the size of VLPs prepared in NaNO₃-Tris (100mM, pH:8) buffer was stable in different serum concentrations (Fig. 4a, c, e, and Supplementary Fig. 4a, b, c). It has been shown that PDI values below 0.7 provide particle size distribution in the midrange^{38,39}. Although the PDI values in all three experiments were below 0.45 and, in the midrange, the effect of different concentrations of serum on PDI variation was evident in NaNO₃ (100mM, pH:7, and 8) solutions (Fig. 4b and d). These changes did not occur for the VLPs produced in NaNO₃-Tris (100mM, pH:8) buffer, with values around 0.3 which are acceptable for drug delivery (Fig. 4f).

RNA extraction and RT-PCR experiments for VLP samples generated in NaNO₃-Tris buffer (pH:8) in the presence of 50% serum concentration revealed the existence of RNA sequences and their stability up to 48 hours at 37 °C (Fig. 4g).

VLPs prepared in NaNO₃-Tris buffer can efficiently enter the cells.

To determine whether the VLPs prepared in NaNO₃-Tris buffer could transduce the cells, we used of Neuro2A cell line as immature neuronal cells (neuroblastoma) which could not transfect easily. Immunocytochemistry was then performed using the MS2 bacteriophage coat protein antibody. Bright intense red spots seen after 1 hour, were then dispersed in the cytoplasm after 4 hours, indicating the VLP entrance and probable disassembly of the VLPs over time (Fig. 5)

Table 1
Primers and oligonucleotides used in this study

name	Oligonucleotide sequence (5'—3')	Products (bp)	Application
MS27.Fw MS27.Rev	CGGGATCCTGGCTATCGCTGTAGGTAGCC CCCAAGCTTATGGCCGGCGTCTATTAGTAG	1680	amplification of MS2 bacteriophage coat protein sequence
shRNA	GCCTACTCGAACCGTTGATTTCAAGAGAATCAACGGTTCGAGTAGGC	-	Checking the stability of VLPs
shRNA-4pac (sense)	AGATCTGGATCCGGCCTACTCGAACCGTTGATTTCAAGAGAATCAACGGTTCGAGTAGGCCATATGTAA CGATCGTAATTGCCTAGAAAACATGAGGATTACCCATGTCTGCAGGTCGACTCTAGAAAACATGAGGAT TACCCATGTCTGCAGGTCGACTCTAGAAAACATGAGGATTACCCATGTCTGCAGGTCGACTCTAGAAAAC ATGAGGATTACCCATGTCTGCAGTATTCGCGGGTTCATTTACGTACTAGCATAACCCCTTGGGGCCTCTAA ACGGGTCTTGAGGGGTTTTTTGGGTACC	-	shRNA Packaging in the VLPs
shRNA-4pac (anti-sense)	CCAAAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTACGTAAATGAACCCGGGA ATACTGCAGACATGGGTAATCCTCATGTTTTCTAGAGTCGACCTGCAGACATGGGTAATCCTCATGTTTTT TAGAGTCGACCTGCAGACATGGGTAATCCTCATGTTTTCTAGAGTCGACCTGCAGACATGGGTAATCCTC ATGTTTTCTAGGCAATTACGATCGTTACATATGGCCTACTCGAACCGTTGATTCTCTTGAAATCAACGGTT CGAGTAGGCCGGATCCA	-	shRNA Packaging in the VLPs
VLP.Fw VLP.Rev	CCTACTCGAACCGTTGATTTCAAGAG TGCAGACATGGGTAATCCTCATG	99, 138, 177,216	Checking the stability of VLPs

Discussion

The MS2 VLP is well known amongst different researchers for its small size and great potential of being a promising gene and drug delivery platform [8, 14, 15, 17, 23, 37]. In this study, we focused on the optimization of the buffers which are used in the precipitation and purification process of VLPs. The ability of different cargos to be encapsulated in VLPs without time-dependent degradation is of great importance ⁴. Thus, the most appropriate buffer and its characteristics including pH and ionic strength were identified. Our results led to a new VLP preparation strategy using NaNO₃-tris (100mM, pH:8). This buffer greatly enhanced the consistency (Fig. 3), homogeneity (Fig. 4e and f), and half-life of VLPs (Fig. 3d).

Along with VLP synthesis, various purification strategies such as dialysis ^{2,9,31}, density gradient by cesium chloride ^{2,4,8,41}, or sucrose ^{2,42}, and PEG precipitation methods have been examined by different researchers. In this study, we used PEG for VLP purification to avoid probable virus aggregation which has been reported in some examples of the CsCl purification method ^{43,44}. Indeed, between all the different purification protocols, polyethylene glycol (PEG) precipitation ⁴⁵, is the less-toxic and low-cost method, appropriate for purifying proteins from various protein sources ^{9,46}. Previous studies have shown that various physicochemical factors can affect the capsid stability and aggregation of VLPs [2, 9, 12, 29, 30, 33]. Among these, pH and ionic strength are the most important factors. Na⁺ containing solutions are used as the most acceptable ionic solutions in the PEG precipitation method ^{2,9,47}, considering the fact that in monovalent electrolytes such as NaCl, KCl, and LiCl, aggregation of MS2 does not occur within a reasonable kinetic time frame, and MS2 was stable even at high salt concentrations ³⁵. Thus, we used the NaNO₃ solution to prepare an appropriate ionic environment. The shape of VLPs was analyzed by the TEM method. Furthermore, the particle size and PDI were measured as pharmaceutical characteristics of particles that influence endocytosis and cellular uptake.

Irrespective of the purification method, the preparation of MS2 VLPs in two concentrations of NaNO₃ solution (1mM and 100mM) in different ranges of pH (2 to 7) revealed higher stability of VLPs in 100mM NaNO₃ (pH:7) because of its high electrolyte concentration ², pH effectiveness ², and minimum mobility ⁴⁷. Despite the previous studies showing the stability of virus nanoparticle suspension against aggregation in ≤ 1M NaCl for more than 10h ³⁵, in this study, the same assessment of two concentrations at the stable pH:8 resulted in different sizes of VLPs (Fig. 1). While the VLPs in

100mM NaNO₃ solution (pH:8) were smaller than those VLPs in 1mM NaNO₃ solution (Fig. 1a and c, $p < 0.001$), PDI of these particles in both concentrations (Fig. 1b) were in the moderate range (0.1–0.3) which could be more acceptable for drug delivery.

The pH of the NaNO₃ solution and concentration of ions drastically affects the stability and aggregation of particles. The pattern of these changes for MS2 bacteriophage is completely different from that of MS2 VLP⁹. VLPs produced in 100mM NaNO₃ at pH:8 displayed a smaller size ($p < 0.0001$) compared to VLPs produced at pH:7 (Fig. 1d). However, even in 100mM NaNO₃ solution (pH: 8) with a smaller size, the size of these VLPs (median: 69.28 nm) is larger than MS2 bacteriophage (27-30nm). Observation of larger VLPs might have resulted from the presence of PEG on the surface of VLPs. Sixty days after VLP preparation in 100mM NaNO₃ solution (pH:7), an electron microscopy assay revealed the presence of isolated and uniform VLPs, while immediately after preparation, VLPs were aggregated. Since MS2 VLPs are stable within a narrow pH range [2, 9, 29, 31, 42], a feasible explanation for VLP dissociation over time could be the decreasing trend of pH of NaNO₃ solution from 8 to 5.5 (Fig. 3f and g).

Tris (pH:8), HEPES (pH:7.2–7.4), and PBS (pH:7.4) buffers, alone or mixed with some electrolytes and chemicals such as MgSO₄, NaCl, and EDTA in different molarities have been chosen in various protocols as a protective buffer for VLP preparation and storage [8, 24, 26, 38, 43–45]. Applying these buffers for MS2 VLP synthesis determined that the VLPs in Tris Buffer (pH:8) are more uniform and smaller than VLPs in HEPES and PBS buffers (Fig. 2). Despite the uniformity of VLPs in Tris Buffer, these VLPs due to incomplete disassembly or inappropriate assembly are smaller (median: 19.96nm) than MS2 bacteriophage (27–30 nm). The diameter of VLPs in the HEPES buffer at pH values of 7.4 and 8 are different. While the particles were larger than VLPs in Tris and PBS buffers at pH:7.4, the particle sizes were undetectable at pH:8 probably due to agglomeration of the VLPs (Fig. 2).

Considering the pivotal role of monovalent cations in VLP formation and stability³⁵ and to improve the VLPs' synthesis process, we employed the new VLP formation strategy by mixing Tris, HEPES, and PBS buffers with NaNO₃ (100mM final concentration). VLPs in Tris buffer (1M, pH:8) containing NaNO₃ (100mM) displayed a similar size to MS2 bacteriophage which is the convenient size for delivering, whereas VLPs in the same buffer at pH: 7.4 showed the maximum size among all the newly developed buffers (Fig. 2d). Interestingly, adding NaNO₃ as an electrolyte to the buffer decreased the size of VLPs, but did not cause a considerable inhomogeneity (Fig. 2e and f). Adding CaCl₂ to the aqueous suspension of MS2 bacteriophage leads to the aggregation of virus nanoparticles³⁵.

In line with the VLPs' time-lapse stability experiments, previous studies have determined that particles are stable just for 3–4 weeks in 10mM HEPES and 100mM NaCl⁴⁰, and remain stable in PBS for > 3 months⁸ at 4 °C. Another study has shown that the stability period of MS2 VLP is at least 4 months at -20 °C in PBS²⁹. While VLPs in NaNO₃ solution (100mM) are stable for 3 months at 4 °C, preparation of VLPs in NaNO₃-Tris buffer (100mM, pH: 8) can increase the stability period to more than 12 months (Fig. 3d). Protein concentration is a reasonable marker for evaluating VLP stability. Contrary to VLPs prepared in NaNO₃ solution which were degraded over time, protein concentration remained invariable for 12 months in NaNO₃-Tris buffer (100mM, pH: 8) (Fig. 3a). The increased stability of VLPs makes them a suitable candidate to be used in vaccination and drug delivery.

VLP's behavior and stability in serum are the most critical parameters for delivery. A previous study has shown that MS2 VLPs kept in serum at 4 °C display the best stability for 3 days²⁹. On the other hand, the stability of some VLPs in the serum-containing medium at 37 °C for 2 hours does not affect VLP's aggregation or disassembly⁵¹. Increasing serum concentration in VLP suspension up to 50%, inhibited VLPs from agglomeration. Although the reduction in VLP's diameter happens probably via serum protein interaction with VLP's coat protein or PEG's removal, increasing serum concentration of VLPs suspension in NaNO₃-Tris (100mM, pH: 8) did not affect the size. Moreover, in the presence of 50% serum at 37 °C, the shRNA sequence inside the VLPs was protected against degradation for 48 hours (Fig. 4g). These results besides the other characteristics of MS2 VLPs prepared and stored in NaNO₃-Tris (100mM, pH: 8), and also the reliable cell entry properties, represents MS2 VLP as a good candidate for gene and drug delivery. Therefore, any attempt at optimizing its generation and enhancing the physicochemical properties would be very helpful. Our results demonstrate that using NaNO₃-Tris (100mM, pH: 8) buffer in the process of MS2 VLPs formation, substantially increases the quality and stability of the generated VLPs.

Material And Methods

No animal was used in this study.

MS2 VLP Plasmid construction and expression system

MS2 VLP expression vector was prepared by amplifying the MS2 Coat and a part of maturase DNA sequences from the pMS27 Plasmid (BCCM/LMBP plasmid Collection, Cabri, Belgium) (Table 1)¹⁸. The amplified sequence was inserted in-frame into pACYCDuet plasmid (Novagen, Gibbstown, NJ, USA) through BamH1 and HindIII restriction enzyme (Thermo Fisher Scientific Inc., USA) sites. The oligonucleotide sequence encoding the shRNA sequence was designed by siRNA Wizard™ software (Invivogen, USA) (Table 1). The synthesized shRNA (MacroGen Inc., South Korea) fused to four repeats of MS2 bacteriophage *pac* sequence (MacroGen Inc., South Korea) was sub-cloned into the other multiple cloning sites of pACYCDuet plasmid through BglII and Kpn1 restriction enzyme (Thermo Fisher Scientific Inc., USA) sites (Fig. 3e). This sequence (shRNA-4pac,

Table 1) was used as a control RNA sequence with the ability to be packed into VLP in each step, plasmid construction was verified by Sanger sequencing (Macrogen Inc., South Korea) and was named the SC Duet.

The SC Duet plasmid was introduced into the BL21(DE3) strain of *E. coli* Bacteria as a prokaryotic expression system. The *E. coli* BL21(DE3)-SC Duet was cultured in a terrific broth medium at 37 °C, supplemented with 34µg/ml chloramphenicol (pACYCDuet antibiotic selection). The expression of the sequences was induced by 1mM IPTG (isopropyl β-D-1- thiogalactopyranoside, Thermo Fisher Scientific Inc., USA) at an OD₆₀₀ =0.6 for 16 hours at 22 °C. Bacteria were precipitated by centrifugation (6000g, 20 min, 4 °C), and the derived precipitate was resuspended in the appropriate buffer (in 1/5th of the initial bacterial culture volume). Lysozyme treatment (0.05 mg ml⁻¹) was performed for 30 min at room temperature followed by sonication (total time: 3 min, 51W). Cell debris was collected by centrifugation (13500g, 20 min, 4 °C). The supernatant containing VLPs was filtered through a 0.22 µm membrane (Jet Bio-Filtration Co, China) and stored at 4 °C for further examination². To eliminate cell debris, the suspension was filtered through a 0.1 µm membrane (Sartorius, Germany) and then MS2 VLPs were purified according to the polyethylene glycol (PEG) precipitation method⁴⁵. In brief, the precipitation of VLPs was performed by adding PEG (MW: 6000, Sigma Aldrich, USA) at 10% of the final concentration (w/v) and 500 mM NaCl. The suspension was incubated at 4 °C for 18h and subsequently was centrifuged at 10000g for 1h, 4 °C. The pellet containing MS2 VLPs was resuspended in the buffer used in the first step of purification (in 1/10th of the initial resuspension volume). Eventually, this MS2 VLP suspension was filtered through a 0.22 µm membrane (Jet Bio-Filtration Co, China). All MS2 VLP samples were kept at 4 °C. Solutions of 1mM, and 100mM NaNO₃ (Merck & Co., Inc. USA), 1M Tris (Sigma Aldrich, USA) buffer, and HEPES Buffer (Biowest, France) were prepared fresh, one day before experiments.

MS2 VLPs disassembly and Western blot analysis

To confirm the expression of VLP coat proteins, purified MS2 VLPs were disassembled by diluting 1:2 in 20mM cold glacial acetic acid for 1 h at 4 °C. To precipitate the free nucleic acids, samples were centrifuged at 4 °C (16000g, 20 min). Then, we assessed the expression of coat protein by SDS-PAGE (12.5% acrylamide gel). Proteins were transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc, USA). The membrane was blocked with 5% non-fat dry milk overnight at 4 °C and then incubated with a polyclonal Anti-Enterobacteria Phage MS2 coat protein antibody (1:7000 dilution, Millipore Sigma Co. USA) for 3 hours at room temperature. Incubation with HRP-labeled secondary antibody (1/18000 dilution, Abcam, USA) for 1 hour was performed to confirm the presence of 14 and 28k Da bands. The membrane was developed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., USA). Signals were detected using the Chemiluminescence Detector system (G: BOX Chemi XT Analyser, SYNGENE, Eur.).

Transmission electron microscopy

The MS2 VLPs were diluted at 1:4 in the buffer. 10µl of samples were dropped onto carbon-coated copper grids and then were negatively stained by 2% uranyl acetate. After drying, VLPs were observed by transmission electron microscopy (Leo912AB, Germany) at 120KV at 4000X, 10000X, 25000X, 31500X, 50000X, 63000X, and 80000X magnifications.

Particle size, polydispersity index evaluation, and Zeta potential evaluation

MS2 VLPs were diluted 1:4 in selected buffers and solutions. Particle size and polydispersity index (PDI) were measured by particle size analyzer (Vasco3-Cordouan, France) based on Dynamic Light Scattering (DLS) at 25 °C. Samples were analyzed by cumulants analysis of 5 consecutive measurements.

Zeta potential for those samples which were not stored in Buffer was estimated by Zeta potential detector (Zeta compact-CAD, France) and reported as zeta potential (mV) line plot at 25 °C. To ensure reliability, it has been performed at least three times.

pH assessment

The pH of all solutions and buffers were measured by a pH meter (Mettler Toledo Co., Switzerland), one day before the experiment. Evaluations were done in triplicates at 0 h, 3 h, 12 h, 24 h, 7 days, and 1-month time points.

Protein spectrophotometry and MS2 VLPs measurement

Protein concentration was measured by absorbance at 280nm (A_{280})-1 Abs in 1cm (1mg ml⁻¹) (Epoch2™ BioTek, Agilent Technologies, USA). Measurements were performed three times at different time points (1, 2, 3, 4, 7, and 12 months). According to the protein concentration and the molecular mass of each VLP particle (14 kDa per coat protein x 180 coat proteins per VLP= 2520 kDa), the number of VLPs in 100mM NaNO₃ solution and NaNO₃-Tris buffer (pH:8) was calculated:

Each VLP molecular mass = 2520 kDa = 4.18e-15mg

Number of VLPs (in each milliliter) = protein concentration of VLP suspension (mg ml⁻¹)/ 4.18e-15

The stability of MS2 VLP containing shRNA-*pac* in serum

To determine MS2 VLP's stability in biological conditions, samples were mixed with fetal bovine serum (Biowest, France) (1:1), incubated in 37 °C for 2, 4, 6, 8, 10, 12, 24, and 48h, and then stored in -80 °C for further RNA isolation, reverse transcription, and PCR. Furthermore, MS2 VLPs were also incubated in different serum concentrations (10, 30, 50%) for 1 hour at 4 °C and particle size and PDI were evaluated diluting 1:4 with additional buffer.

RNA extraction, reverse transcription, and polymerase chain reaction

300ul of MS2 VLP suspension in different solutions or buffers (after purification by PEG precipitation method) were subjected to RNA isolation using the Total RNA Isolation Kit (DENArist Asia Co., Iran) according to the manufacturer's instructions. The quantity of RNA was determined by a spectrophotometer (spectroEpoch2™ BioTek, Agilent Technologies, USA). A total of 1 µg RNA was used for cDNA synthesis using random hexamer and MMLV reverse transcriptase (Thermo Fisher Scientific Inc., USA). To identify and check the integrity of shRNA-4pac RNA, polymerase chain reactions were carried out by Taq DNA Polymerase Master Mix (Ampliqon, Denmark) and designed primers (Table 1). Polymerase chain reaction steps were 94 °C for 5 min, followed by 35 cycles of 30sec at 94 °C, 15sec at 70 °C, 15sec at 72 °C, and a final extension period of 10 min at 72 °C. PCR products were run on 2% agarose gel.

Cell culture and Immunocytochemistry

To confirm the VLP entrance, Neuro2A cells (Mouse Neuroblastoma cell line, ATCC) were cultured in Dulbecco's modified minimal essential medium (DMEM, low glucose) (Gibco: Thermo Fisher Scientific, USA) containing 10% (v/v) of FBS (Gibco: Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Biowest, France) at 37°C, 5% CO₂ and 95% humidity. Cells were grown on poly D-lysine (Sigma Aldrich, USA) coated coverslips in four groups (1hour and 4hours after VLP treatment, sham (without VLP), and blank negative control (with VLP treatment and without primary antibody). Briefly, cells were treated with 0.05µg/ml of VLP suspension 24hours after seeding. The cells were fixed by 2% paraformaldehyde (w/v) for 10min at room temperature. Subsequently, after washing with cold PBS, the cells were permeabilized with 0.25% Triton X-100 in PBS) for 10min at room temperature and blocked with PBS containing 1% BSA. Cells were then incubated with primary antibody (anti- Enterobacteria Phage MS2 coat protein, 1:5000, Millipore Sigma Co. USA) for 3 hours at room temperature. The coverslips were washed with cold PBS and then incubated for 1hour at room temperature in darkness with a secondary antibody (Monkey anti-rabbit Cy3 conjugated, 1:200, Jackson ImmunoResearch Laboratories Inc., USA). The coverslips were mounted on slides after washing with cold PBS. Stained cells were visualized using fluorescent microscopy (Nikon, Japan).

Statistical analysis

Data analysis was performed by GraphPad Prism (version 8.0.2, GraphPad Software Inc., CA). Results were analyzed using the Mann-Whitney and Kruskal Wallis test analysis.

Abbreviations

PEG: polyethylene Glycol, VLP: Virus-Like Particle, shRNA: short hairpin RNA, PDI: Poly Dispersity Index, TEM: Transmission Electron Microscopy, RT-PCR: Reverse transcription-polymerase chain reaction

Declarations

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions

KH: Conceptualization, Investigation, Formal analysis, Methodology, Writing- Original Draft, MMGS.: Writing- Review and editing, MRA.: Writing- Review and editing, Resources, BM-N: Writing- Review and editing, Formal analysis, MRB: Conceptualization, HD: Validation, Methodology, Writing-

Review and editing, Resources, AA-G: Supervision, Project administration, Validation, Methodology, Formal analysis, Writing- Review and editing, Funding acquisition.

Competing interest

Other Authors do not have competing interest.

A patent on the NaNO₃-Tris (100mM, pH:8) Buffer has been filed in The Organization of Registration of Documents and Real Estate of Iran by the Ferdowsi University of Mashhad with Kh. Hashemi, H. Dehghani, and A. Afkhami-Goli as inventors.

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Figures

Figure 1

NaNO₃-Tris (100mM, pH:8) buffer provides the best condition for shape, particle size consistency, and dispersity of VLPs.

Figure 2

The severe pH fluctuation of NaNO₃ solutions, during MS2 VLP production and purification, different buffers were used to stabilize the pH-dependent changes in particle size and PDI.

Figure 3

Figure 3

MS2 Virus-like particles produced in the NaNO3-Tris (pH:8) were more stable than VLPs produced in the NaNO3 solution (pH:8).

Figure 4

Figure 4

VLPs prepared in NaNO₃-Tris buffer remained stable in the presence of serum

Figure 5

Figure 5

VLPs prepared in NaNO₃-Tris buffer can efficiently enter the cells.

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