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SHORT REPORTS

Cloning and expression of truncated ORF2 as a vaccine candidate against hepatitis E virus

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Abstract

Hepatitis E virus infection is responsible for acute viral hepatitis and associated with high mortality and still birth in pregnant women in developing countries. We report expression of truncated forms of HEV ORF2 as potential vaccine candidates for nanoparticle-based delivery. These two truncated ORF2 proteins (54 kDa and 26 kDa) have been reported to be highly immunogenic and can be used as nanoparticle-based vaccine candidate. The bacterial expressed protein was purified by affinity chromatography and further confirmed by western blot using anti-HEV antibody. The chitosan nanoemulsion was synthesized using ultrasonic waves. The nanoparticle size was found to be 120–160 nm and the entrapment efficiency of purified truncated ORF2 proteins within these nanoparticles was 70% (26 kDa) and 59% (54 kDa). In cell cytotoxicity analysis, 100 µg/mL nanoemulsion was found suitable for cell viability in both HeLa and THP1 cell lines. Release kinetics of encapsulated proteins at physiological pH 7.4 showed 26–59% and 9.7–40% release of 26 kDa and 54 kDa protein within 1 h that gradually increased with time (48 h). Encapsulated proteins were found to be unstable at pH 1.2.

Keywords Chitosan · Nanoemulsion · Hepatitis E virus · Truncated ORF2

Introduction

Hepatitis E virus (HEV) infection is the major cause of acute viral hepatitis with 20 million new infection cases 70,000 deaths and 3000 stillbirths worldwide (Rein et al. 2012). HEV infection is self-limiting and resolves within 4 weeks. In pregnant women, HEV-related mortality rate is very high (25%) (Khuroo et al. 1981).This calls for due attention and need for vaccine development. Due to lack of cell culture system to grow HEV, it is not possible to develop traditional live attenuated or killed vaccine, so we rely either on protein or DNA for vaccine development.

HEV is single stranded, positive sense RNA virus with a genome size of approximately 7.2 kb (Yamashita et al. 2009). HEV genome contains four open reading frames (ORFs), of which ORF2 encodes 72 kDa capsid protein which is immunogenic in nature (Tam et al. 1991; Nair et al. 2016). Various truncated forms of ORF2 encoded proteins have been used as vaccine candidates (Purdy et al. 1992; Zhang et al. 2001; Li et al. 2004, 2005; Purcell et al. 2003; Arankalle et al. 2009; Huang et al. 2009). In all these studies, FDA approved-alum was used as adjuvant to increase the immunogenicity with exception of lipo-NE-DP (aa 458-607) and pE2 (aa 394-604) that employed liposomes and Freund's adjuvant. In addition to these, chitosan and polycaprolactone nanoparticles have also been used as adjuvant for other vaccines (Jesus et al. 2017). However, only two vaccines HEV 239 (26 kDa) and 56 kDa have progressed to human clinical trials. The larger ORF2 fragment encoding amino acid (aa) 112-607 (54 kDa) retains all the properties of ORF2 and also sufficient to form VLPs when expressed in baculovirus vector (Li et al. 1997, 2005), whereas the smaller ORF2 fragment encoding 368-607 aa (26 kDa) is highly immunogenic and sufficient to elicit immune response (Li et al. 2005). The HEV 239 (termed Hecolin®), is licensed for use in China (Taherkhani and Farshadpour 2015).

We have selected these two fragments for nanoparticle-based vaccine development. In this study, chitosan nanoemulsion synthesis, electron microscopy, uptake and release of candidate vaccine in different physiological pH and at different time points were studied. The suitable dose of

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chitosan nanoemulsion for toxicity was analyzed by cell viability assay.

Materials and methods

Cloning of truncated HEV ORF2

The ORF2 region (GenBank accession no. AF444003.1) of HEV genotype 1 was commercially synthesized. Amplification of truncated 112-606aa and 368-606aa regions was carried out using primers (NdeHEV112aa-334F:ggCATATGgcggtcgctccggcccatgac, *Nde*I-HEV368aa-1102F: ggCATATGatagcgcttaccctgtttaac and *Xho*I-ORF2-606aa-1821R:ccCTCGAGcacagagtggggggctaaaac). The PCR amplified products were cloned in pET30b vector at *Nde*I-*Xho*I site by directional cloning. The ligation products were transformed in DH5α *E. coli* competent cells and positive clones were screened by RE digestion and sequence confirmed.

Expression and purification of recombinant protein

pET30b-ORF2 was transformed into BL21 (DE3) *E. coli* competent cells followed by inoculation of single colony in Luria Bertaini (LB) media at 37 °C with stirring at180 rpm overnight. This was followed by subculturing in fresh LB and allowed to grow until absorbance (OD at 600 nm) reaches 0.4–0.8 before induction with 0.2 mM IPTGfor 2.5 h at 37 °C. The induced bacterial culture was centrifuged at 8000 rpm for 10 min at 4 °C. The expression of recombinant protein was analyzed on 10% SDS-PAGE.

The bacterial pellets were resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 0.2% sarkosyl) and sonicated for 2 min with pulse cycle of 10 s on and 20 s off. After centrifugal separation, supernatant containing recombinant proteins were purified using Ni-NTA spin columns from G-Biosciences following product manual. The eluted proteins were analyzed on 10% SDS-PAGE and confirmed by western blot using anti-HEV-positive serum and goat anti-human IgG/HRP and 3,3′-diaminobenzidine (DAB) as chromophore.

Synthesis of chitosan nanoemulsion

An (o/w) emulsion of chitosan was prepared by addition of 0.1% chitosan solution into oil phase containing olive oil and surfactant (PluronicF-127) followed by dropwise addition of cross-linking agent glutaraldehyde under continuous ultrasonication (Akbuğa and Durmaz 1994). Synthesized o/w nanoemulsion was observed under optical microscope 100× and transmission electron microscope (TEM).

Toxicity analysis of chitosan nanoemulsion

HeLa and THP1 cells were seeded in 96-well plates at cell density of 1×10^3 in each well and grown to subconfluent (70% confluency) stage. The chitosan nanoemulsion (CNE) of concentrations 25, 50, 100, 150, 175, 200, 250, and 275 µg/mL were added to these cells and incubated for 24 h. MTT assay was performed to test toxicity of CNE on HeLa and THP1 cell lines (Meerloo et al. 2011). Percentage of viable cells were evaluated using formula:

$$\% \text{ Cell viability} = (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100.$$

Protein release kinetics and stability analysis

Twenty microgram (µg) of the purified protein was incubated with 100 µg/mL of CNE for overnight at 4 °C. The entrapment efficiency was evaluated by quantifying percentage of protein remaining in supernatant after centrifugation of protein-loaded CNE (CNP) at 9000 rpm for 15 min. Finally, CNP was incubated in 10 mL of simulated intestinal fluid (SIF) pH (6.8) (KH_2PO_4 6.8 g/L, NaOH 0.89 g/L), phosphate buffer (PB), pH (7.4) (KH_2PO_4 3.4 g/L, Na_2HPO_4 3.5 g/L) and simulated gastric fluid (SGF) pH (1.2) [NaCl 2.0 g/L, HCl (concentrated) 7.0 mL/L]. This was followed by centrifugation at 9000 rpm for 15 min at 4 °C and protein estimation in supernatant at after 1, 3, 5, 24 and 48 h.

Results and discussion

The truncated ORF2 region was PCR amplified using gene-specific primers and was observed as 719 bp and 1487 bp band on 1.5% agarose gel. The gel purified PCR products were further subcloned in bacterial expression vector pET30b at *Nde*I and *Xho*I site. The colonies obtained after transformation were screened for positive clone by RE digestion analysis.

Protein expression and purification-truncated ORF2

The recombinant clones were expressed in BL21 (DE3) *E. coli* competent cells by induction with 0.2 mM IPTG at 37 °C for 2.5 h. The expressed proteins were analyzed on 10% SDS-PAGE. Figure 1a shows overexpression of induced proteins of 26 kDa and 54 kDa in Lane 1 and Lane 4, respectively, as opposed to the uninduced culture pellet in Lane 7. After lysis, approximately 55% of 26 kDa (Lane 3) and 80% of 54 kDa (Lane 6) proteins are solubilized while remaining proteins (Lane 2, 5) remains in insoluble fraction. Figure 1b shows Ni-NTA purified eluted fractions of both 54 kDa (Lane 1–3) and 26 kDa (Lane 8–9) proteins with

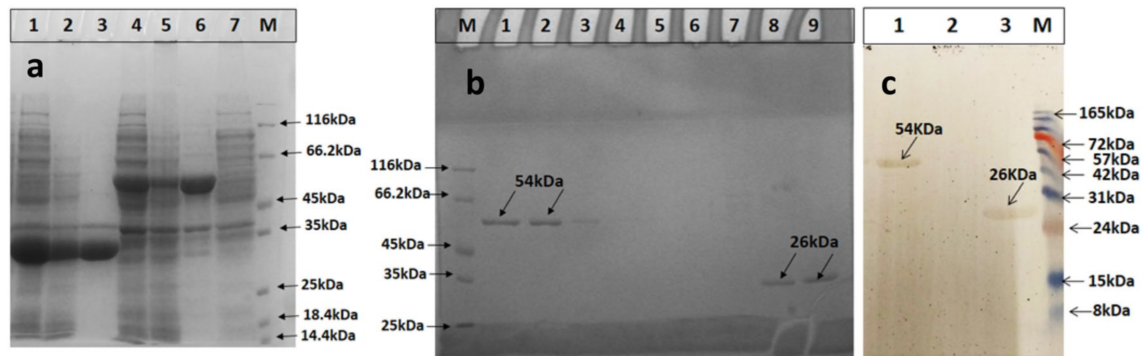

Fig. 1 **a** Analysis of truncated ORF2 protein expression on 10% SDS–PAGE, Lane 1, 4: induced sample of 26 kDa and 54 kDa, Lane 2, 5: insoluble and Lane 3, 6 soluble fraction of 26 kDa and 54 kDa proteins after lysis, Lane 7: uninduced sample, Lane M: protein Marker; **b** Ni-NTA purification fractions on 10% SDS-PAGE with Lane M: protein marker; Lane 1–3: 54 kDa eluted fractions, Lane 4–6: third, fourth and fifth washing sample; Lane 7: flow through; Lane 8, 9: 26 kDa eluted fraction; **c** Western blot image—Lane M: prestained protein molecular weight marker, Lane 1 and Lane 3: 54 kDa and 26 kDa purified proteinsand Lane 2: negative control (uninduced sample)

flowthrough in Lane 7 and final washing step sample in Lane 4–6 while Lane M shows marker protein. Finally, purified proteins were confirmed by western blot using HEV-positive human sera. Figure 1c shows 54 kDa and 26 kDa brown-colored band developed in Lane 1 and Lane 3, respectively, after incubation with HRP-tagged secondary antibody and exposure with DAB while no band was observed in uninduced sample (Lane 2). Lane M shows prestained protein molecular weight marker.

Nanomaterial cytotoxicity, protein entrapment and release analysis

Chitosan nanoemulsion synthesized using ultrasonic waves were characterized using optical microscopy and transmission electron microscopy (TEM). Optical image of chitosan nanoemulsion (Fig. 2a) shows spherical particles. Further characterization using TEM shows size of CNE to be 120–160 nm (Fig. 2b).

Dose optimization and toxicity analysis of nanoemulsion was evaluated for on HeLa and THP1 cell lines. Cells subcultured in a 96-well plate at 70% confluency, were treated with different concentrations of chitosan nanoemulsions (in triplicate) for viability testing by MTT assay. In MTT assay, optimum cell viability (>90%) was observed (Fig. 2c) 100 µg/mL of CNE-treated cells. The cell viability further reduced to 80% beyond 150 µg/mL and remains only 50% at 275 µg/mL of CNE treatment. Further, toxicity of particles was also evaluated on the in vitro model for immune modulation, i.e., THP1 cell lines. THP1 cells treated with 50 µg/mL of CNE shows 85% of viable cells, while further increase in CNE upto 275 µg/mL shows 75% of viable cells. Hence, based on the results from both cell lines, 100 µg/mL concentration of nanoparticles for in vivo studies may be considered for less toxicity.

Protein entrapment efficiency was calculated by incubating the 20 µg of each protein with 100 µg of CNE overnight in separate tubes. The suspensions were centrifuged and supernatant evaluated for the amount of protein. Results showed 70% and 59% entrapment of 26 kDa and 54 kDa proteins. Finally, protein release kinetics and stability analysis under different pH conditions was evaluated in simulated intestinal fluid (SIF) pH 6.8, phosphate buffer (PB) pH 7.4 and simulated gastric fluid (SGF), pH1.2 for vaccine studies. Figure 2d shows an initial quick release of 26% of 26 kDa, while only 9.7% of 54 kDa protein. A gradual increase upto 59% (26 kDa) and 40% (54 kDa) protein release upto 48 h was observed. However, in SIF after initial protein release of 100% protein, the protein percentage gradually decreases to less than 30% suggestive of instability. While in SGF, no protein peak was observed indicative of either no release or complete degradation of protein within 1 h.

Conclusion

In the present study, two immunogenic fragments of HEV ORF2 were cloned and expressed proteins of 26 kDa and 54 kDa were purified. CNE of 120–160 nm size negligible toxicity upto 150 µg/mL with 90% cell viability. Protein entrapment, release and stability analysis showed instability at pH 1.2, thus eliminating oral vaccine possibility.The capsid fragment used for bacterial expression belongs to HEV genotype 1. This candidate vaccine can possibly cross protect to other genotype as this region share amino acid homology among genotypes. The efficient delivery in host and antibody production will be checked in our future study.

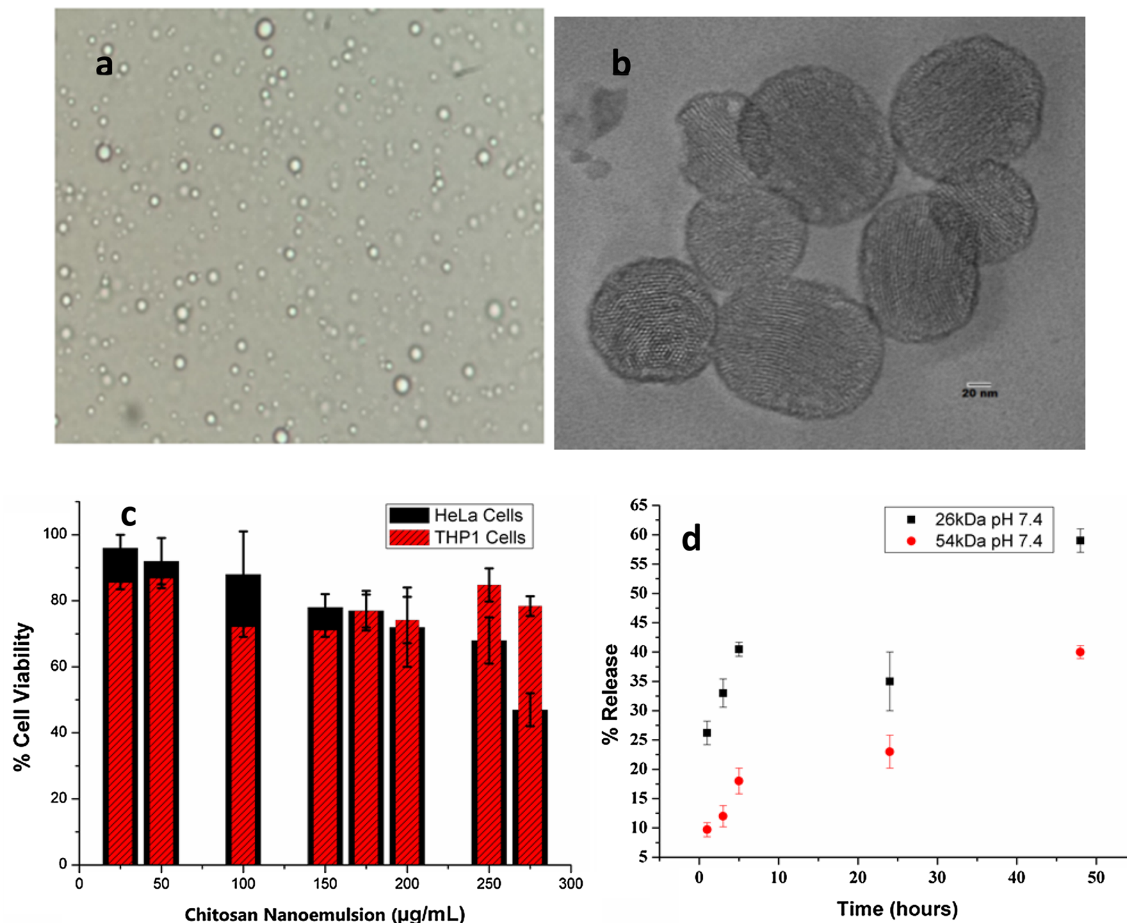

Fig. 2 **a** Optical microscopic image of o/w chitosan nanoemulsion at ×100. **b** Transmission electron micrograph of chitosan nanoemulsion at ×50,000, **c** percentage viability of HeLa cells (black bars) and THP1 cells (red bars) on treatment with varying concentration of chitosan nanoemulsion. **d** Percent protein released with time in SIF, SGF and PB

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Compliance with ethical standards

Conflict of interest All authors hereby declare that there is no conflict of interest.

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