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Abstract Using human papillomavirus (HPV) as subunit vaccine and its manipulation of surface loops is current trend in research. Since the atomic model of L1 protein conformations were deciphered, their manipulations of epitopes bring multivalent vaccines. Here, in the present study we had expeditiously manipulated antigenic loops of HPV 6b L1 capsid proteins in the amino acid regions 174–175 (L1:174EGFP) and 348–349 (L1:348EGFP) with whole enhanced green fluorescent protein (EGFP), expressed in the silkworm larva using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid technology. The expressed proteins were partially purified using sucrose density-gradient centrifugation and size-exclusion chromatography (SEC). The display of EGFP in virus-like particles (VLPs) was confirmed by immuno-fluorescence microscopy, western blots and immune-transmission electron microscopy (immuno-TEM). There was higher expression of EGFP incorporated L1:174EGFP than L1:348EGFP. Hydrodynamic diameter of VLPs was corroborated by dynamic light scattering, confirming the size of expected range of around 160 nm and substantiating the incorporation of EGFP. From immuno-TEM, each L1:EGFP VLP formed small particles, suggesting that small particles of L1:EGFP fusion protein were aggregated. Our study illustrates that incorporation of whole protein can efficiently form chimeric VLPs, without hindering the

conformation. HPV L1 protein accommodated a whole protein on its antigenic loop as a small particle, but inserted whole protein was unstable.

Keywords: virus-like particle, human papillomavirus, L1 protein, silkworm, BmNPV bacmid, display of protein

1. Introduction

Human papillomavirus are group of double stranded DNA viruses, causing low risk warts to cervical cancers in humans. Human papillomavirus 6b L1 (HPV6b L1) virus infects mucosal epithelial tissues in genital areas causing benign lesions [1]. The structural protein of HPV consists of L1 and L2 capsid proteins, L1 having functions of virion formation and cell association where the later involved in membrane penetration, post-entry trafficking, and genome encapsidation. The L1 is the primary capsid protein approximately of 55 kDa, where 360 monomeric capsomere proteins assemble as 72 pentamers to form T=7 icosahedral lattice. The fully assembled L1 virus particle has a size of around 60 nm in diameter. Conformation of L1 capsid protein assembly and its five surface antigenic loops BC, DE, EF, FG and HI were solved using crystallographic studies [2, 3].

Recombinant L1 capsid protein alone can be expressed in expression systems to form VLPs, which can elicit immune response when used as subunit vaccines [4-8]. Also, display of foreign protein in VLPs makes elicit immune response against both the proteins [9]. Fusing or inserting epitopes or proteins on the terminal regions of capsid protein doesn't interfere with the conformations of VLPs. It is proved that a single VLP can be used as a multivalent drug against disease and tumor targeting entities [10–13].

Previously, we had showed that the expression of full length HPV 6b L1 capsid protein could form efficiently VLPs when expressed in silkworm-bacmid system [14]. In this work, we present incorporation of whole EGFP into two antigenic loops of HPV 6b L1, expression using bacmid technology, and characterization of the VLP assembly.

2. Materials and Methods

2.1. Expression of HPV 6b L1 proteins

Two different surface loops incorporated with EGFP in HPV 6b L1 capsid protein was constructed as described previously [14]. Briefly, each HPV 6b L1:EGFP fusion gene was obtained by two-step PCR. In addition, an EGFP gene was amplified by PCR and, a second PCR was performed using the amplified EGFP and the two amplified truncated L1 genes as a template and primers, respectively, to obtain L1:174EGFP fusion gene. The EGFP gene was inserted into EF loop between 174 and 175 amino acids in an L1-coding gene. Moreover, linker-region coding sequences (GGGGSGGGGS) were also added between L1 and EGFP genes. By this two-step PCR, the EGFP gene was inserted into HI loop between 348 and 349 amino acids in an L1-coding gene (L1:348EGFP) was obtained. Each amplified fusion gene was inserted at *Eco*RI-*Kpn*I site in pFastBac1. The recombinant BmNPV CP^- bacmid containing each HPV 6b L1:EGFP fusion gene was constructed according to the protocol described previously [14, 15]. The fifth instar

Bombyx mori larvae were individually injected with 4 µg of chimeric HPV 6b L1:EGFP bacmids and reared in 25°C incubator [15]. After 7 days, fat body is collected from each silkworm and stored at -80°C until use.

2.2. Purification of HPV 6B L1:EGFP chimeric VLPs

Extraction of expressed protein was achieved by sonicating the fat body using PBS (pH 7.4) containing 0.1% Triton-X100. The lysates were clarified using centrifugation at 30000 g for 15 min. The clarified lysates were loaded on top of 25–60% sucrose density-gradient cushion and ultracentrifuged at 122 000 g for 3 h at 4°C, and ultracentrifuged samples were collected in every 0.5 ml of fraction from the top of the tube to the bottom. The fractions containing target bands were detected by western blots. The fractions containing HPV 6b L1:EGFP chimeric VLPs were collected and loaded into HiLoad Superdex™ 16/60 200 prep grad column which was equilibrated with 3 column volumes of PBS (pH 7.4). The samples were eluted with PBS (pH 7.4) plus 0.5 M NaCl; 5 ml fractions were collected in fresh tubes and stored at 4°C for further analysis.

2.3. Immunoblotting

Fractions from sucrose density-gradient and size exclusion chromatography were subjected to immuno-analysis. Thirty µl of the fraction was mixed with 10 µl of sample

buffer and 20 µl were loaded to SDS-PAGE gels, respectively. For western blot, the gel was transferred to PVDF membrane using Trans-blot® semi dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) at 15 V for 1 h. After transfer the membrane was blocked with 5% skim milk in Tris buffered saline with 0.5% tween 20 (TBS-T) for 1 h. Washed three times with TBS-T and incubated with 1:5,000-diluted mouse anti-EGFP antibody (Clontech, Mountain View, CA, USA) for overnight at 4°C. Anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) (1:10,000 diluted, GE Healthcare Japan, Tokyo, Japan) was used against primary antibody, and incubated for 1 h at room temperature. Detection was carried out using Versa Doc Imager and analyzed using Quantity-One software (Bio-Rad).

2.4. Size analysis of chimeric VLPs

The partially purified HPV 6b L1:EGFP chimeric variants were analyzed for hydrodynamic property and homogeneity using Dynamic Light Scattering (DLS) assay. One ml of PBS (pH 7.4) was mixed with L1:174EGFP and L1:348EGFP individually and diluted to 0.1 mg/ml. The samples were analyzed and calculated based on 10 successive measurements at 25°C using Zetasizer nano ZS DLS analyzer fitted with 532 nm laser lamps (Malvern Instruments, UK).

2.5. Immuno-transmission electron microscopy (Immuno-TEM)

The chimeric L1:EGFPP VLPs were immobilized on the carbon-coated grids. Dried for 15 min and blocked using 3% BSA for 30 min. Primary EGFP antibody (Clontech) diluted to 100-fold in blocking buffer (Tris-BSA) and adsorbed to grids for 1 h. After washing 5 times with TBS, the grids were soaked in 10 nm gold conjugated 1: 200 diluted mouse anti-IgG+IgM antibodies (BBI International, Ltd., Cardiff, UK) for 1 h. After washing, the grids were stained with 2% phosphotungstic acid and dried for 30 min. VLPs were then observed by TEM (JEM 2100, Japan) at 40,000× magnifications.

2.6. Confocal laser scanning microscopy

Fresh fat body cells containing HPV 6b L1 and L1:EGFP chimeric VLPs were washed three times in PBS, and fixed with 4% formaldehyde solution for 20 min at room temperature. Cells were permeabilized using 0.1% Triton-X100 in PBS for 20 min. Then the cells were subjected to blocking with 3% BSA for 1 h and incubated with 1:100 dilution of EGFP primary antibody. Alexa fluor® 594 goat anti-mouse IgG (H+L) (Life Technol. Japan Co. Tokyo, Japan) was used as a secondary antibody. The cells were counterstained for nuclei using 4,6-diamidino-2-phenylindole (DAPI). Samples were observed using LSM 700 microscope (Zeiss, Jena, Germany) and images were analyzed by Zen2010 software.

3. Result and discussion

3.1. Designing incorporation of EGFP in HPV 6b L1 VLPs

Chimeric HPV 6b L1 VLPs displaying EGFP as a model protein was designed in such a way that the incorporation was done in surface antigenic loops where the incorporated protein can be displayed. PCR was performed to insert the whole EGFP protein into the HPV 6b L1 EF and HI epitopes by specific primers (Fig. 1A). Previously, it was demonstrated that bovine papillomavirus (BPV) L1 protein inserted human mucin 1 (MUC1) peptide into its HI loop can assemble into VLP, but BPV L1 protein inserted the same peptide into BC and DE loops can not be assembled [16]. In addition, EF loop localizes the outermost of L1 pentamer [17]. Therefore, EF and HI loops were selected to insert EGFP in this study. Moreover, It was previously reported that use of flexible linkers to allow the incorporated whole protein to flexibly move to take conformation [18]. Therefore, we added a double GGGGS linker sequence that was placed in the N-terminal and C-terminal of the EGFP protein. Since EGFP has green fluorescence when expressed EGFP has correct conformation, whether expressed L1:EGFP chimeric proteins have correct conformation or not can be easily confirmed by its green fluorescence. The chimeric constructs were ligated in the *Eco*RI and *Kpn*I regions of

pFastbac I plasmid and transformed to BmNPV CP^- bacmid to express in silkworm larvae (Fig. 1B).

3.2. Expression and purification of HPV 6b L1:EGFP chimeric VLPs

Expression of each HPV 6b L1:EGFP chimeric VLPs was confirmed in silkworm larvae by observing under UV transilluminator (data not shown). The fat body samples were sonicated and the clarified lysates were applied to 25–60% sucrose density-gradient ultracentrifugation and the fraction (Fractions 3–6 in all 10 fractions) containing 83 kDa of HPV 6b L1:174EGFP and L1:348EGFP chimeric VLPs along with degraded proteins were confirmed by western blot (Fig. 2). The expression levels of the two constructs varied, and L1:174EGFP (150 μg/larval hemolymph ml) was comparatively higher than L1:348EGFP (62 μg/larval hemolymph ml). Fractions containing each L1:EGFP chimeric VLP were collected and further purified by size exclusion chromatography using Hiload superdex™ 16/60 200 prep grade column. L1:174EGFP and L1:348EGFP chimeric VLPs were eluted at 35–55 ml and 40–50 ml, respectively, on this SEC column. Partially purified fractions were recovered.

3.3. Confirmation of size and structural integrity

To determine the conformation of HPV 6b L1:EGFPP chimeric VLPs, dynamic light scattering was carried out [19]. The sizes of chimeric L1:174EGFP (Fig. 3A) and L1:348EGFP (Fig. 3B) were 156 nm and 155 nm, respectively (at pH 7.4, 25°C), which are bigger than HPV 6b L1 VLP alone (~ 60 nm). Size of HPV L1 VLP is normally 55 nm in diameter. In this study, each purified L1:EGFP chimeric VLP has approximately 155 nm in diameter. From immuno-TEM images, these purified chimeric VLPs formed small particles and these particles were aggregated. In addition, gold nanoparticles were observed on the small particles and aggregates composed of each chimeric VLP, indicating that EGFP were displayed on the surface of these particles.

3.4. Localization of HPV 6b L1:EGFP chimeric VLPs in fat body cells of silkworm larvae

To prove that the HPV 6b L1 chimeric VLPs with EGFP incorporation expressed in silkworm larvae are having native conformation, the fat body was immuno-labeled with EGFP antibodies [20]. As shown in Fig. 4, the EGFP fluorescence at 488 nm was observed in cytoplasm of cells where L1:174EGFP or L1:348EGFP was expressed. With immuno-labeling with Alexa Fluor® 594, we can confirm that its red fluorescence was coinciding with the EGFP fluorescence, indicating that each expressed L1:EGFP chimeric VLPs had EGFP fluorescence. This result is contradictory to previous report,

which mentions these L1:EGFP chimeric VLPs did not show EGFP fluorescence [14]. In previous study, the fat body homogenate was applied to HiTrap heparin affinity column chromatography and Mono S 5/50GL column chromatography in series. But after purification any fluorescence of each purified L1:EGFP chimeric VLP was not detected (Data not shown). In this study, the purification was modified as follow; the VLPs were separated from the fat body homogenate by sucrose density-gradient ultracentrifugation, and were applied to HiLoad Superdex 16/60 200 prep grad column. However, any fluorescence of each purified L1:EGFP chimeric VLPs was not detected. This indicates that purification of HPV 6b L1 VLPs using columns affects the structural integrity or immune response. Kim *et al.* also reported that choice of resin-bound ligand affects the structural and immunogenity of column-purified human papillomavirus type 16 VLPs [21]. On the other hand, fat body cells and silkworm larvae where each L1:EGFP chimeric VLP expressed showed EGFP fluorescence. It suggests that L1:174EGFP and L1:348EGFP chimeric VLPs had EGFP fluorescence *in vivo*, but after extraction and purification processes, its EGFP fluorescence disappeared because of its instability or conformational change *in vitro*.

4. Conclusion

Display of foreign proteins on the surface of VLPs has many advantages, for example use as multiple antigenic vaccines or trafficking studies of signaling. Our study suggests that incorporating whole protein in VLPs can be helpful in future incorporations like other L1 capsid protein/epitopes or mutualistic/opportunistic diseases associated with papillomavirus. This technology can provide the formation of chimeric VLPs using epitope loop without compromising the conformation of VLPs.

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Figure legends

Fig. 1. Schematic representation of chimeric HPV 6B L1:EGFP construction. Using PCR technology whole protein EGFP was constructed (A), the constructed HPV 6b L1:EGFP chimeric VLPs were ligated with pFastbac1 *Eco*RI and *Kpn*I restriction site and transformed to *E. coli* BmDH10 Bac CP^- and the bacmid was injected to silkworm larvae for expression.

Fig. 2. Immuno-analysis of chimeric HPV 6b L1:EGFP. Sucrose density-gradient fractions (3, 4,5and 6) of HPV 6b L1:174EGFP (A) and HPV 6b L1:348EGFP (B) were collected separately in fresh collection tubes and analyzed by western blots.

Fig. 3. Hydrodynamic size, negative staining and immuno-TEM of chimeric VLPs. Partially purified HPV 6b L1:174EGFP (A) and HPV 6b L1:348EGFP (B) were measured for mean size by dynamic light scattering. Electron microscopy of partially purified HPV 6b L1:174EGFP and HPV 6b L1:348EGFP stained with 2% phosphotungstic acid. VLPs were immuno labeled with colloidal gold nanoparticles of 10 nm against EGFP primary antibody. Bars denote 100 nm.

Fig. 4. Expression of chimeric VLPs in fat body cells. Expressed L1:174EGFP (A), L1:348EGFP (B) and HPV L1 (C). Fat body were fixed and permeabilized using 0.1%

284 Triton-X100 and probed with EGFP primary antibody and labeled using Alex Flour

285 594®; counterstained with DAPI and observed under confocal microscope. Bars denote

286 20 µm.

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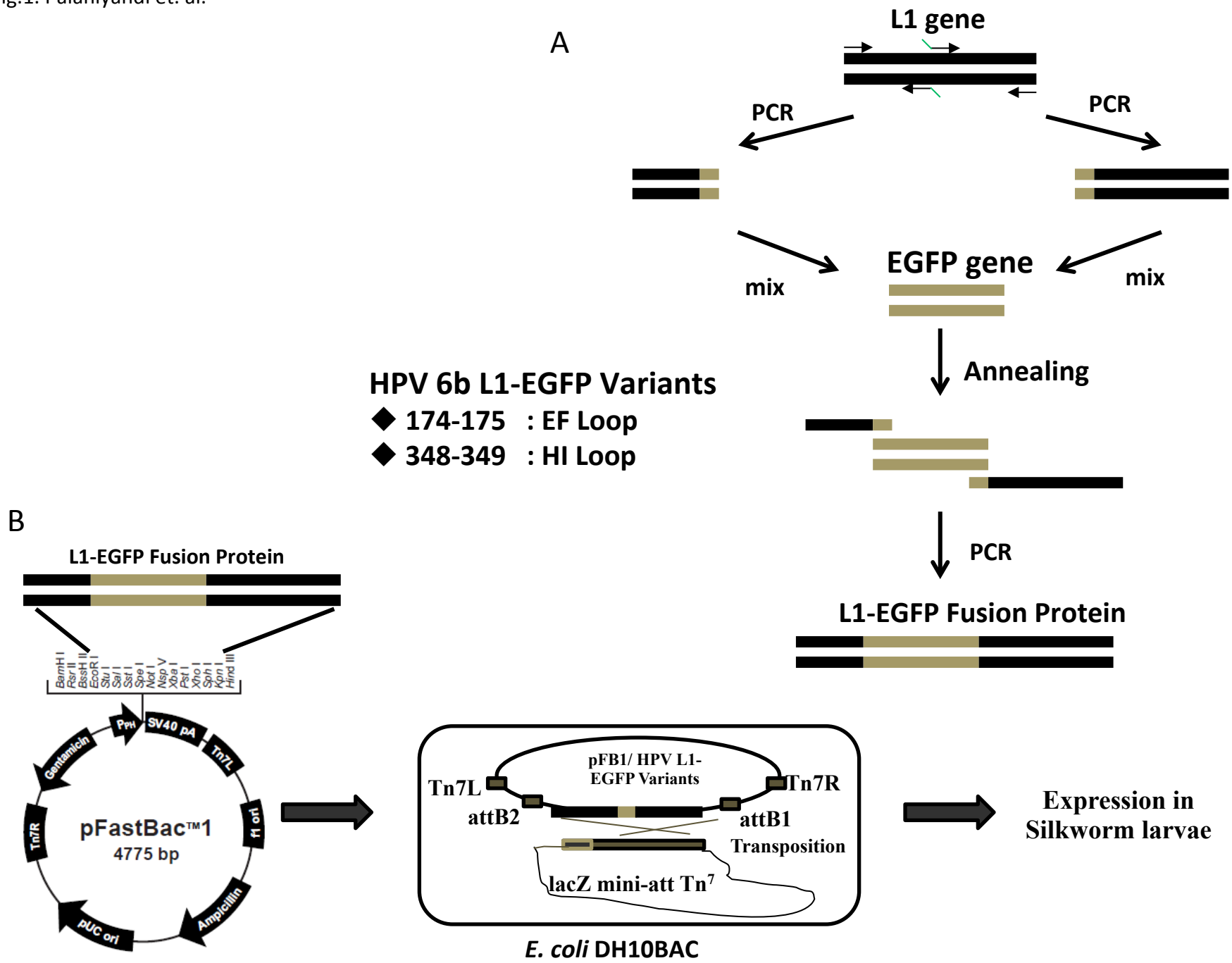

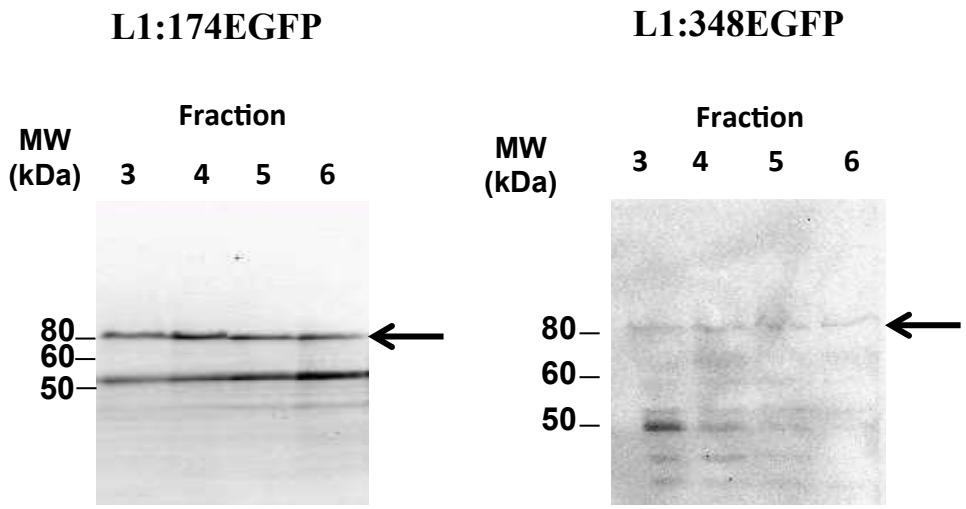
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Fig. 3. Palaniyandi et. al.

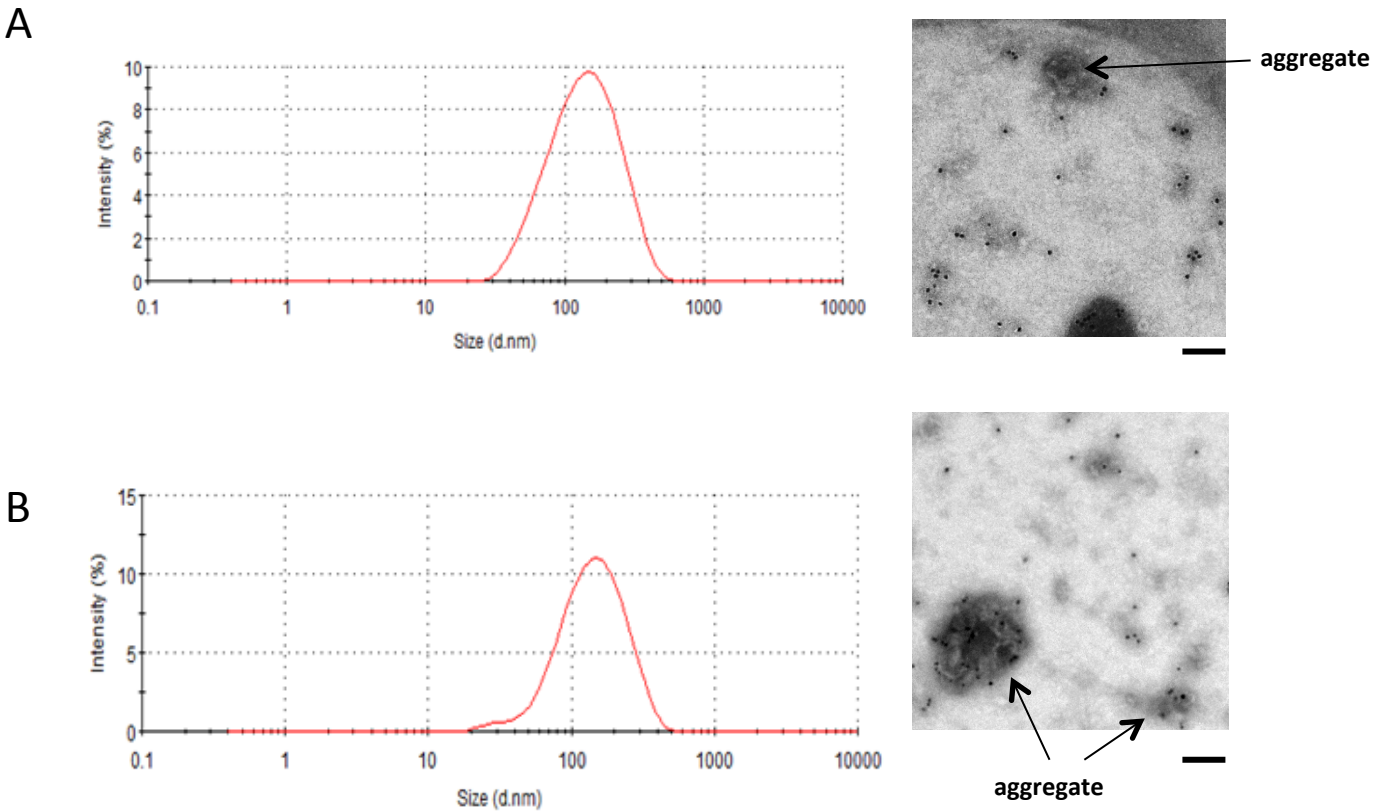

Fig. 4. Palaniyandi et. al.