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Cationic Liposome-Mediated Delivery of Leishmania Donovani Soluble Antigen: Physicochemical Analysis and Induction of an IgG Response in BALB/c Mice.

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Abstract Visceral leishmaniasis is common in certain areas. The liver and spleen are affected by this illness caused by the Leishmania donovani parasite in humans and animals. This research aimed to develop a new SLA-based liposome vaccination. The purpose is unclear. Improved illness detection and treatment. SLA antigen was encapsulated in a positively charged liposome for vaccine formulation. Stability and immune cell interaction are promoted by egg lecithin, cholesterol, and stearylamine. The vaccine was well-prepared and targeted the antigen, according to testing. A test showed a concentration of 2.1 ± 0.3 mg/min for SLA antigen. The SLA-containing liposome particles were of outstanding size, measuring around 245 ± 10 nm. Particle sizes were consistent with a dispersion value of 0.21 ± 0.05. The zeta-potential investigation found 61.16 mV more positive charge than empty liposomes. Improved cellular absorption and stability should boost immunization efficacy. FTIR and XRD studies revealed that the liposomes contained the SLA antigen, indicating that the loading technique was effective and did not modify the liposomes. Before exposing mice to harmful compounds, their immune reaction has to be assessed. The liposome-SLA group had a stronger antigen-specific IgG response than the SLA-only or empty liposome groups. It seems that liposomes boosted the immunological response. The SL1 group's final count was 1/160, double the S2 group's 1/80. Since it was their first encounter to the virus, the unimmunized group exhibited the greatest antibody response. Even at 1/320 concentrations, the SL1 group had robust and sustained reactions with higher values. This consistent outcome shows that the liposome-SLA vaccination prepares the immune system to respond promptly to the illness. Visceral leishmaniasis (VL) vaccination, apparently.

Keywords: Liposome, SLA, *Leishmania*, ELISA

Introduction The *Leishmania* parasite causes leishmaniasis. Animal and human immunizations are not yet permitted (1). Visceral leishmaniasis is severe. It affects around 98 nations and 380 million individuals annually (2). As the second-leading infectious murderer and fourth-leading disease killer, it is a neglected tropical illness. These parasites' soluble antigens are mostly lipophosphoglycans. In addition to a secreted acid phosphatase and repeated phosphoglycan chains, they bind albumin. Soluble leishmaniasis antigen (SLA) comprises gp63, KMP-11, PSA, and gp46,

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making it a vaccine candidate. 5 SLA was used as a model for early vaccines because of its immunogenicity against Leishmania. With the correct assistance, SLA may activate a Th1-type immune response to control the infection. This antigen is simple, affordable, and versatile, making it a promising vaccine ingredient (7).

Nanoparticle delivery strategies? They are vital in biology and medicine. In this aspect, liposomes are crucial. Due to their phospholipid and cholesterol membranes, they can easily handle hydrophilic and hydrophobic substances. This allows them to gather several chemicals (8). Liposomes are attractive because to their biocompatibility, great carrying capacity, and simplicity of manufacturing. If desired, I can have them perform additional things. They perform effectively in vaccines when their size, charge, and flexibility are considered (9). Its stability makes liposomes ideal for encasing antigens and providing long-term protection against *Leishmania donovani.* Recent vaccine manufacturing advances allow bulk production of strong adjuvant liposomes. Thus, we need reliable and efficient protein content measurement methods to ensure its efficacy and purity.

BCA works effectively for measuring total protein concentration. Many detergents work with it. This is done via protein peptide linkages. Protein reduces copper ions to Cu^{+}. Change depends on protein quantity. The colorful, light-absorbing combination of copper ions and bicinchoninic acid peaks at 562 nm. Protein may be measured by absorption strength. The test works well for a series of microplate studies, even if lipids may affect the findings (12). The immune system relies on antibodies to fight infections. The humoral immune system generates these chemicals, which may hinder or activate defenses against invaders. Antibody-producing B cells are crucial to leishmaniasis (13). Visceral leishmaniasis patients have many Leishmania antibodies, but they don't protect them. Higher antibody levels suggest worse illness (11). Researchers are testing blood tests like ELISA for cutaneous leishmaniasis (CL) detection (1). We employ synthetic proteins, peptide fragments, and soluble Leishmania antigen in these experiments. All of these factors impact exam performance (13).

Material and Methods

Ethical approval

The investigation was ethically certified by the College of Veterinary Medicine ethics committee at University of Al-Qadisiyah, Iraq, resulting in the issuance of an ethical approval number, 1890 in 28/8/2023.

Experimental animals and parasite culture

Female BALB/c mice aged 6 to 8 weeks were used in the experiment and randomly divided into four groups of ten animals each. This design allowed for tracking lesion size in four mice per group throughout the study, while four duplicate subgroups from each group were dedicated to assays assessing parasite load and immune response characteristics.

All media preparations were conducted under sterile conditions within a laminar flow hood. The culture medium consisted of 440 ml RPMI 1640 supplemented with 50 ml heat-inactivated fetal bovine serum (FBS), along with penicillin (10,000 units) and streptomycin (10 µg/ml) to prevent microbial contamination. The medium was sterilized through a disposable bottle-top filter and stored at 4°C (14).

Soluble *Leishmania donovani* Antigens (SLA) preparation

SLA for vaccination was prepared following the method described previously (15), with some modifications. In brief, promastigotes were collected from a mixed culture of *L. donovani* parasites, counted using a hemocytometer, washed three times with cold phosphate-buffered saline (PBS), and resuspended in PBS. Up to 5×10^6 cells were transferred into a 1.5 ml microcentrifuge tube or a 15 ml centrifuge tube and centrifuged at 300 × g for 5 minutes. After completely discarding the supernatant, 400 µl of DR Buffer and 4 µl of β-mercaptoethanol were added, and cells were resuspended by pipetting or vortexing. Protein extraction was then performed using a kit from Geneaid following the manufacturer's instructions. The lysate was incubated at room temperature for 5 minutes, then centrifuged at 12,000–16,000 × g for 2 minutes . For protein precipitation, four volumes of ice-cold acetone were added to the flow-through (e.g., 2.8 ml

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acetone to 700 µl flow-through) and incubated on ice or at −20°C for 30 minutes. The sample was centrifuged at 14,000–16,000 × g for 10 minutes, and the supernatant was discarded. The protein pellet was washed with 100 µl ice-cold 70% ethanol, the supernatant discarded, and the pellet air-dried at room temperature. Finally, the protein pellet was dissolved in up to 100 µl of DV buffer (8 M urea) or another compatible buffer for downstream applications.

BCA Assay (Bicinchoninic Acid Assay)

Measuring protein concentration is a critical step in many biological experiments, especially when you want to quantify the amount of protein in a sample before proceeding with further analysis (16) The BCA assay quantifies protein concentration by exploiting the biuret reaction, where proteins reduce Cu^{2+} to Cu^{+} in an alkaline medium. The resulting Cu^{+} ions form a purple complex with bicinchoninic acid, which absorbs light at 562 nm. For the assay, a working reagent is prepared, and a standard curve is generated from serial dilutions of a 1 mg/ml protein standard. Cell samples, such as promastigotes, are homogenized and centrifuged to obtain a protein supernatant. Subsequently, standards and samples are mixed with the BCA working solution, incubated at 37°C for 30 minutes, and the absorbance is measured to determine concentration against the standard curve.

Liposomes preparation and coated with SLA

Coating soluble Leishmania antigen (SLA) with liposomes composed of octadecylamine (ODA) that called Stearylamine involves forming lipid vesicles that encapsulate or adsorb the antigen, Soluble Leishmania antigens (SLA) with coated liposomes positive charge were prepared according to a previously established method (S. Bhowmick et,.al 2007) with some modifications. Add Octadecylamine (ODA): 1 mg/mL (10% molar ratio relative to PC). Then Adjust the ratio of ODA based on the desired surface charge (e.g., 5-20% molar ratio). After that Liposomes with a positive charge were formulated using egg lecithin (27 mol) and cholesterol at a molar ratio of 7:2 (Sigma-Aldrich). Alternatively, a combination of egg lecithin, cholesterol, and stearylamine (Fluka, Buchs SG) was used at a 7:2:2 molar ratio. The preparation followed a modified version of the Afrin method (17).

Initially, The lipid mixture was first dissolved in chloroform, then the solvent was evaporated under reduced pressure using a rotary evaporator, leaving a thin, dry lipid film. This film was then resuspended in either 1 mL of PBS (for empty liposomes) or 1 mL of PBS containing 1 mg/mL SLA (for antigen-loaded liposomes). The suspension was vortexed and sonicated for 30 seconds using a probe sonicator (Misonix).

Physicochemical Characterization Techniques for liposomes.

The following characterization tests was used including, UV-Visible spectroscopy (UV-Vis), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), field-emission scanning electron microscopy (FESEM), and zeta potential analysis to analyze liposomes and determine their physical and chemical properties.

Immunization of the experimental animals

This research examined immune system augmentation using liposomes, soluble *Leishmania* antigen (SLA), and imiquimod. We evaluated 18 vaccination combinations to discover the best. SLA and imiquimod liposomes were intended to boost immune response and enhance subsequent vaccines. The mouse population was split into multiple fifteen-member groups and immunized three times every two weeks using intraperitoneal injections. In the lab, liposomes with 50 µg of soluble leishmaniasis antigen, empty liposomes, SLA antigen alone, and HEPES-sucrose buffer solution were generated.

Elisa analysis of the mice IgG

The basic ELISA protocol for detecting antibodies against SLA of *Leishmania donovani* was performed according to Piyasiri (19) with some modification. This protocol can be adapted based on specific requirements or reagents available. Include positive and negative controls in assay and optimize the antigen concentration and incubation times based on preliminary experiments. The material was using in ELISA kit analysis are SLA of L. donovani antigen, ELISA plates (96-well), Coating buffer (carbonate-bicarbonate buffer, pH 9.6), Blocking buffer (1% BSA in PBS), Sample diluent (PBS with BSA), Primary antibodies (serum samples),

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Secondary antibodies (HRP-conjugated anti-IgG), Substrate solution (TMB), Stop solution (1N HCl) and Washing buffer (PBS with Tween-20)

Dilute the SLA in coating buffer to the appropriate concentration, Add 100 µL of the antigen solution to each well of the ELISA plate. Then Incubate overnight at 4°C at room temperature. Remove the coating solution and wash the wells 3 times with washing buffer. Then add 200 µL of blocking buffer to each well and incubate for 1-2 hours at room temperature. Remove the blocking solution and wash the wells 3 times. then dilute the serum samples or antibodies in sample diluent and add 100 µL to each well. Incubate overnight at 4°C. Wash the wells 3 times with washing buffer. Add 100 µL of HRP-conjugated secondary antibody to each well and incubate for 1 hour at room temperature. Wash the wells 3 times with washing buffer. Add 100 µL of substrate solution to each well and incubate until color develops (usually 15-30 minutes). Stopping the Reaction: Add 50 µL of stop solution to each well to halt the reaction. Reading the Plate: Measure the optical density (OD) at 450 nm using an ELISA plate reader.

Results

Diagnostic of soluble *leishmania* antigens (SLA) by BCA Assay (Bicinchoninic Acid Assay)

Measuring protein concentration is essential in biological experiments for quantifying protein amounts prior to further analysis, and the BCA (Bicinchoninic Acid) assay is a widely used method for this purpose.

The measure of OD value the absorbance at 562 nm using a spectrophotometer, and compared the absorbance of samples to the standard curve to determine the protein concentration (figure 1) The graph illustrates a standard curve obtained through the Bicinchoninic Acid (BCA) assay for determining total protein concentration. Absorbance was measured at 560 nm, revealing a polynomial correlation between optical density and protein concentration (µg/ml). The curve is defined by the second-degree polynomial equation: **$y = 7046.2x^2 - 2924.3x + 274.85$**, with an **$R^2$ value of 0.9012**, suggesting a strong fit between the data points and the model. This standard curve serves as a reference for estimating the protein concentrations of SLA using their absorbance readings at 560 nm.

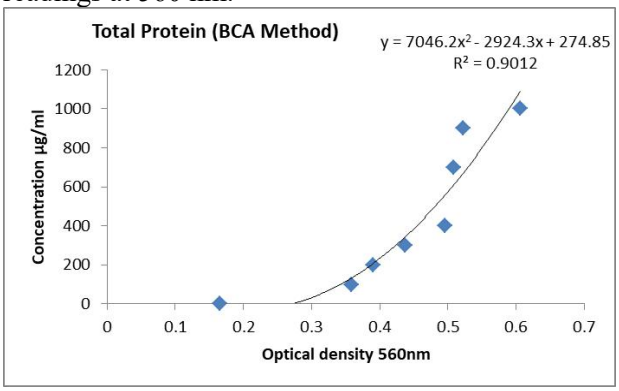

Figure 1: total protein in BCA method which show the curve is defined by the second-degree polynomial equation: $y = 7046.2x^2 - 2924.3x + 274.85$, with an R^2 value of 0.9012,

The results showed concentration of SLA in each sample are 1.74 mg/ml and 0.54 mg/ml respectively for sample 1 and sample 2 table (1). each concentration diluted with BPS in 50 µl according to calculate c1v1=c1v2.

Table (1): BCA protein purification results OD The data indicates protein quantification. The *Leishmania* sample shows successful purification with measurable SLA levels. Verification of buffer names and units is recommended.

BCA protein purification results OD:562nM			
	X	Y	
BSA Standard	O.D	Concentration µg/ml	
ST1	0.606	1000	
ST2	0.523	900	
ST3	0.508	700	
ST4	0.496	400	
ST5	0.438	300	
ST6	0.391	200	
ST7	0.359	100	
ST8	0.166	0	
Leishmania donovani proteins purification			
	O.D	Concentrations (µg/ml)	mg/ml
Sample 1	0.709	1743.41	1.74
Sample 2	0.492	541.68	0.54

Diagnosis of liposomes as well as SLA-liposome by Physicochemical Characterization Techniques.

The following tests UV-Visible spectroscopy (UV-Vis), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), field-emission scanning electron microscopy (FESEM), and zeta potential analysis were performed to analyze liposomes

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and determine their physical and chemical properties

UV-Visible spectroscopy (UV-Vis) tests

Liposomes alone (without SLPI) typically show low absorbance in the UV-vis range (200–800 nm) because they primarily consist of phospholipids, which lack strong chromophores, a slight absorbance may appear near 200–300 nm due to lipid carbonyl groups or unsaturated bonds in the phospholipid tails (figure 3-6)

SLPI is a protein, and proteins absorb strongly in the UV region (200–300 nm) due to peptide bonds (absorption peak at ~205 nm) and aromatic amino acids like tryptophan/tyrosine (peak at ~280 nm). If SLPI is successfully encapsulated, the absorbance spectrum should show: A prominent peak at ~205 nm (peptide bond absorption). A smaller peak at ~280 nm (aromatic amino acids). The absorbance intensity at these wavelengths will correlate with the concentration of SLPI inside the liposomes (figure 2)

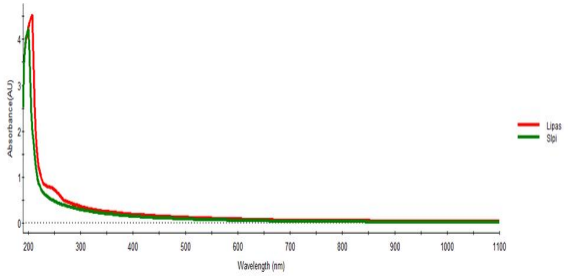

figure (2) The provided UV-visible spectrophotometry image is a template without actual absorbance data, but typical results for liposomes (lipases) and SLPI-loaded liposomes (SLA –liposomes)

Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of Lipos (plain liposomes) and Slai (SLPI-loaded liposomes) reveal distinct molecular interactions and structural changes. Lipos exhibits characteristic lipid peaks: ~2925 cm^{-1} and 2854 cm^{-1} (CH_2 asymmetric/symmetric stretching of acyl chains), ~1743 cm^{-1} (C=O ester carbonyl of phospholipids), and ~1466 cm^{-1} (CH_2 bending), confirming the lipid bilayer structure. The broad peak at ~3437 cm^{-1} indicates O-H/N-H stretching, likely from hydration or residual water (figure 3)

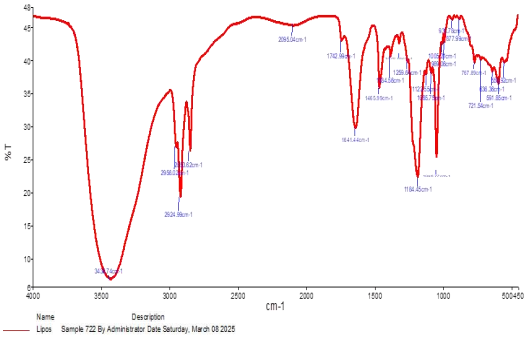

Figure 3 FTIR analysis of Lipos (plain liposomes)

In contrast, Slai shows additional features: a shifted O-H/N-H peak (~3401 cm^{-1}), altered intensities in CH_2 stretches, and a prominent ~1643 cm^{-1} peak (amide I, C=O stretching of SLPI), confirming successful protein encapsulation. The reduced transmittance (%T) in Slai's amide and CH_2 regions suggests SLPI-lipid interactions, possibly via hydrogen bonding or hydrophobic insertion. Missing peaks in Slai (e.g., ~2095 cm^{-1}) may reflect conformational changes or masking by SLPI signals. These results demonstrate SLPI's integration into the liposomal bilayer, critical for drug delivery applications (figure4)

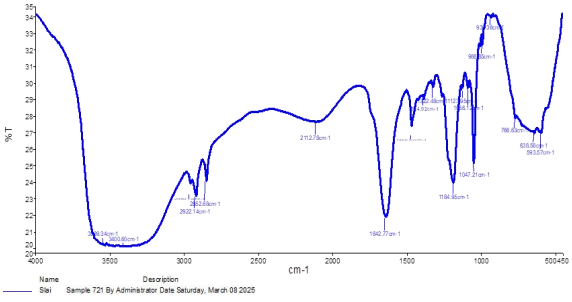

Figure (4) FTIR spectra of Slai (SLPI-loaded liposomes)

X-ray diffraction (XRD)

The XRD results from Liposomes alone (figure 5) and SLA loading liposomes suggest that the Liposomes loading sample likely contains proteins or antigens (figure 6) while the liposome sample (LIPOS) shows typical lipid

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bilayer characteristics without clear protein signatures.

The SLA (loading liposomes) data reveals multiple sharp diffraction peaks (e.g., at 21.36° 2θ, 20.70° 2θ, and 22.17° 2θ), which are consistent with crystalline or semi-crystalline structures often observed in protein or antigen-containing systems (figure 6). In contrast, the LIPOS sample (liposome alone) displays only broad peaks (e.g., 19.49° 2θ), characteristic of lipid lamellar phases without long-range order.

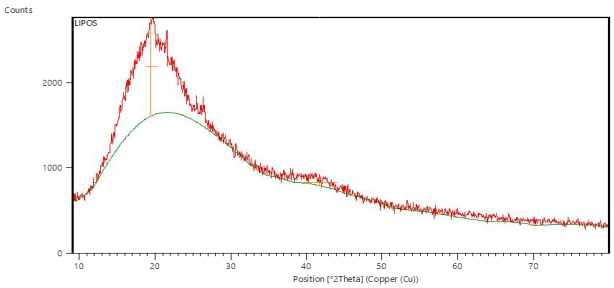

Figure (5): LIPOS (liposomes alone) show only broad peaks at (19.49° 2θ)

The presence of well-defined peaks in SLA 1 (loading liposomes) particularly at lower angles may indicate protein-lipid interactions or antigen crystallization, whereas the simpler pattern in LIPOS reflects pure
lipid self-assembly.

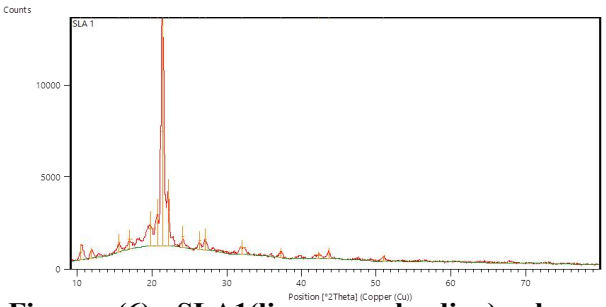

Figure (6) SLA1(liposome loading) show multiple sharp diffraction peaks (e.g., at 21.36° 2θ, 20.70° 2θ, and 22.17° 2θ)

Field-emission scanning electron microscopy (FESEM)

Based on the FESEM image of the liposomes, the sample displays a relatively uniform distribution of spherical vesicles with smooth surfaces (figure 7). The liposomes appear well formed with consistent morphology, suggesting successful synthesis. No significant aggregation or structural deformation is observed, highlighting good stability and dispersion quality.

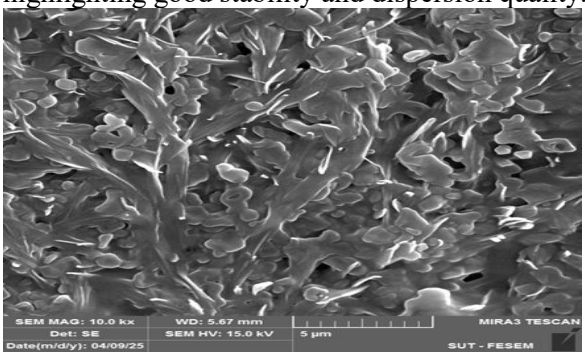

Figure (7) FESEM for liposome with show spherical vesicles with smooth surfaces

FESEM images of SLA-loaded liposomes at 135 kX show their smooth surfaces and spherical form. Various particle sizes were found. Some liposomes were huge, others little. This suggests that the self-assembly process was inconsistent or that molecules adhered differently during preparation. To produce a clearer surface image, we modified the imaging settings to 15.0 kV acceleration and 5.67 mm working distance. The 200 nm scale bar in the photographs makes particle sizes easy to quantify. The results show we generated SLA liposomes of various sizes. Figure 8

shows that this increases the liposomes' varied distribution, suggesting they may be valuable in various biological situations.

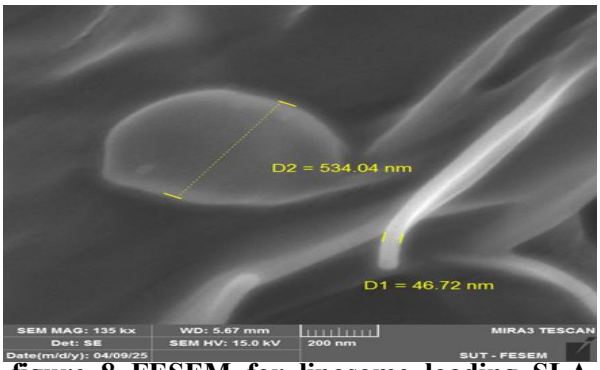

figure 8 FESEM for liposome loading SLA show multiple spherical vesicles with smooth surfaces Zeta Potential for SLA with Liposomes

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Zeta potential tests showed that the two samples had different surface behaviors. Basic spherical particles like polystyrene latex have little variability and good colloid stability, as seen by the unmodified liposomes' 22.28 mV average potential. SLA liposomes exhibited a higher average potential of 61.16 mV with a standard variation of 13.8 mV. The result has two peaks at 8.53 and 53.78 mV, higher than the pure material. Bipartite distribution suggests surface topology or antigen binding efficiency differences. When SLA is loaded, the particle surface changes substantially and electrostatic stability increases. These qualities presumably increased electrostatic stability and surface modification due to particle antigens. This is corroborated by SLA-Liposomes' enhanced conductivity and wall zeta potential (table 2).

Table (2) The table compares key zeta potential parameters of two polystyrene latex samples. with greater deviation and dual peak values, indicating a more heterogeneous surface with a surface charge and stability.

Parameter	Liposomes	SLA-Liposomes
Zeta Potential (Mean, mV)	22.28	61.16
Zeta Peak One Mean (mV)	-1.58	8.53
Zeta Peak Two Mean (mV)	24.08	53.78
Conductivity (mS/cm)	0.01511	0.0456
Wall Zeta Potential (mV)	3.457	-11.18
Zeta Deviation (mV)	8.378	13.8
Mean Count Rate (kcps)	130,200	277,700
Reference Beam Count Rate	1732	1646
Quality Factor	4.29	3.89

Immunization Response Analysis by Elisa test in mice affected with *leishmania Donovan* determination of IgG titer

Immunization Response Analysis of this study evaluated the humoral immune response through IgG titer levels following immunization with different *Leishmania donovani* antigen formulations. The experimental groups included: SL (Soluble Leishmania Antigen combined with Nanoparticle liposome), L (Nanoparticle liposome alone), S (Soluble Leishmania Antigen alone), along with negative control (Buffer) before and after challenge. Before the challenge, the mean absorbance values across various serum dilutions from 1/10 to 1/640 show that the SL1 group had the highest mean absorbance (0.564 ± 0.07), followed by groups S2 (0.453 ± 0.04), L1 (0.437 ± 0.03), and Buffer (0.348 ± 0.01). These values reflect serum antibody binding levels to the antigen in each group. The Least Significant Difference (LSD) test indicated a statistically significant difference between groups (LSD = 0.089, $P < 0.05$), but no significant interaction (LSD = 0.236). The buffer-treated control group exhibited the lowest optical density (OD) values across all dilution levels, providing a stable reference for calculating the cutoff value. The calculated cutoff value defined as the mean plus twice the standard deviation of the control group—was 0.418, allowing for a clear and robust separation between the inoculated and control groups .The highest terminal titers were recorded in the SL1 group at a 1/160 dilution, followed by the S2 group at 1/80, and then the L1 group at 1/40, reflecting a hierarchy in the strength of the generated immune response (see Figure 9). These results clearly indicate that immunization with liposome-encapsulated SLA resulted in a more robust and effective serological response compared to unencapsulated SLA or liposome-only adjuvant use, even prior to pathogen exposure.

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Figure 9. Antigen-specific IgG titers prior to challenge. Serum collected from immunized mice was subjected to serial dilution and evaluated by ELISA. The dashed horizontal line represents the predetermined positive cutoff (OD = 0.418). Data points are presented as mean ± standard error.

Absorbance changed after the challenge. In terms of mean absorbance, the Buffer group exceeded SL1 (0.814 ± 0.10), followed by S2 (0.526 ± 0.04) and L1 (0.476 ± 0.04). The antibody responses of the groups after exposure varied greatly regardless of serum dilution. The antibody response in each group post-infection was shown by ELISA curve analysis with a threshold of 0.418. Even after diluting the serum to 1/320, the SL1 group—whose antigen is in liposomes—had the strongest response to vaccination. This indicates a powerful, long-lasting antibody response. This shows that the vaccination worked and that viral exposure improved it. S2 (antigen alone, liposomes omitted) had a clear antibody response at 1/160. However, the response was weaker than liposomes, resulting in little immunity. The data reveal that liposomes stabilize and make the antigen simpler to identify, improving antibody response. In the liposome-only L1 group, antibody levels were lowest and narrowly above the limit at 1/80. The liposomes' adjuvant properties and pathogen exposure during the challenge may have caused this non-specific response. Look at plot 10.

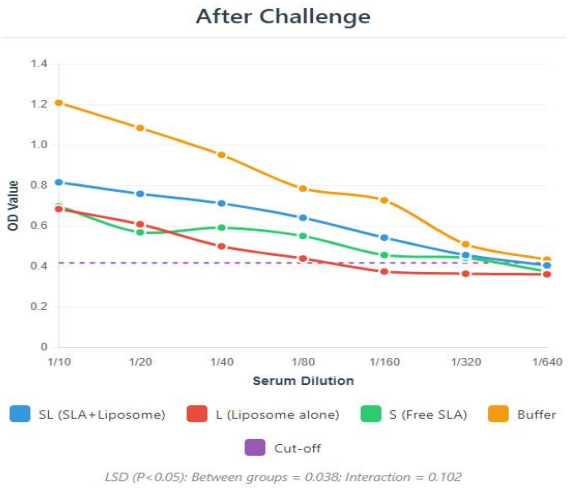

Figure 10: Antigen-specific IgG antibody titers measured after challenge. Serum samples from immunized mice were serially diluted and tested by ELISA. The dashed horizontal line represents the positive cutoff value (OD = 0.418). Data points indicate the mean ± standard error.

Discussion

The BCA Protein Assay is a colorimetric technique designed for the accurate measurement of total protein and is compatible with detergents. It relies on the principle that proteins reduce copper ions (Cu^{2+} to Cu^{+}) in an alkaline environment. The resulting cuprous ions (Cu^{+}) are then detected using a reagent that contains bicinchoninic acid (BCA), which forms a purple-colored complex, allowing for sensitive and specific protein quantification (figure 2).Our results show concentration of protein by BCA are 1.74 mg/ml and 2.75 mg/ml respectively which agree with the study (20) that show 1.85 mg/ml , The BCA assay is a valuable tool for high-throughput screening, especially when assessing numerous samples with identical liposome concentrations and formulations (20). Although rapid quantification methods for small molecules are well established, the capacity to quickly quantify protein encapsulation within liposomes is still limited. Many traditional protein quantification techniques, including the BCA assay, provide a basis for such measurements.

Soluble Leishmania Antigen (SLA) is a crude preparation acquired from lysed of *Leishmania* species promastigotes and is commonly utilized

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in the study and diagnosis of visceral leishmaniasis (VL), a serious disease resulting from *Leishmania donovani* infection . This antigen mixture includes various immunogenic proteins that can trigger both cellular and humoral immune reactions, making SLA a key element in vaccine research and immunodiagnostic tools. Its efficacy stems from the diversity of antigens it offers, closely resembling those encountered during natural infection, which facilitates effective uptake and presentation by macrophages and dendritic cells, which function as antigen-presenting cells (21). Immunizing BALB/c mice with SLA boosted their Th1-type immune response and protected them against VL. Liposomes containing Soluble *Leishmania donovani* Antigen were made from ODA and cholesterol. This approach encapsulated 82%–83% of the SLA, making vaccine delivery efficient. These liposomes are typically multi-layered and possess a positive zeta potential (~22–61 mV), which enhances their stability and uptake by antigen-presenting cells (APCs). Particle sizes range from ~246–526 nm. liposomal SLA induces a strong Th1 response significantly increased IFN-γ and IgG levels, along with nitric oxide production, have been observed in vaccinated (22). In murine visceral leishmaniasis models, such formulations reduce parasite burdens in spleen and liver and limit lesion development compared to controls Collectively, these physicochemical and immunological characterizations demonstrate that cationic liposomal SLA is a stable, potent vaccine candidate for visceral leishmaniasis.

In our study the physiochemical techniques observed this study using UV-Visible (UV-Vis) spectroscopy which given the intrinsic spectral properties of both liposomes and proteins, spectral analysis in the 200–800 nm range was employed Which agree with previous study (23) The UV-visible absorption, normalized to the same nominal mass concentration, measured at 800 nm was 0.1 for the 40 nm PS sample, 57.5 for the 125 nm PS sample, and 182.4 for the 200 nm PS sample. The FTIR spectra of Lipos (plain liposomes) and Slai (SLPI-loaded liposomes) reveal distinct molecular interactions and structural changes. Lipos exhibits characteristic lipid peaks: ~2925 cm^{-1} and 2854 cm^{-1} (CH_2 asymmetric/symmetric stretching of acyl chains), ~1743 cm^{-1} (C=O ester carbonyl of phospholipids), and ~1466 cm^{-1} (CH_2 bending), confirming the lipid bilayer structure. The broad peak at ~3437 cm^{-1} indicates O-H/N-H stretching, likely from hydration or residual water (figure 3-7), Which agree with a previous study (24) The spectral region between 3000 and 2900 cm^{-1} exhibits strong vibrational activity due to the stretching vibrations of C–H bonds in the hydrocarbon chains of phospholipids. In the case of liposomes, the asymmetric stretching of CH_2 groups , A signal is observed at approximately 2920 cm^{-1}, while symmetric stretching occurs close to 2850 cm^{-1}. Both the position and width of the diffraction peaks are highly sensitive to any change in the composition of the liposome's lipid chains. Comparative X-ray diffraction (XRD) analysis of simple and SLA-loaded liposomes shows that the loaded forms are more likely to contain proteins or antigens associated with the structure, while the unloaded liposomes retain a bilayered lamellar pattern without characteristic peaks indicative of protein presence. SLA-loaded liposomes (SLA1) exhibited several sharp peaks at 21.36°, 20.70°, and 22.17° (2θ), suggesting the formation of crystalline or semi-crystalline structures, often attributed to the incorporation of a protein or antigen into the liposome system. Regular liposomes had wide, short peaks around 19.49° (2θ). This is typical for lipid layers that aren't very crystalline. X-ray diffraction is helpful because it doesn't ruin the sample, and it tells us a lot about how crystalline the sample is and when the lipid layers change phases. Understanding membrane behaviour may enhance molecular loading and medical liposome safety (25).
SL, L, S, and a control group were examined in this research. Each group received different antibody doses before and after pathogen exposure. Antibody response before exposure averaged 0.564 in SL and 0.348 in control. The L and S groups had pretty similar average responses. This means that the immunization got their immune systems going way better than the untreated control group. After exposure, something interesting happened. The average response in the control group jumped up to 0.814,

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the highest we saw. This tells us that they were seeing the pathogen for the first time and their bodies were reacting. In contrast, the pre-immunized groups (SL, L, and S) maintained stable or slightly lower levels of reaction, a pattern consistent with a more efficient secondary immune response capable of limiting pathogen replication without requiring a substantial increase in circulating antibody levels . These results confirm the effectiveness of the immunization protocols used, and the data relating to the SL group support the immunological superiority of the antigen encapsulation technology within nanoliposomals, possibly by enhancing antigen stability, improving its delivery to antigen-presenting cells, and achieving a gradual release of the antigen that enhances the sustainability of the immune response. These results align with prior studies where liposomal vaccines elicited stronger humoral responses against *Leishmania major* antigens in murine models. For example, research involving sphingomyelin liposomes encapsulating soluble Leishmania antigens also demonstrated significantly elevated total IgG antibodies before infection compared to control groups, confirming the adjuvant effect of the liposomal system (26). The ELISA results showed that Immunization with SLA formulated in positively charged liposomes elicited the highest total IgG antibody levels relative to all other groups ($p < 0.001$) , while free SLA also stimulated a significant response compared to controls. Over the course of infection, total IgG levels became similar across groups, , indicating a strong Th1-biased response.The antibody response pattern, correlated with the level of protection achieved, demonstrating that a mixed Th1/Th2 response with a bias toward Th1 is critical for protection against *Leishmania donovani*. Thus, ELISA total IgG analysis served as important correlates of vaccine-induced immunity, with positively charged liposomal SLA showing the strongest humoral response and protective effect (27). However, such studies also highlighted a key immunological caveat. Even though antibody levels went up, the immune response leaned towards the Th2 pathway. So, just because there are more antibodies from the liposome thing doesn't automatically mean it works well. Most crucial are their effectiveness and which immune cells they protect. Similar to how immunizations improve IgG levels in Leishmania-infected animals, liposomes may promote parasite immunity (28)

Mice were infected with a modified *Leishmania tarentolae* gene that encodes glutamylcysteine synthase had robust antibody responses and superior protection against *Leishmania major* and *L. donovani.* Lower parasite counts were linked to increased IgG antibodies (29) A mouse DNA and peptide injection research underlined the relevance of the cellular immune response (30) The study found that both injections increased IgG levels, but the primary protective effect was from activating T cells that generate IFN-γ. There is enough data to imply that the SL injection increases humoral immunity, but not enough to determine its protective efficacy (31) Attenuated experiments show that most vaccinations need a significant Th1 cell response, predominantly IgG antibodies, and a low parasite load. Although hopeful, higher antibody levels may not indicate everything. Thus, further studies are needed to determine whether SL destroys parasites like it did in previous successful testing.

The cationic liposome approach delivers SLA to its target region and boosts the immune response. After priming the immune system with the SLA-liposome vaccination, a powerful and long-lasting antibody response defeated the infection. Thus, the liposome SLA vaccine may prevent visceral leishmaniasis in the future.

Conflict of interest

The authors declare that there is no conflict of interest in the current study.

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