Kinetoplastid membrane protein (KMP-11) of *Leishmania donovani* form transient pore in artificial lipid membrane

Under supervision of

DR. KRISHNANANDA CHATTOPADHYAY

Chief Scientist & Head

Structural Biology & Bioinformatics Division

CSIR-Indian Institute of Chemical Biology

Kolkata, India

Submitted By

SATARUPA DEB SINHA

Roll No: 234111008

MTech, Final Year

Department of Biosciences & Bioengineering

Indian Institute of Technology

Guwahati, India

Acknowledgement

I would like to thank Dr. Krishnananda Chattopadhyay, Chief Scientist & Head, Structural Biology & Bioinformatics Division, CSIR-Indian Institute of Chemical Biology, Kolkata for giving me the opportunity to do this project work under valuable guidance and guiding me thoroughly to write this dissertation.

I would also like to thank Rajdip Roy, Research Scholar, Structural Biology & Bioinformatics Division, CSIR- Indian Institute of Chemical Biology, Kolkata for constant support, guidance, and motivation to complete this project work, without which it would have been impossible for me to complete this dissertation. I would also like to thank Rajeev Jain, Souradip Paul, Sumangal Roychowdhury, Ashmita Mukherji, Sampa Mandal, Saradindu Mondal, Pratip Mukherjee and all other lab members for their help.

My sincere thanks to Dr. Sachin Kumar (Associate Professor), and Dr. Rakhi Chaturvedi (Head of the Department), Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati for allowing me to do this project work as a part of course curriculum.

Last but not the least I would like to thank my family for providing constant support and strength.

Date:

Signature

Place: (Satarupa Deb Sinha)

Abstract

Kinetoplastid membrane protein-11 (KMP-11), of size 11 kDa is a small, conserved flagellar protein amongst all kinetoplastid parasites. It is highly expressed throughout the *Leishmania* life cycle and is a potential leishmaniasis vaccine candidate. This study focuses on the interactions between the KMP-11 protein of the Leishmania parasite and artificial lipid membranes using an unsaturated 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and saturated 1,2-didecanoyl-sn-glycero-3-phosphocholine (DPPC) lipid. LUVs (Large unilamellar vesicles) were made with DOPC and SUVs (Small unilamellar vesicles) were made with DPPC lipids respectively. Calcein release assay was performed and as observed from the fluorometric experiments, the fluorescence intensity was seen to increase with increasing concentrations of KMP-11 in both calcein infused LUVs and SUVs. This assay, which evaluated the effect of KMP-11 on vesicle disruption, indicated that the protein might have pore-forming capabilities, causing the release and fluorescence of calcein encapsulated within the vesicles.

Keywords: KMP-11, kinetoplastid parasites, artificial lipid membranes, DPPC, DOPC, calcein, fluorescence

1 Introduction

Leishmaniasis, a vector-borne disease caused by parasites from the genus *Leishmania*, presents in various clinical forms depending on where the parasites infect. It can be broadly classified into tegumentary leishmaniasis, affecting the skin or mucosa, and visceral leishmaniasis (VL), affecting internal organs like the spleen, liver, bone marrow, and lymph nodes. Tegumentary leishmaniasis includes cutaneous (CL), diffuse cutaneous (DCL), and mucosal (ML) forms, with CL being the most common. [1,4] One of the most lethal forms is visceral leishmaniasis (VL), caused by *Leishmania donovani*. The estimated annual incidence is 0.2–0.4 million cases of VL and 0.7–1.2 million cases of CL, with around 20,000–40,000 deaths per year due to VL, though these numbers are likely underestimated. More than 90% of global VL cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil.[5] These parasites employ several strategies to evade the host's immune response, including – (i) Modifying the complement system and phagocytosis, (ii) Altering the Toll-like receptor pathway (iii) Inhibiting phagosome maturation (iv) Impairing antigen presentation and co-stimulation (v) Altering host cell signalling (vi)Modifying T-cell responses.[2] Among these, the impairment of antigen presentation is particularly notable due to the sequestration of antigenic components from being loaded onto MHC class-II molecules. KMP-11, a highly abundant and immunogenic protein on the parasite's surface, plays a crucial role in this process.[3] This protein, which is 11 kDa and highly conserved across all stages of the *Leishmania* life cycle, serves as an effective B and T cell immunogen during infection.

KMP-11 is predominantly found on the cell surface, particularly around the flagellar pocket, intracellular vesicles, and flagellum. It is expressed in both the promastigote (sand fly) and amastigote (human) phases of the parasite, with higher surface expression in the amastigote phase.[2] This suggests a significant role of KMP-11's molecular structure in the parasite's interaction with the mammalian host. KMP-11, essentially an α-helical protein, exhibits significant sequence homology with human apolipoprotein A-1 (ApoA1), sharing an amphiphilic character.[6] This similarity includes an abundance of tryptophan residues, typically found in membrane proteins and thought to anchor the protein near the lipid-water interface.[7] This characteristic supports the potential of KMP-11 as a vaccine candidate against visceral leishmaniasis, leveraging its immunogenic properties during infection.

The interaction of KMP-11 with phospholipid membranes has been explored using various biophysical methods. For instance, studies have shown that peptides can insert into the hydrophobic core of phospholipid bilayers, affecting membrane permeability and integrity.[8]

This is relevant for understanding how KMP-11 might interact with cellular membranes and contribute to its biological functions. In this study, we thoroughly investigated the interaction between KMP-11 and phospholipid membranes.

2 Objective

i. Purification of KMP-11 protein from transformed *E.Coli* BL21cells
ii. Preparation of Large Unilamellar Vesicles (LUVs) and Small Unilamellar Vesicles (SUVs)
iii. To check the interaction of KMP-11 protein upon binding with lipid vesicles by calcein release assay

3 Materials and Methods

3.1 Materials used

LB(Luria-Bertani) media (from Sigma Aldrich), Calcein dye (from Sigma Aldrich), Sodium dihydrogen phosphate (Sigma Aldrich), Di-Sodium hydrogen phosphate (Sigma Aldrich), Potassium dihydrogen phosphate (Sigma Aldrich), Imidazole (Sigma Aldrich), SnakeSkin dialysis tubing-10 MWCO (Thermo Scientific), Hi-Media Glass plates, Thermo Scientific GeneJET Plasmid Miniprep Kit, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Avanti Polar Lipids, 1,2-didecanoyl-sn-glycero-3-phosphocholine (DPPC) from Avanti Polar Lipids, Ni-NTA (Qiagen), Sodium dodecyl sulphate (SDS) from Amresco, SDS Loading Buffer 2x (from G-Biosciences), Invitrogen DNA Ladder, PageRuler Protein Ladder Phenylmethanesulfonyl Fluoride (PMSF) from Sigma Aldrich, Ammonium persulfate, TEMED (Bio-Rad) , Pipetteman, Micropipette tips, Eppendorfs, Falcons (from Tarson), Syringr filter 0.22 µm (from Sartorius)

3.2 Preparation of competent cells and transformation

100 µl of DH5α cells from 20% glycerol stock was added to 50 ml of autoclaved LB media and allowed to incubate overnight at 37°C at 200 rpm. Next day 400 µl of the above culture was added to 20ml autoclaved LB media and kept for incubation at 37°C at 200 rpm for 2 hrs. The culture was then centrifuged at 6000 rpm for 10 mins at 4°C. Supernatant was discarded carefully inside laminar followed by addition of 4.5ml of 100 mM chilled $CaCl_2$(filtered) to cell suspension. After keeping the suspension on ice for 30 mins it was again centrifuged at 6000 rpm for 10 mins at 4°C. The supernatant was thereafter discarded and 1.5 ml of $CaCl_2$ was added and resuspended. This mixture was then kept on incubation for 2 hrs in ice. Further 200 µl of $CaCl_2$ and cell mixture was taken in an Eppendorf and 2µl plasmid (pET28a vector

containing gene encoding for KMP-11) was added to it followed by tapping on table top vigorously. The sample was then kept in ice for 30 mins followed by heat shock at 42°C on water bath for 90s and then cold shock for 3 mins in ice. This cell suspension was then added to 1 ml of chilled LB media inside laminar and then allowed to incubate at 37°C for 2 hrs at 200rpm. Henceforth supernatant was discarded after pelleting down for 5 min at 6000rpm and fresh 200µl media was added. 200µl of media containing the transformed cells were plated on Kanamycin resistant agar plates. Another 200µl of competent cells without containing the plasmid was plated on Kanamycin resistant agar plates for control. These plates were kept at 37°C overnight allowing multiplication of cells.

Fig.1. LB Agar plates containing Kanamycin showing transformed DH5α *E.Coli* plate (left) and Control DH5α plate (right).

3.3 Plasmid purification

Plasmid was purified from DH5α cells following a standard protocol from Thermo Scientific GeneJET Plasmid Miniprep Kit with slight modifications. The bacterial culture was first grown overnight in 5 mL of LB broth containing the appropriate antibiotic (Kanamycin). The cells were then harvested by centrifugation at 6,000 rpm for 2 minutes at 4°C. The bacterial pellet is then resuspended in 250 µL of the Resuspension Solution (containing RNase A) and transferred to a microcentrifuge tube. Next, 250 µL of Lysis Solution is added and the tube is gently inverted 4-6 times to mix. Subsequently, 350 µL of Neutralization Solution is added and the tube is inverted 4-6 times to mix thoroughly. The lysate is then centrifuged for 5 minutes to

pellet cell debris and chromosomal DNA. The clear supernatant is transferred to a GeneJET spin column by pipetting, avoiding the pellet. The column is centrifuged for 1 minute, the flow-through is discarded, and the column is placed back into the same collection tube. The column is washed by adding 500 µL of Wash Solution (diluted with ethanol) and centrifuging for 1 minute. The flow-through is discarded, and the wash step is repeated. After the second wash, the column is centrifuged for an additional 1 minute to remove any residual wash solution. The column is then transferred to a clean microcentrifuge tube, and 50 µL of Elution Buffer is added to the centre of the membrane. The column is incubated for 2 minutes at room temperature, followed by centrifugation for 2 minutes to elute the plasmid DNA.

The purified plasmid DNA was then run on 0.8% agarose gel to assess the purity and size of plasmid.

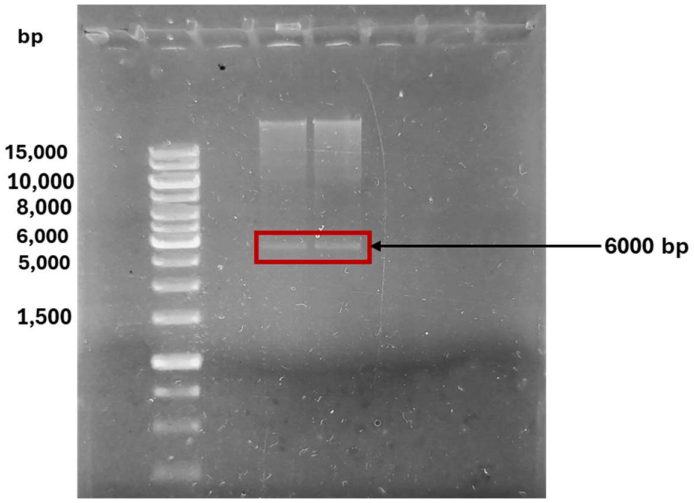

Fig.2 Image shows purified KMP-11 plasmid run on 0.8% agarose gel. Lane 1 is empty, lane 2 consists of Invitrogen DNA ladder (1 kb), lane 3 is empty whereas lane 4, and 5 was loaded with 8 µl and 10µl of plasmid respectively.

3.4 Transformation of purified KMP-11 plasmid to BL21 cells

To transform the purified plasmid into E. coli BL21 cells, the chemically competent E. coli BL21 cells were thawed on ice. 1-5 µL of the purified plasmid DNA was added to 50 µL of the competent cells and gently mixed. The mixture was incubated on ice for 30 minutes, followed by a heat shock at 42°C for 45-60 seconds. The cells were immediately placed back on ice for 2 minutes. Subsequently, 450 µL of Recovery LB media was added, and the cells were incubated at 37°C with shaking at 200-250 rpm for 1 hour. Following incubation, 100 µL of the transformation mixture was spread onto LB agar plates containing the appropriate antibiotic (Kanamycin). The plates were incubated overnight at 37°C.

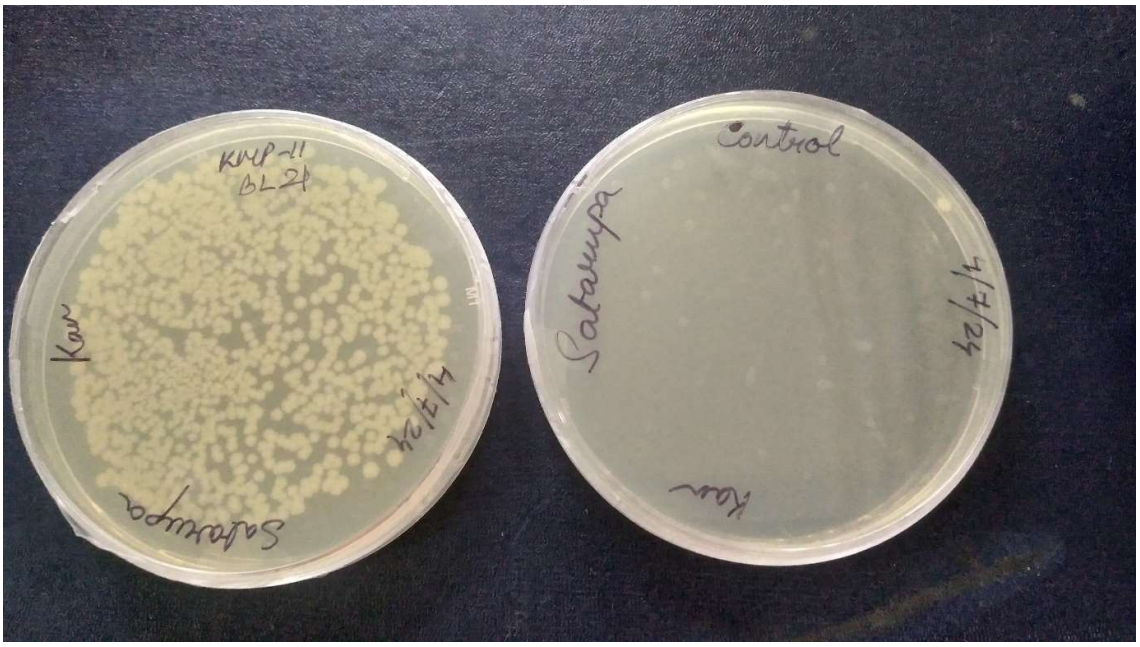

Fig.3. LB Agar plates containing Kanamycin showing transformed BL21 *E.Coli* plate (left) and Control BL21 plate (right).

3.5 Purification of KMP-11 protein by Ni-NTA affinity chromatography

Recombinant KMP-11 constructs, were expressed and purified through Ni-NTA affinity chromatography following Qiagen protocol with little modifications.[9] 200 µl of the strain was taken from glycerol stock (transformed BL21 cells) and added to 50ml LB along with 50µl of Kanamycin and was allowed to incubate overnight in 37°C at 150rpm. For secondary culture 10 ml of primary culture was added to 500ml of LB. Additionally 500µl of Kanamycin was added and was kept for incubation in 37°C at 150rpm for 3-4 hrs. OD value was measured at 280 nm and when it reached 0.6, the culture was induced with IPTG and allowed to incubate for 2 more hours. After overexpression of the protein on inducing with IPTG, the culture was divided into 2 oak ridge tubes and centrifuged at 5000rpm for 20 mins. Supernatant was discarded and pellet was stored in the oak ridge tubes at -20°C overnight. Next day the pellet was lysed using 10 ml of lysis buffer and 200µl of PMSF solution, followed by sonication for 2 hours. It was then centrifuged at 12,500 rpm for 45 mins at 4°C. Supernatant was collected and stored in falcon kept on ice.

Column chromatography was performed using Bio-Rad's PD-20 column. Column was first washed with lysis buffer and then the supernatant was added and allowed to bind for 1hr 30 mins in shaker in cold room. The flow through was collected by passing the above through the column. This step was followed by washing with 50 ml low salt buffer to remove non-specifically bound proteins. KMP-11 protein was then eluted using high salt elution buffer.

After extracting KMP-11 protein from BL21 cells using column chromatography, the eluted protein was dialyzed using SnakeSkin dialysis tubing with a 10 kDa molecular weight cut-off (MWCO). The protein solution was placed into the dialysis tubing and dialyzed against 1x, 20mM Sodium phosphate buffer 24 hours. Buffer was changed 3 times and fresh 1x Sodium phosphate buffer was added during the procedure. The dialyzed protein was then aliquoted and stored in Eppendorf in -20°C.

3.6 Determining molecular weight of KMP-11

In order to ensure the purity and size of the protein, SDS PAGE was performed. 10µL of 2x SDS Loading buffer consisting of Tris-HCl, glycerol, SDS and bromophenol blue (BPB) was added to the protein samples and denatured by boiling in a water bath for 15 minutes. After cooling, different concentrations of protein- 25µM, 50 µM, 80 µM, 100 µM, 200 µM were loaded onto an 18% polyacrylamide gel prepared according to the protocol. Electrophoresis was carried out at a constant voltage of 200V until the bromophenol blue dye front reached the bottom of the gel. Post-electrophoresis, the gel was carefully removed and stained with Coomassie Brilliant Blue R-250 for 40mins, followed by destaining in a solution of 10% acetic acid and 40% methanol until clear bands were visible. Protein bands were visualized (Fig. 4) and their molecular weights estimated by comparison with a pre-stained protein ladder run concurrently. The purity of KMP-11 was determined based on the presence of a single prominent band at the expected molecular weight (11kDa).

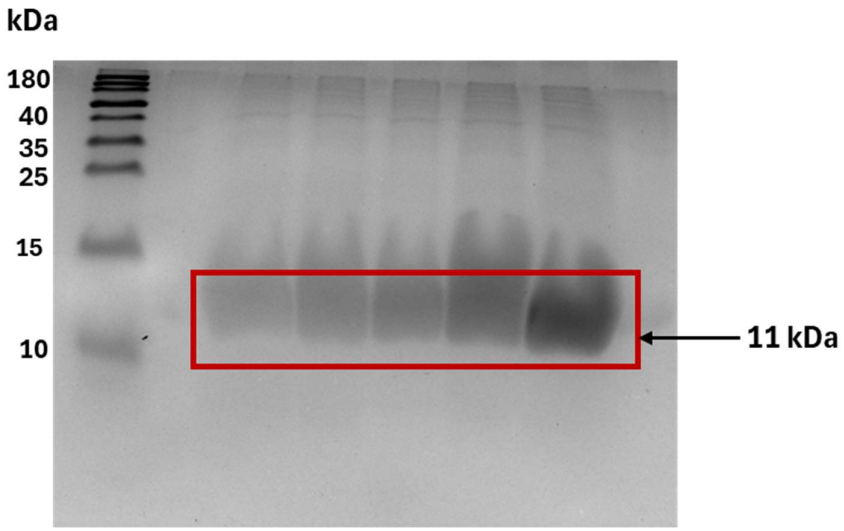

Fig 4. 18% SDS-PAGE gel showing desired band of different protein concentrations at the expected position that is 11 kDa. Lane 1 consists of PageRuler protein ladder, lane 2 is empty whereas lane 3, 4, 5, 6 and 7 respectively consists of protein in the concentrations-25µM, 50 µM, 80 µM, 100 µM and 200 µM.

Further MALDI-TOF was also performed to verify the purity and size of the obtained protein. (Figure 5)

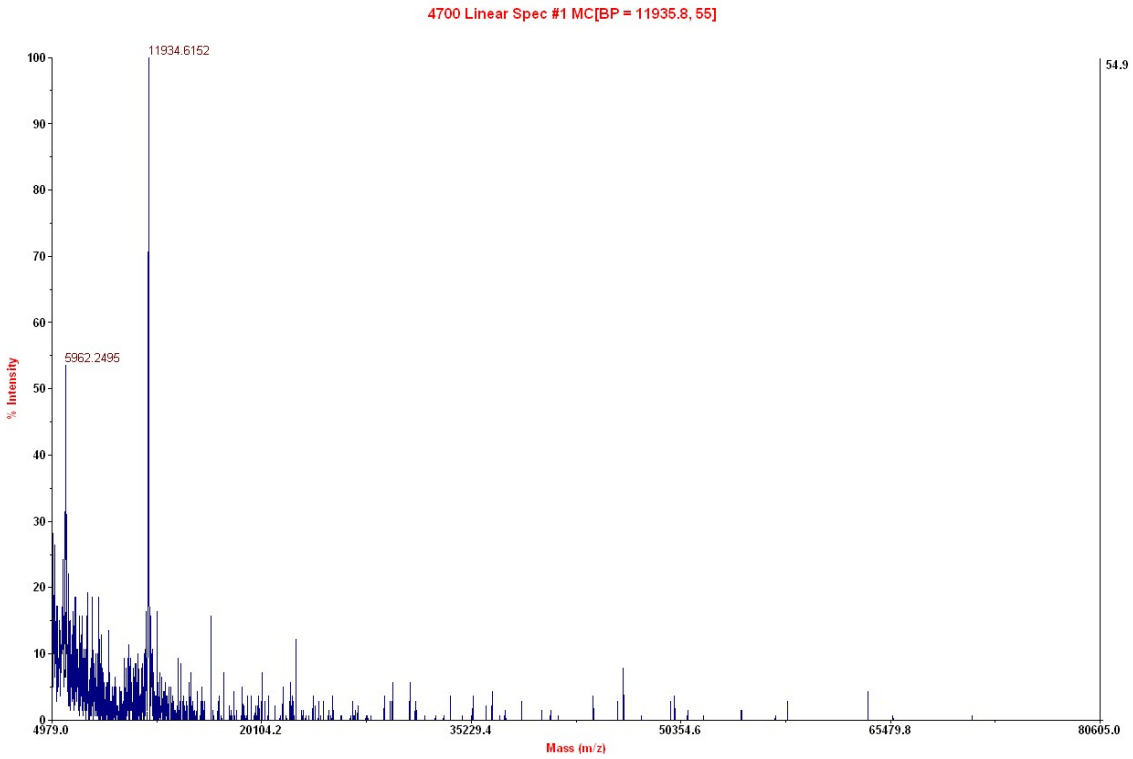

Fig. 5. MALDI-TOF mass spectrum for detecting molecular weight of KMP-11

3.7 Preparation of calcein dye

A 70 mM calcein dye solution was prepared by dissolving 0.0187g of calcein in 1.7 mL of Sodium phosphate buffer. The solution was mixed thoroughly by vortexing for 30mins until the calcein was completely dissolved. The pH of the solution was adjusted to 7.4 using 300µl of 3N NaOH as needed. The prepared calcein dye solution was poured into SnakeSkin dialysis tubing with 10 MWCO and dialysed against 20 mM Sodium phosphate buffer for 3 days to get rid of the free dye. It was then filtered through a 0.22 µm syringe filter to remove any particulate matter and ensure sterility. The filtered solution was collected in sterile falcon tubes and stored at -20°C until use.

3.8 Preparation of calcein loaded Small and Large Unilamellar Vesicles (SUVs and LUVs)

Small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) were prepared using DPPC and DOPC, respectively. For SUVs, 12 mM DPPC and 8 mM cholesterol was dissolved in 200 µl of chloroform and dried under a stream of nitrogen gas to form a thin lipid film, which was then placed under vacuum overnight. The dried lipid film was hydrated with 4ml Sodium phosphate buffer and sonicated for 2 hours in a bath sonicator at 60°C. For LUVs, we first

dissolved DOPC in a 5 mL glass vial. The working lipid concentration was 5mM. The chloroform was evaporated using a gentle stream of dry nitrogen gas, followed by placing the sample in a desiccator connected to a vacuum pump for 3 hours to remove any residual solvent. and to form a thin lipid film. The glass vial containing the dried lipid film was hydrated with prepared calcein solution and sonicated for 3 hours in bath sonicator at 60°C followed by probe sonication for 1.5 hours at 60°C in order to infuse calcein dye within the vesicles. The final vesicle suspensions were stored at 4°C until further use. The average diameter of LUVs(>100nm) and SUVs(<100nm) were determined by performing dynamic light scattering (DLS).

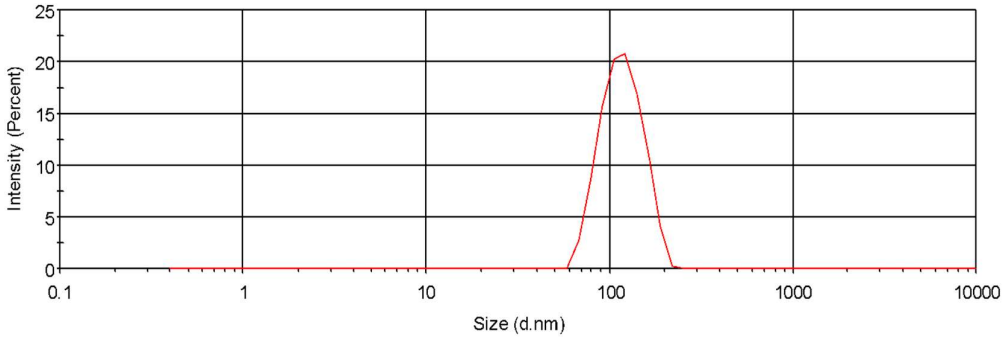

Fig.6. DLS measurement showing average diameter of the prepared LUVs

3.9 Determining secondary structure of KMP-11

The secondary structure of protein KMP-11 was determined using Circular Dichroism (CD) spectroscopy by following a specific protocol. Protein samples were prepared in an appropriate buffer, typically 10 mM sodium phosphate. A quartz cuvette with a path length of 1mm was used. The CD spectra were recorded in the far-UV region (200-250 nm) using a JASCO J720 spectropolarimeter (Japan Spectroscopic Ltd.). The spectra were collected with a bandwidth of 1 nm, a scan speed of 100 nm/min, and a response time of 4 seconds. Multiple scans (e.g., 3 runs) were accumulated to improve the signal-to-noise ratio, averaged, and the buffer baseline was subtracted. The CD spectra were analyzed to identify characteristic minima and maxima `corresponding to different secondary structures, such as α-helices, β-sheets, and random coils.

4 Results and Discussion

4.1 Calcein release assay

Calcein, a fluorescent dye, is commonly used in calcein leakage assays to assess membrane integrity and permeability. Encapsulated within liposomes, calcein exhibits strong fluorescence quenching at high concentrations. Upon membrane disruption, calcein is released, resulting in

dilution and a corresponding increase in fluorescence.[10] The excitation and emission maxima of calcein are at approximately 495 nm and 515 nm, respectively. This property makes calcein a suitable marker for real-time monitoring of membrane damage and the effects of membrane-active agents, such as peptides or detergents.

A fluorometric assay was employed to study the binding of KMP-11 with large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs) composed of DOPC and DPPC-cholesterol mixtures. All samples were prepared in 20 mM sodium phosphate buffer at pH 7.4. The steady-state fluorescence emission spectra of the dye were measured with an excitation wavelength of 485 nm. The peak intensity values at 520 nm for DOPC and at 550 nm for DPPC-cholesterol were plotted against protein concentration.

4.1.1 KMP-11 interaction with calcein infused LUVs

A set of four samples were prepared using fixed amount of uniformly synthesized lipid vesicles and increasing protein concentrations - 10 µM, 20µM, 25 µM, and 40µM respectively in Sodium phosphate buffer. Time-based fluorescence scan was carried out. Fluorescence intensity of calcein released from LUVs were plotted against different concentrations of protein by subtracting the blank. Result shows that the fluorescence intensity increases over time with increasing concentrations of protein.

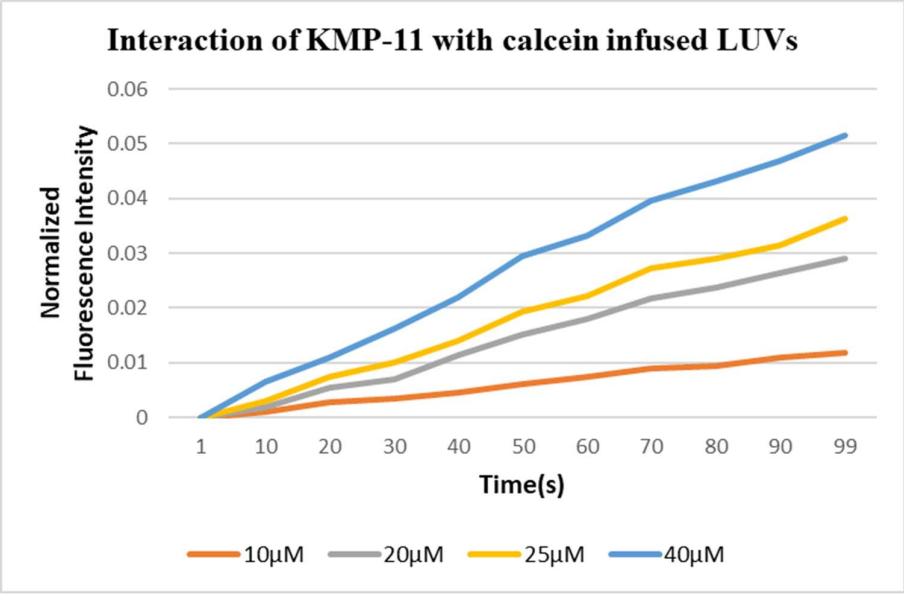

Fig.7. Fluorescence intensity originated from calcein in the presence of DOPC LUV were plotted against the concentration of KMP-11

4.1.2 KMP-11 interaction with calcein infused SUVs

A set of three samples were prepared using fixed amount of uniformly synthesized lipid vesicles and increasing protein concentrations - 10 µM, 20µM, and 30µM respectively in Sodium phosphate buffer. Time-based fluorescence scan was carried out and normalized fluorescence intensity were plotted against different concentrations of protein by subtracting the blank. Result reflects that the fluorescence intensity increases over time with increasing concentrations of protein.

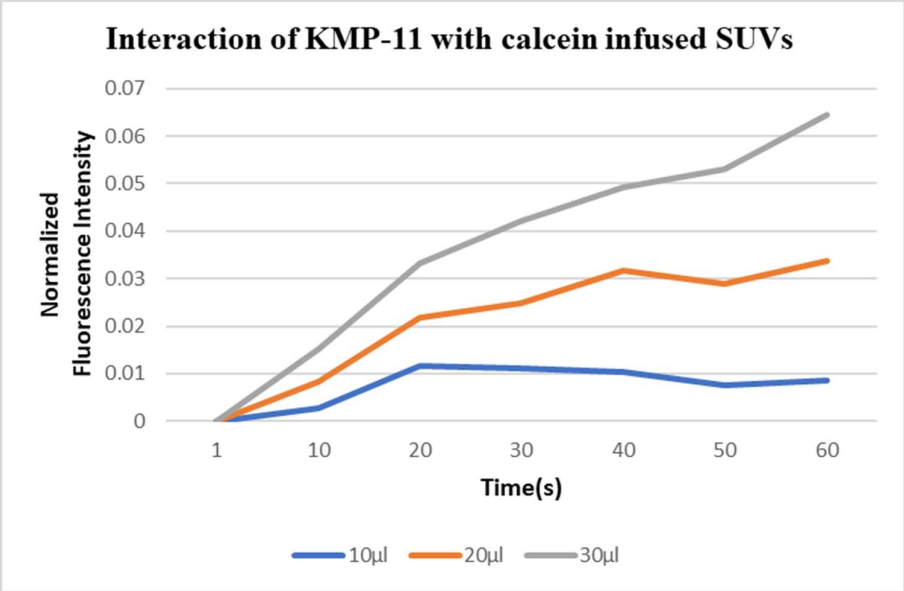

Fig.8. Fluorescence intensity originated from calcein in the presence of DPPC SUV with cholesterol were plotted against the concentration of KMP-11

4.2 CD spectroscopy analysis

Far UV CD spectroscopy is a widely used technique for determining the secondary structure of proteins. In Far UV CD spectroscopy, the presence of double minima at 208 nm and 223 nm (Figure 9) is indicative. The CD profile observed suggests that KMP-11 has an alpha-helical structure.

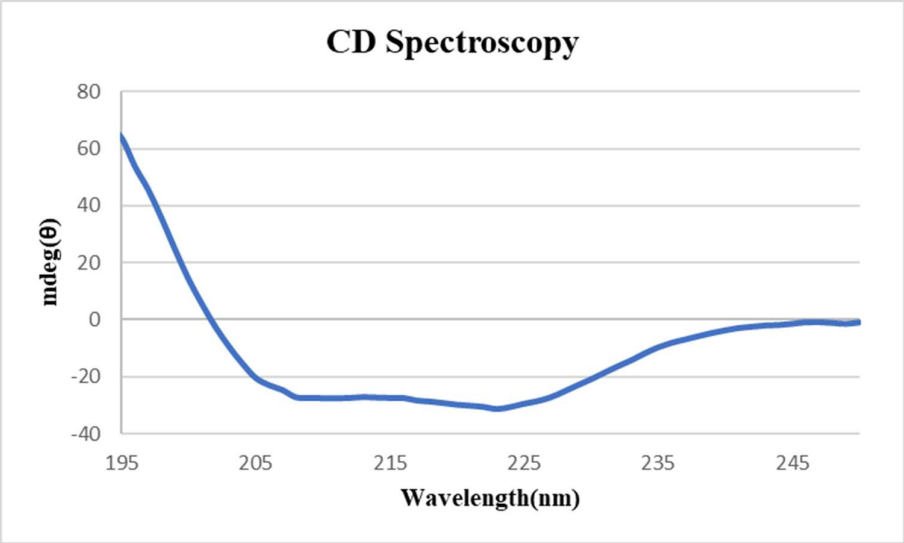

Fig 9. Far UV-CD spectra of KMP-11 protein.

5 Conclusion

The water-soluble fluorophore calcein is highly suitable for dye leakage assays due to its self-quenching behaviour at high concentrations within the lumenal space of synthetic vesicles. Upon dilution in the extra-liposomal buffer following membrane disruption, there is a significant increase in fluorescence emission (Figure 7 & 8). The assay employed to assess the impact of KMP-11 protein on vesicle disruption suggests that the protein may possess pore forming ability as a result of which calcein infused within the vesicles got released and showed fluorescence.

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