EBOLA LIPOSOME VIRAL-LIKE NANOPARTICLES: CHARACTERIZATION AND PCR DETECTION

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Abstract

Viral-like particles that express Ebola glycoprotein on surface are very sophisticated and risky techniques for developing diagnostics and study of the virus biology. On the other hand, liposome nanoparticles are easy to prepare and have customized lipid content to encapsulate preferred cargo inside while chemically modified to carry other molecules on their surface. The aim of this study was to prepare Ebola liposome viral-like nanoparticles encapsulated with nucleic acid and to develop a polymerase chain reaction (PCR) method to quantitate liposomes without extraction. Liposomes containing equal ratios of three lipids (cholesterol, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) and phosphatidylcholine) and carrying two fragments of Ebola partial and whole GP gene (700 bp and 2031 bp, respectively) were synthesized. Liposome size was estimated in the range of 80-100 nm. Zeta potential analysis showed a negative charge of liposome particles in the ranges of -30 and -70 mV. Direct PCR method was developed to avoid fragment loss during extraction. Several additives were tested to improve PCR detection of liposomes including DMSO, glycerol, triton X-100, tween 20 and tween 80. The addition of triton X-100 (as low as 0.5% per reaction) has showed significant improvement in amplification. The addition of $MgCl_2$ (>50 mM per reaction) in presence of triton X-100 also improved amplification. Although a complete specific product was not obtained, the amplification was semi-quantitative at 5 orders of serial dilution. By employing PCR with shorter product sizes, we believe it is possible to develop more accurate method in the future.

Keywords: Liposome, Ebola, Viral-Like Particles, PCR, PCR additives

1. INTRODUCTION

The first Ebola outbreak occurred in 1976, and since then several sporadic incidents followed in west African countries [1]. The last epidemic of Ebola (2014-2015) resulted in over 11,000 deaths, yet it has raised worldwide attention to the seriousness of this disease [2]. Because of its high mortality rate and lack of approved vaccines and treatments, Ebola virus is classified as a biosafety level-4 which requires sophisticated facilities and high levels of expertise [3, 4].

Genetically modified Ebola virus [3] as well as vesicular stomatitis virus (VSV)-based Ebola-like particle systems [5] are two of the most advanced techniques in study of dangerous pathogens; due to their abilities to simulate the real virus in process of infection, however they are also sophisticated and require high levels of expertise.

Virus-based nanoparticles (VNPs) have been shown to be useful platform for several applications like imaging and drug delivery [6]. However, VNPs employ classes of viruses that only have capsids and lack the outer membrane layer of influenza and Ebola viruses.

Membrane lipids of a host and virus play major role in viral life cycle [7]. The lipid content of genetically modified virus or VSV-based virus particles are dependent on host cell lines which they are propagated, and thus it is difficult to study the direct role of virus membrane lipids using these models. On the other hand, liposome

nanoparticles are very customizable and easy to synthesize. They can carry and deliver different types of cargo to cells ranging from drugs to nucleic acids. The chemical nature of liposomes can be also modified to carry different surface molecules [8].

The aim of this study was to prepare liposomes encapsulated with Ebola nucleic acid and to develop a PCR method to quantitate liposomes without extraction.

2. RESULTS AND DISCUSSION

Liposomes containing equal ratios of three lipids (cholesterol, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) and phosphatidylcholine) and carrying two DNA fragments of partial and whole Ebola *GP* gene (700 bp and 2031 bp, respectively) were synthesized. Scanning (SEM) and transmission (TEM) electron microscopy showed liposome nanoparticles of lower than 200 nm size (**Figure 1**). Particle size estimation of liposomes in water and PBS buffer medium were in the range of 80 - 100 nm (**Figure 2a**).

Zeta potential analysis showed a very stable negative charge of liposome nanoparticles in the ranges of -30 and -70 mV (**Figure 2b**) which leads to high colloidal stability. This negative charge also indicates electrophoretic capability.

To visualize liposomes, direct electrophoresis of liposomes showed mobility of liposome 700 bp in 1% agarose but not liposome 2031 bp (**Figure 3**). Liposome 700 bp made a tunnel trail through the gel because the pore size of 1% agarose gel was smaller than mobile liposomes. The relative mobility (R_f) of liposome 700 bp was equivalent to R_f of DNA ladder at 1500 bp which is nearly twice the size DNA inside the liposome.

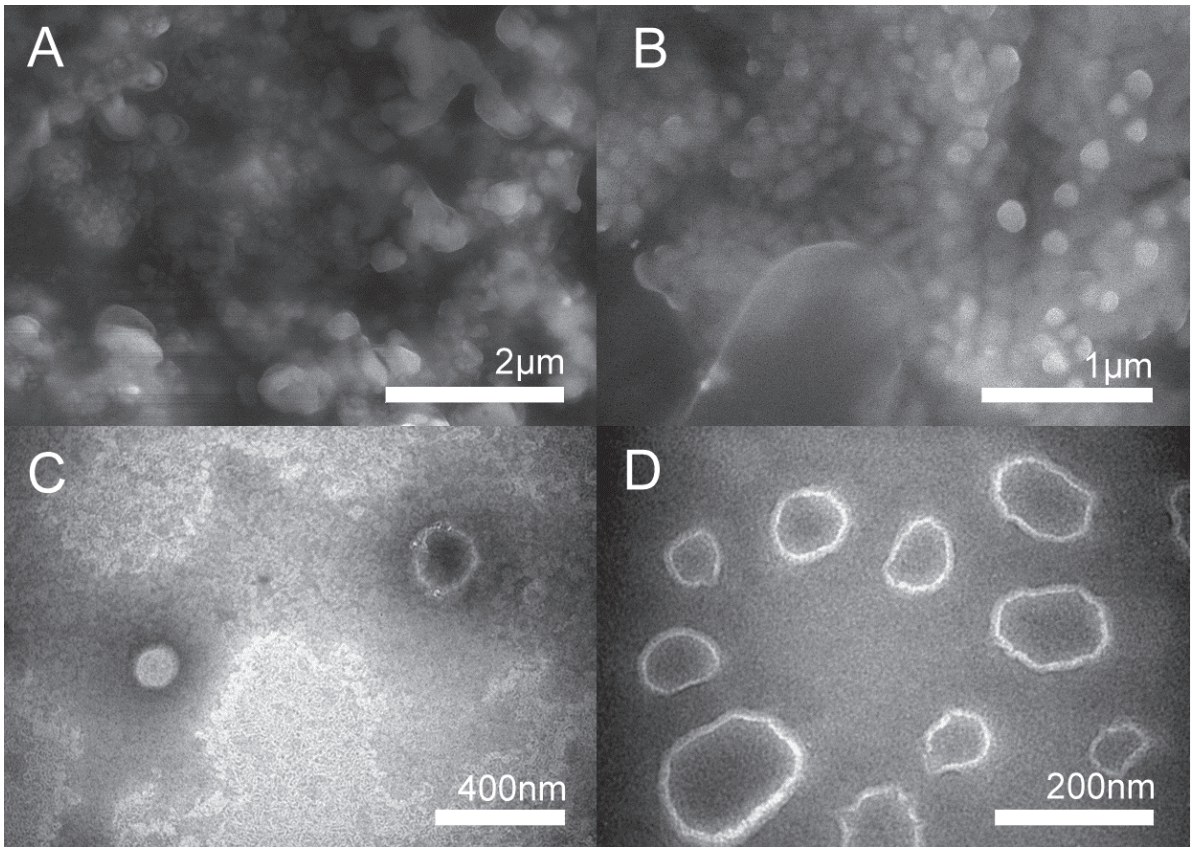

Figure 1 Electron microscopy of liposomes. (**A**) SEM image of Liposome 700. (**B**) SEM image of Liposome 2031. (**C**) TEM image of Liposome 700. (**D**) TEM image of Liposome 2031

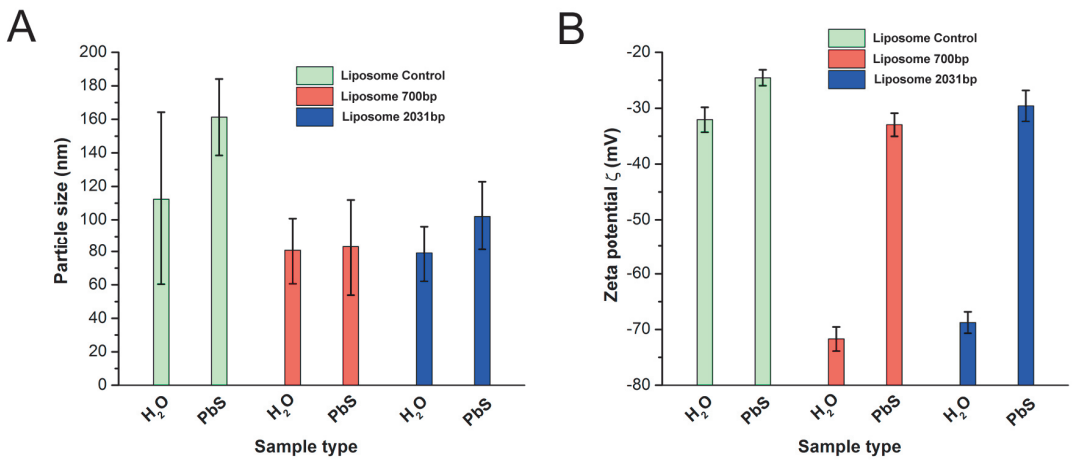

Figure 2 Particle size and zeta potential analyses of liposomes. (**A**) Particle size. (**B**) Zeta potential

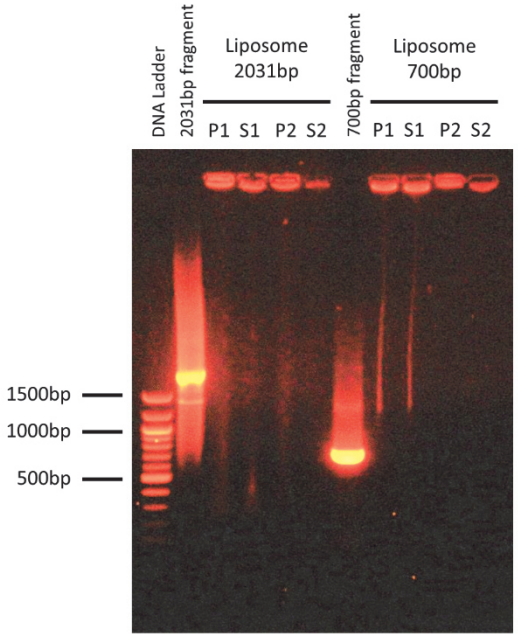

Figure 3 Direct electrophoresis of liposomes. Liposomes in ACS water were centrifuged at 2000×g/1 min and suspended in ACS water then centrifuged again. The precipitate of first centrifugation (P1), supernatant of first centrifugation (S1), precipitate of second centrifugation (P2), and supernatant of second centrifugation (S2) are shown

Direct PCR method was developed to avoid fragment loss during extraction (**Figure 4**). Several additives were tested to improve PCR detection of liposomes including DMSO, glycerol, triton X-100, tween 20 and tween 80 (**Figure 4a**). The addition of triton X-100 (as low as 0.5% per reaction) has showed significant improvement in amplification (**Figure 4a** and **4b**). Tween 20 and Tween 80 showed primer dimer formation, which is prospective for improvement of PCR at higher concentrations (**Figure 4a**). The addition of $MgCl_2$ (>50 mM per reaction) in presence of triton X-100 also improved amplification. Although a complete specific product was not obtained, the amplification was semi-quantitative at 5 orders of serial dilution (**Figure 4d**). Since quantitative realtime PCR using SYBR green only requires binding of double stranded DNA [9], this method can be reproduced by realtime PCR without the amplification of a complete product. However, by employing PCR with shorter product sizes, we believe it is possible to develop more accurate method in the future.

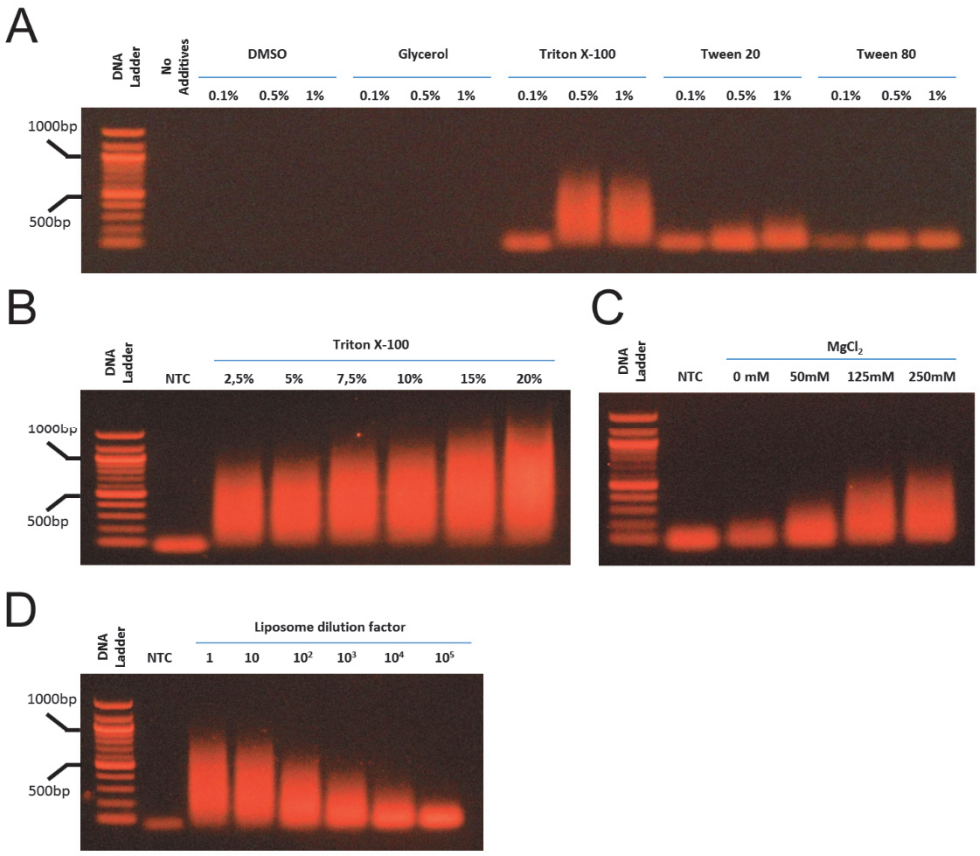

Figure 4 Development of method for direct PCR detection of liposome. Amplification of liposome 700 bp in presence of additives (**A**) showing improved amplification in presence of Triton X-100. (**B**) PCR at different concentrations of Triton X-100. (**C**) PCR with addition of $MgCl_2$ at different concentrations in the presence of 10% Triton X-100. (**D**) PCR for 10 fold serial dilution of liposomes in presence of 10% Triton X-100. NTC: No template control

3. EXPERIMENTAL PROCEDURES

3.1. Ebola Glycoprotein Gene DNA fragments

Large amounts of DNA were prepared using PCR to amplify 700 bp and 2031 bp products from the Ebola glycoprotein *GP* gene (EBOV subtype Zaire, strain Mayinga 1976). The 700 bp fragment covered the last third of *GP* cDNA whereas the 2031 bp covered the whole *GP* cDNA. The following primers were used for amplification of Ebola GP DNA from the pCMV3-Zaire EBOV-U23187-GP-FLAG vector (Sino Biological Inc., Beijing, China): 700 bp-Forward: GACCCCCAAAAGCAGAGAAC. 700 bp-Reverse: ACGCCTGTAACTCCAATACCTG. 2031 bp-Forward: CTAAAAGACAAATTTGCATATACAGA. 2031 bp-Reverse: ATGGGCGTTACAGGAATATTG. Protocol for reaction is described in section 3.6. After the purification of DNA fragments using MinElute kit (Qiagen, Germany), the concentration of DNA was estimated using Infinite200PRO NanoQuant instrument (Tecan, Switzerland).

3.2. Liposomes Nanoparticles

Cholesterol, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG) and phosphatidylcholine (PC) were purchased from Sigma-Aldrich. Liposome film was synthesized in a ratio of cholesterol:DOPG:PC (1:1:1) according to previously modified method [10]. Briefly, 100 mg of each lipid were dissolved in 4.5 ml

chloroform. A lipid film was obtained by rotary evaporation of chloroform in a 2 ml Eppendorf tube, and residual solvent was flushed out by nitrogen.

Liposomes in ACS water or PBS were prepared for two fragments of DNA; namely 700 bp and 2031 bp fragments. Approximately 10^{11} copies of DNA, suspended in 500 µl ACS water or PBS, were added to Eppendorf tube covered with lipid film, and sonicated for 2 min then incubated with heating at 60°C for 15 min with shaking. The liposome solution was allowed to cool down to room temperature then stored at 4°C until analysis. Control liposomes were synthesized by suspending 500 µl ACS water or PBS in lipid film tube.

3.3. Electron Microscopy

Liposomes visualization transmission electron microscopy (TEM) was done by negative staining technique using organotungsten compound Nano-W (Nanoprobes, USA). Samples (~4 µl) were deposited onto 400-mesh copper grids coated with a continuous carbon layer. Dried grids were imaged by a Tecnai F20 microscope (FEI, USA) at 80 000× magnification.

Liposomes were also visualized by scanning electron microscopy (SEM). Images of their external structure were taken using a MIRA II LMU (Tescan, Czech Republic) instrument equipped with an In-Beam SE detector. A beam current of approximately 1.0 nA was used with accelerating 15,000 volt.

3.4. Zeta Potential and Particle Size Analyses

The zeta potential measurements were performed on Zetasizer MALVERN (Malvern Instruments Ltd. UK), considering the same refraction index, absorption coefficient temperature and viscosity as described in particle size measurements. Calculations considered the diminishing of particles concentration based Smoluchowsky model, with a F(κa) of 1.50 and an equilibrating time of 120 s. For the measurements, a disposable cell DTS1070 was employed. In each case, the measurement duration depended on the number of runs, which varied between 20 and 40. The measurements were triplicate and were performed under the automatic setting of attenuation and voltage selection.

The particle size measurements were performed considering a refraction index of the dispersive phase of 3.00 and 1.333 for the dispersive environment. The absorption coefficient in both cases was 10 and 3 respectively. The measuring temperature was set at a constant value of 25°C, while the viscosity was 0.8872 cP. For each measurement, disposable cuvettes type ZEN 0040, were used, containing 40 µl of sample. The equilibration time was 120 s, at a measurement angle of 173° backscatter. The measurements were triplicate.

3.5. Direct Electrophoresis

Liposomes in ACS water were centrifuged at 2000×g/1 min and supernatant was isolated by careful pipetting. Approx. 500 µl ACS water were added to dissolve pellet, then centrifuged again at 2000×g/1 min. The precipitate of first centrifugation (P1), supernatant of first centrifugation (S1), precipitate of second centrifugation (P2), and supernatant of second centrifugation (S2) were visualized by electrophoresis for liposomes carrying both types of fragments. Glycerol-based sample loading dye was used (5 µl sample was mixed with 3 µl loading dye). The 1% Agarose gel was run at 85 volt for 110 min and visualized using in-gel ethidium bromide under UV light.

3.6. PCR and Electrophoresis

Taq polymerase chain reaction kit from New England BioLabs (Ipswich, USA) was used. Approximately 2.5 µl standard buffer, 0.5 µl dNTP, 3 µl of each primer, 0.2 µl Taq polymerase, 1 µl of liposome solution, and the additives were added accordingly to a final volume of 25 µl PCR reaction. The PCR reaction was carried out using Mastercycler EP gradient S instrument (Eppendorf AG, Germany). The thermal profiles were as follows: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min

and extension at 68°C for 1 min; with final extension at 68°C for 7 min and hold at 4°C. PCR products were visualized on 1% Agarose gels using ethidium bromide under UV light.

4. CONCLUSION

Ebola viral-like liposome nanoparticles carrying *GP* gene fragments are easy to prepare, visualize, and quantify. Our findings suggest that nucleic acid encapsulated liposomes were single layered (size estimate was 80-100 nm) with very stable charge in the ranges of -30 and -70 mV which lead to high colloidal stability. Direct PCR method was developed to avoid fragment loss during extraction procedures. After testing several additives, triton X-100 (>10% per reaction) demonstrated significant improvement in amplification. The addition of $MgCl_2$ (<50 mM per reaction) in presence of triton X-100 also improved amplification. Although a complete specific product was not obtained, the amplification was semi-quantitative at 5 orders of serial dilution. By employing PCR with shorter product sizes, we believe it is possible to develop more accurate method in the future.

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