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Design and characterization of Tacrolimus monohydrate loaded core shell lipid polymer hybrid nanoparticle

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ABSTRACT

Tacrolimus monohydrate (TAC) loaded core shell lipid polymer hybrid nanoparticle (CS-LPHNs) composed of polymer core (PLGA) surrounded by lipid shell (lecithin). DSPE-PEG_{2000} was used as stabilize the nanoparticles. TAC-CS-LPHNs were prepared by single step modified nanoprecipitation method. Field emission electron microscope and Transmission Electron Microscope reveals a spherical shape nano-sized particle coated with a lipid layer. The nanoparticles ranged in size from 82nm±0.2-195±0.143nm which increase with increase the polymer quantity. The entrapment efficiency also affected by the polymer quantity that ranges from 55±1.23%-76±1.93%. XRD and DSC studies show conversion of TAC from crystalline into amorphous when imbedded inside the CS-LPHNs and FTIR study shows no chemical interaction between TAC and other components. The Cytotoxicity study of TAC and TAC-CS-LPHNs show a decrease in cytotoxicity of loaded TAC (IC_{50}=1.18067±0.045µg/ml) in compare with free TAC(IC_{50}=0.697±0.02µg/ml). Finally, the uptake study shows complete internalization of nanoparticle by Vero cell and accumulation of nanoparticles inside nucleus and cytoplasm. The results suggested CS-LPHNs is a good drug delivery system for TAC which is safe, effective and promising a better clinicals outcome.

ARTICLE HISTORY

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KEYWORDS

lipid polymer hybrid, Tacrolimus monohydrate, IC_{50}, PLGA, Lecithin, LPHNs.

INTRODUCTION

lipid Polymer hybrid nanoparticles (CS-LPHNs) are core-shell nanocarriers made by hybridization of lipid and polymeric materials to form a nanocomposite that having properties of both lipid (liposome) and polymer (polymeric nanoparticles). Liposome are bilayer lipoidal spherical vesical of amphiphilic lipid in which both hydrophilic and lipophilic drugs can be incorporated and carried as a delivery system. A lot of limitation of this system has been reported like low entrapment efficiency, low stability, batch to batch variability and sterilization difficulty.[1,2]

Physical and chemical stability are the main problem that limited the spread manufacturing of liposomes. The chemical instability may be due to oxidation of the unsaturated bond in the lipid or may be due to hydrolysis of an ester bond. The physical instability may happen as a result of liposomal aggregation and drug leakage out of the liposome which affect the efficiency of the delivery system.[2]

Polymeric nanoparticles are biodegradable and biocompatible solid nanoparticles that can entrap and encapsulate hydrophilic and hydrophobic drugs by physical adsorption or chemical linkage to the polymers.[3] The major drawbacks of polymeric nanoparticles are the leakage of the drug from the particles that lead to high burst rate, rapid clearance by a reticular endothelial system and lysis of polymeric components in the presence of serum.[3]

In an effort to eliminate the limitation of liposome and polymeric nanoparticle, a novel hybrid drug delivery system known as lipid polymer hybrid nanoparticles have been invented which combine both of them in one nanoparticles.[3] It composed of three distinct components, the polymeric core which is mostly hydrophobic in nature, where the drug is entrapped inside and the outer shell that is lipids in nature and help in retarding the drug inside

and impart mechanical strength and control release.[4] The outer most layer is the lipid-PEG layer that surround the lipid shell help in stabilization of the nanoparticles by decrease the aggregation through a steric hindrance also prolong in vivo circulation of CS-LPHNs.[5,6] The lipid should be cationic or zwitterionic in nature which is opposite in charge to the polymer in order to build up an electrostatic interaction between lipid and polymer.[5]

Tacrolimus (TAC) is an immunosuppressive medication that is primarily used for various immune related disorder especially organ transplantation to suppress the immune system and therefore reduce the risk of organ rejection. It is also used in topical preparation in the treatment of severe atopic dermatitis, uveitis, vitiligo and bone marrow transplants.[7] Due to the lipophilic nature of TAC, It is difficult to solubilize in a solution and that make it uneasy to uptake by the cell.[8] Because of low water solubility and a serious side effect originated from oral use limited its use, so it's important to manipulate Tacrolimus solubility by loading into CS-LPHNs.

EXPERIMENTAL

Materials

Tacrolimus monohydrate was purchase from Hangzhou Hyper Chemicals Limited (China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000} and Soya bean phosphatidylcholine were purchase from Avanti Polar Lipids, USA. Poly (D, L-lactide-co-glycolide) lactide:glycolide (PLGA) (75:25), mol wt 66,000-107,000 was purchased from Sigma-Aldrich, Chemie GMBH, Germany. All the other chemicals were of analytical quality.

Preparation of Tacrolimus monohydrate loaded lipid polymer hybrid nanoparticles (TAC-CS-LPHNs)

Core shell LPHNs were synthesize by self-assembly of PLGA, lecithin and DSPE-PEG_{2000} through a single-step nanoprecipitation method.[9]

Stock solution of TAC, PLGA, lecithin and DSPE-PEG_{2000} were prepared in concentration 1mg\ml, 5mg\ml, 0.1mg\ml and 0.1mg\ml respectively. PLGA and TAC were dissolved in acetonitrile (ACTN) whereas DSPE-PEG_{2000} dissolved in deionized water. The stock solution of lecithin was prepared by using deionized water contain 4% ethanol to help in solubilization of lecithin. The ethanolic aqueous solution was heated beyond the gel-liquid transition temperature for about 65˚C.[9]

The organic phase was prepared by mixing PLGA and TAC at different ratio while aqueous phase was prepared by mixing lecithin and DSPE-PEG_{2000} at different ratio. The organic phase was added to aqueous phase drop wise in rate of (1ml\min) under gentle moderate stirring (500 rpm) and temperature 25˚C. The volume of organic and aqueous solution was adjusted as 2 ml and 20 ml respectively so that the ratio of organic: water kept at 1:10. The resulting solution was sonicated for 3 min at frequency of 42 kHz and power of 50 W. The free drug, PLGA, lipids and organic solvent were removed by washing TAC-CS-LPHNs solution three times using an Amicon® Ultra-4 centrifugal filter unite (MWT 10000 Dalton) at rate 1000 rpm which may resuspend again in deionized water to obtain desired concentration. The resulting TAC-CS-LPHNs were stored at 4˚C, or freeze dried and lyophilized for storage at -20˚C.[9]

Characterization of TAC-CS-LPHNs

The particle size and PDI were estimated using a Zetasizer Nano ZS (Malvern, Worcestershire, UK), with a detection angle set on 173° and the temperature set at 25°C. The experiment was repeated three times and the data were expressed mean±standard deviation as a function of the number, intensity, and volume.[10]

Zeta Potential

Electrophoretic light scattering is the most frequently used method for estimating zeta potential since it is used to measure particle velocity in an electric field. The zeta potential was measured at a temperature of 25°C using electrophoretic light scattering in a Zetasizer Nano ZS (Malvern, Worcestershire, UK) apparatus.[10]

Drug loading (D.L.) and Entrapment efficiency (E.E.)

D.L. and E.E. of TAC-CS-LPHNs were determined directly by weighing a known quantity of lyophilized TAC-CS-LPHNs and calculating the amount of drug entrapped within the nanoparticles. To calculate D.L., the amount of loaded drug divided by lipid-polymer mass, is given by

$$\text{D.L. (\%)} = (\text{drug}) / \text{lipid-polymer} \times 100$$

Entrapment efficiency, which is proportional to the amount of drug that can be incorporated into TAC-CS-LPHNs, is expressed as

$$\text{E.E. (\%)} = (\text{entrapped drug}) / \text{total drug} \times 100$$

The entrapped drug was measured through dispersing 1 mg of lyophilized powder of TAC-CS-LPHNs in 5 ml ACTN and left under magnetic stirrer for 5 hrs.[11,12]

A certain volume of the dispersed solution was diluted with mobile phase and centrifuged for 45 minutes at 5000 x g and 4°C. Following that, a free drug was collected in the aqueous phase, filtered through a 0.22 µm membrane, and TAC was determined using HPLC.[12,13]

X-Ray Diffraction

Lyophilized dry powder formulation and pure TAC, PLGA, DSPE-PEG_{2000}, the lecithin and physical mixture were analyzed by X-ray diffractometer equipped with CuK radiation at 40 kV and 15 mA. The 2θ scan range was 3–50° with a 0.02° step size and a 1 s scan speed. [14]

Differential scanning calorimetry

A DSC-60 is a differential scanning calorimeter (Shimadzu, Japan) was used to conduct the analysis. TAC, lecithin, DSPE-PEG_{2000}, PLGA, physical mixture, and lyophilized TAC-CS-LPHNs (4-5mg) were placed in hermetically sealed aluminum pans, with empty pans used as control. The samples were heated at a rate of ten degrees Celsius per minute from 25 to 700 degrees Celsius while being purged with nitrogen at a rate of thirty milliliters per minute. Parameters such as the onset temperature (T), melting point, and enthalpy (H) were determined using Software (Shimadzu, Japan).[15]

Thermogravimetric analysis (TGA)

TGA studies of Tacrolimus, lecithin, PLGA, DSPE-PEG_{2000} and optimized formula of TAC-CS-LPHNs were done so that we can study the physicochemical properties of TAC-CS-LPHNs with the help of TGA-4000 Perkin.

Additionally, TGA is used to assess properties such as absorption, adsorption, sublimation, weight loss and vaporization. TGA is frequently used to evaluate selected properties of decomposed samples that have lost or gained weight. After tarring, a limited number of samples were taken in a crucible, the weight of the crucible was kept in assembly, and software was written to run. The number of graphs depicting weight loss was gathered and reported .[24]

The thermogravimetric analysis (TGA) was performed at a temperature range of (0-700) ˚C by a temperature increment interval of 10˚C under argon gas pressure.[16]

Fourier transform infrared (FTIR)

Infrared spectroscopy is an efficient technique to investigate CS-LPHNs structural properties. By comparing the spectra obtained from sample and reference, it can detect changes or alteration from the original. Spectra of pure drug, lecithin, PLGA, DSPE-PEG_{2000}, Physical mixture and lyophilized formula were analyzed using FTIR. A mixture of 2 mg of sample and 200mg KBr was pressed for 4 minutes in a hydrostatic press at a pressure of 40 psi. At resolution of 4 cm^{-1} and 400-4000 cm^{-1} scanning range.[17]

Field Emission Scanning Electron Microscope (FESEM)

Field emission Scanning electron microscopy is the technique for observing the materials' morphology and surface structure. 5 µl of TAC-CS-LPHNs solution were dropped onto a silicon wafer to prepare samples for FESEM. Following an overnight drying period at 25˚C, the droplet was coated with chromium and imaged using a Phillips XL 30 Field emission SEM.[18]

Transmission Electron Microscopic (TEM)

Transmission electron microscopy is a technique used to analysis a nanoparticle's internal structure. To visualize the internal structure of hybrid nanoparticles, a drop of TAC-CS-LPHNs solution at a concentration of 10 µg/mL was dropped onto carbon-coated copper grids. The grid was then washed with 10 drops of distilled water five minutes after the sample was deposited. The grid was then stained with a drop of 1% uranyl acetate stain. The grid was visualized using TEM after drying, using a 100 KVA accelerating voltage.[19,20]

Cell viability study

The morphological feature of nanoparticles like shape, porosity, surface chemistry and flexibility can have marked effect on the interaction of the nanoparticles with biological membrane and cell toxicity.

Due to this interaction with biological system, it may reveal harmful and toxic effect. Therefore, in order to prove safety and efficacy of TAC-CS-LPHNs, cytotoxicity test should be done.[21]

The cell viability study was conducted in the Institute of Pharmaceutical sciences (TIPS)\Tehran university for medical science.

Cell type

Vero cells are an epithelial cell derived from the kidney of an African green monkey obtained from the Iranian cell line national bank. Vero cells are one of the most common surfaces attached normal mammalian continuous cell lines used in research.[22]

Cell culture

The Vero cell line was cultured in complete DMEM (Dulbecco's Modified Eagle's Medium) containing 100 µg/mL streptomycin, 100 IU/mL penicillin and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. At 80% confluence, the cells were trypsinized, resuspended in complete medium and counted to evaluate the cell density. Cells were observed under a phase-contrast microscope (Olympus CKX41) for evaluation of their overall appearance.[23]

MTT cytotoxicity test

Vero cells were seeded into a 96-well plate at a concentration of 2×10^4 cells/well. Following 24 hours of incubation, cell culture medium was replaced with medium containing 0, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/ml TAC. The supernatants were removed after 72 hours of incubation. subsequently, 50 µL of fresh prepared medium (DMEM and FBS) was added to each well, followed by 50 µL of 5-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) at a 5 µg/mL for 3-4 hours with a temperature of 37°C and a moist atmosphere of 5% CO_2. Further to that, 150 L DMSO was added to each well and shaken for 15 minutes. Using a microplate reader (Biotec, Tecan US, Inc.), the absorbance was recorded at 570 nm. The treatment groups' viability was determined by the percentage of controls that achieved 100%. The mean±standard error (SEM) of cell viability were estimated for each treatment. Additionally, probit regression analysis was used to determine the sample's (IC_{50}).

In the next step, the IC_{50} of TAC and TAC encapsulated in CS-LPHNs against Vero cells was investigated using MTT assay. Simultaneously, control cells were cultured in simple medium DMEM containing 1% penicillin/streptomycin) without TAC for the same time span (72 h). Additionally, biocompatibility of CS-LPHNs was determined by treating cells with blank CS-LPHNs.[24]

Percentage of cell activity was calculated as:

Cell viability (%) = Ab (treatment group) \Ab (control group) *100

Cellular uptake study

Qualitative study (Confocal laser scanning microscopy)

For the qualitative study, a microscopic slide in 6-well plate was seeded with vero cell (5×10^5 cells per well) and cultured for 1 day until complete adhesion of the cells was achieved. Then the cells were treated with 6-C-CS-LPHNs (0.25 mg/ml). After 2 and 4 h incubation at 4 and 37°C, the cell washed three time after culture media removed with cold phosphate buffer saline. After 15-10 min of fixation with 4% paraformaldehyde solution, the cells were stained with Hoechst (2µg/ml) for another 5-10 min, followed by three time washing with phosphate buffer saline. At the end, the cell was visualized and the fluorescence image was captured using confocal laser scanning.[25]

Quantitative uptake study (ELISA analysis)

For a quantitative evaluation of cellular uptake, 24-well plate were seeded with vero cell at density of 1×10^5 cells/well. When the cells become mature, they were incubated with 100 and 200 µg/ml of free 6-C and 6-C-CS-LPHNs in a DMEM medium at 37°C for 4 h. The solution was withdrawn at predetermined intervals and the wells were washed three times with cold PBS. Following that, each well was treated with 0.5% Triton X-100 to lyse the cells. The fluorescence intensity of each well was determined using a microplate reader (Biotec, Tecan US, Inc.) set to 485 nm excitation and 528 nm emission.[26]

Statistics study

All data were conducted in triplicate and provided as mean±S.D. Statistical significance was determined using two-way ANOVA in Minitab program, with a significance level of $p<0.05$ considered important.

RESULT AND DISCUSSION

Before optimizing the formulation parameters, the fundamental process parameters such as the rate of organic phase addition and the speed of the magnetic stirrer were standardized. The rate of dropping was maintained at 1ml/min, and the stirring rate was maintained at 500 rpm.[27] At a high and low rate of stirring aggregation seen on the surface of solution. The drop wise addition of organic phase using insulin syringe should be done carefully. The needle of the syringe should be inserted inside the solution to avoid the aggregation of the polymer on the surface of the solution. This may be due to the surface tension that created between the droplet of organic phase and solution surface.[28]

The lecithin and DSPE-PEG_{2000} should be dissolved in deionized water contain 4% ethanol to help in solubilization of lipid. The solution should be heated beyond 65˚C which is the sol-gel-transition temperature of lecithin.[29]

As a drop of organic phase injected inside aqueous phase, the acetonitrile will migrate and diffuse to the aqueous phase leaving a protoparticle of PLGA that entrapping TAC inside which grows following nucleation process.[30] The E.E. was found to be between 55±1.23%-76±1.93% with D.L. up to 8.1%. A significant ($P<0.05$) increase in E.E. of TAC was observed with an increase in polymer amount. The carboxylic group of PLGA improving the entrapment efficiency of TAC by two ways. First, carboxylic groups make the polymer more rigid and help interaction with TAC. Second, regarding the structure of TAC, carboxylic group bind with TAC by hydrogen bond rather than ionic bond.[31]

The influence of the ratio of lipid\polymer (L\P) on particle size and zeta potential of TAC-CS-LPHNs have been studied in ratio ranging from 9%-42%. Figure (1) shows an effect of (L\P) on Zeta potential (ζ), increase in (L\P) will lead to a significantly decrease in the magnitude of ζ ($P<0.05$) as the cationic lipid will neutralize the negative charge of PLGA.[32]

Figure (1) shows the effect of L\P on the particle size, an increase in lipid content lead to increase in the particle size which may be due to the formation of multilayer lipid CS-LPHNs.[33] Formulas having L\P less than 18% aggregated and precipitated after 24hrs. storage at 4˚C.

The effect of the drug amount on ζ are shown in figure (2) in which there was a significantly decrease in the magnitude of zeta potential (P<0.05) as the drug amount increase from -28 to -0.2 mv for 1 and 3 mg respectively. The particle size affected significantly (P<0.05) as drug amount changes from 1mg to 3mg while insignificant changes when the drug amount changes from 2 to 3 mg. Also, the PDI affected significantly (P<0.05) by changing the drug amount from 1 mg to 3 mg as shown in figure (3).

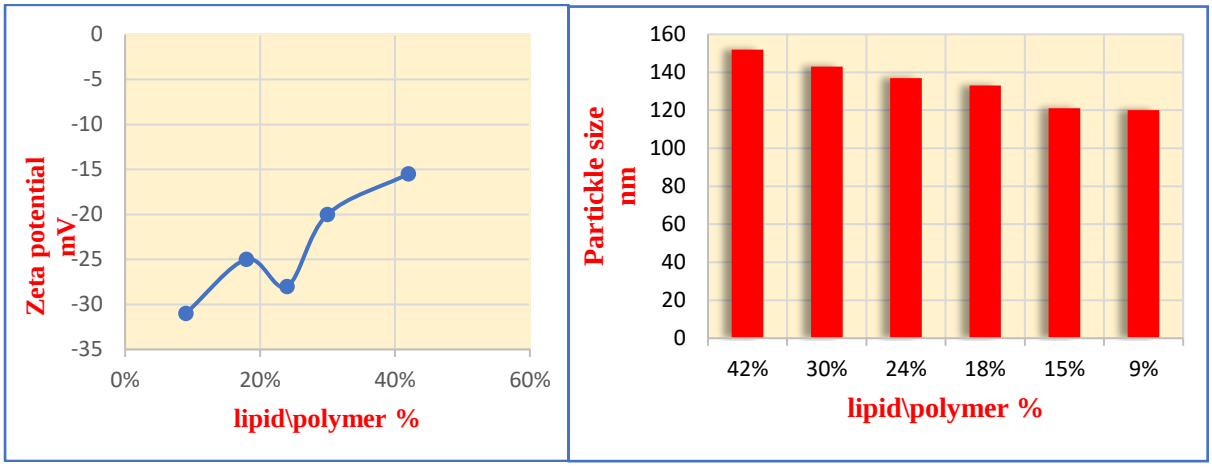

Fig.1: The influence of lipid\polymer on zeta potential and particle size

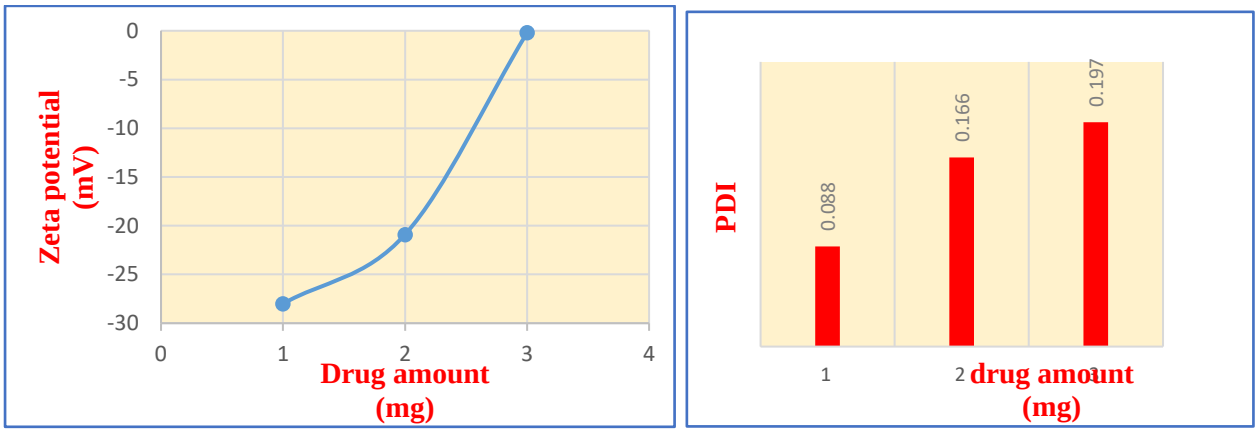

Fig.2: The influence of the drug amount on Zeta potential and PDI

The polymer amount has a significant effect on TAC-CS-LPHNs characteristics like particle size. Figure () predicted a significant change in particle size (P<0.05) when the polymer amount increased from 5 to 10 mg. zeta potential didn't change significantly (P>0.05) because L/P and DSEPE-PEG/lecithin mass ratio was kept constant.

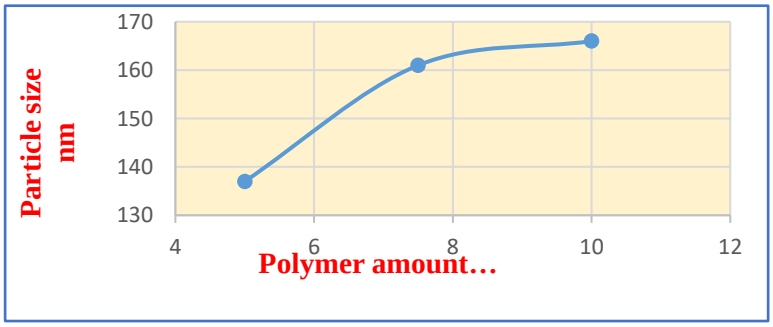

Fig.3: The influence of polymer amount on particle size

Thermal analysis

DSC and TGA study were conducted to understand the physical nature of the drug in the TAC-CS-LPHNs, as this could impact the drug's in vitro and in vivo release from the system. The DSC-TGA graph of TAC shows endothermic events from 40˚C to 134˚C which may be attributed to evaporation of water bounded to TAC with losing 2.3% w/w as shown in thermogram curve in figure (4), follow with melting of anhydrous TAC at 135.69 ˚C. after cooling for -50˚C and reheated again, the endothermic melting point disappear with the appearance of endothermic peak at 74.10˚C which represent the glass transition temperature Tg. This indicating the conversion of TAC into amorphous solid and did not crystallize again with homogenous dispersion in PLGA matrix. [34]The optimal formula thermograms shows an endothermic peak at 84˚C and 117˚C. The disappearance of TAC melting point indicated conversion into amorphous or completely loaded inside polymer with losing crystallinity due to deformity of the structure. The data show changing of the polymorphic form of the drug during the CS-LPHNs formulation.[34]

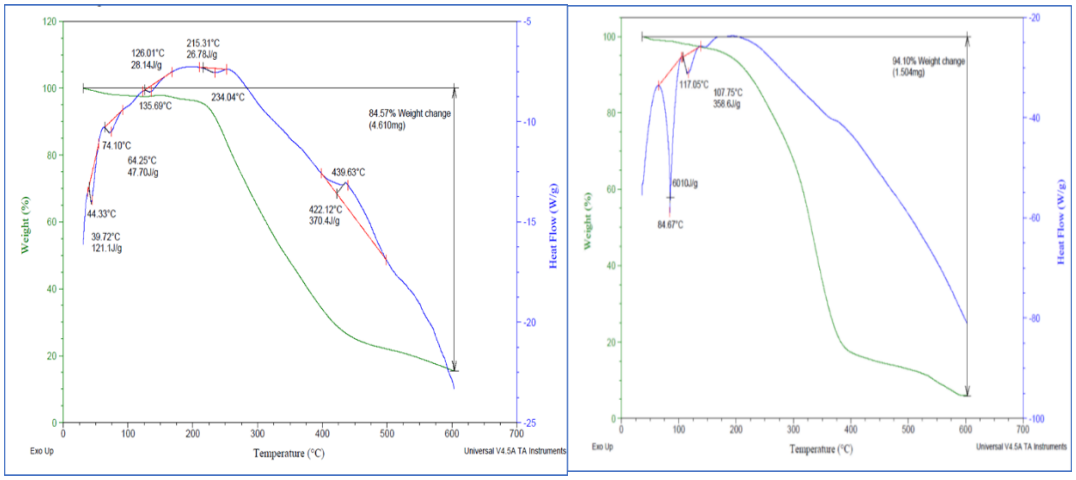

Fig.4: DSC and TGA thermogram of TAC. lyophilized optimal formula

Crystallinity

XRD allows for deep characterization of TAC-CS-LPHNs crystallinity behavior while DSC reveals the crystallinity of TAC and its conversion into amorphous in formulation.

The XRD of a free drug TAC shown in figure (5, A) display the characteristic crystalline peaks at 2Θ of 8.45,10.2,11.05,12.45,13.65, 15.2,17, 18, 18.85, 23.35. Peaks of XRD disappears in the TAC-CS-LPHNs optimal formula which indicated conversion of TAC into amorphous and completely dispersed in PLGA polymer bed as shown in figure (5, B).

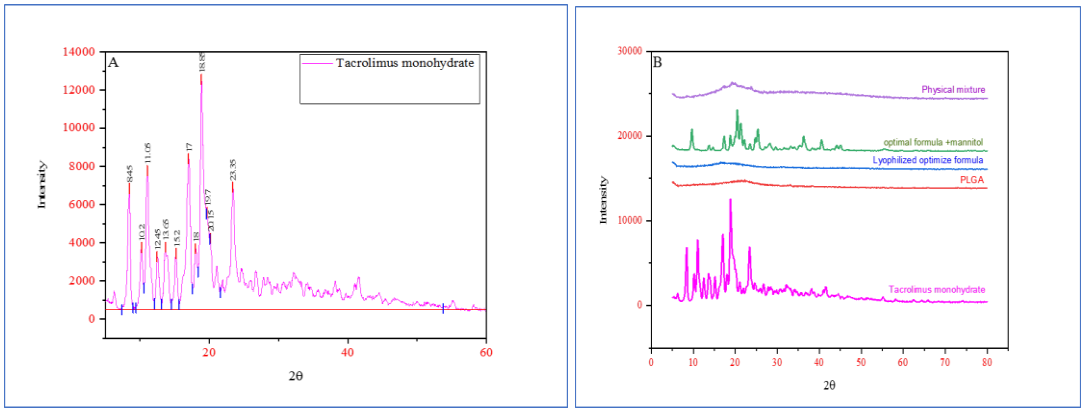

Fig.5: X-Ray diffraction of (A) Tacrolimus monohydrate, (B) Overlay of XRD for PLGA, physical mixture, optimal formula. Optimal formula with mannitol and Tacrolimus monohydrate.

Fourier transform infrared (FTIR)

The FTIR spectra of TAC, PLGA, Lecithin, DSPE-PEG_{2000} and the functional group and FTIR spectra of lyophilized formula with and without mannitol have been shown in figure (6). The carbonyl group (C=O) of Tacrolimus shows strong stretching peaks at 1730 cm^{-1} which is changed to 1736 cm^{-1} in optimized formula may be due to the influence of carbonyl stretching for PLGA. Tacrolimus monohydrate spectrum showed absorption bands of O-H stretch at 3442 cm^{-1} which decrease in intensity and downshifts to 3434 cm^{-1}. The main

reason for that many be due to interaction of hydroxyl group with phospholipid hydroxyl group and C=O group through hydrogen bonding. The lyophilized formula that contain mannitol shows downshifting of OH group to 3381 cm^{-1} and decrease the intensity of C=O group with upshifting to 1755 cm^{-1} due to the little amount of formula in compare with mannitol quantity.

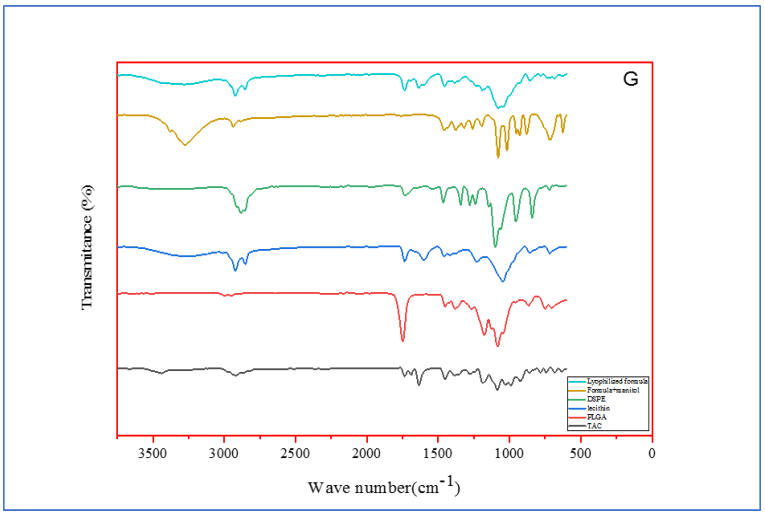

Fig.6: The overlay of FTIR spectra.

Preparation of 6-coumarin loaded CS-LPHNs

Nanoparticles containing 6-C where designated using the same nanoprecipitation method for formulating TAC. This fluorescence dye acts as probe for CS-LPHNs which offers a sensor for a quantitative and qualitative determination of nanoparticles cellular uptake.[34] Briefly 75µg was dissolved in 5 mg of polymer (PLGA) using 2 ml of ACTN. The organic phase was injected into an aqueous phase containing lecithin and DSPE-PEG_{2000}.

Field Emission Scanning Electron Microscope (FESEM) Analysis

The FESEM was performed to get a better understanding of TAC-CS-LPHNs morphology and confirm the particle size obtained from DLS. DLS was not an accurate way to study the particle size specially when the shape of a particle is irregular or high poly dispersity index where FESEM can produce a 2D-images.[35]

Figure (7) shows the spherical shape of nanoparticles with a nanometer size has been obtained.

The particle size obtained by FESEM and TEM less than that obtained by DLS. This difference may be explained by the presence of a hydration layer surrounding particles, DLS measure the hydrodynamic radius of particles which represented the actual size of nanoparticles with solvent layers that created on particles as a result of charge and double layer formation.[36]

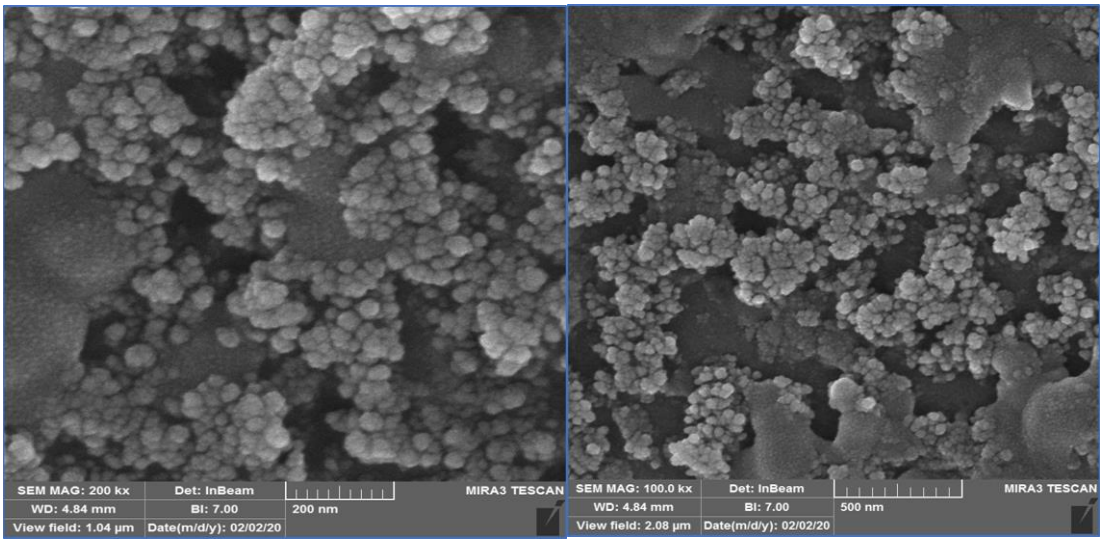

Fig.7: FESEM of optimize TAC-CS-LPHNs formula.

Transmission Electron Microscopic (TEM) Analysis

In order to characterize TAC-CS-LPHNs internal structure, the nanoparticles were stained with a negative stain uranyl acetate. The dye will stain the TAC-CS-LPHNs lipid shell with high electron density so that made it easy to recognize from the polymer core as seen in figure (8). The pictures of TEM reveals that the TAC-CS-LPHNs are spherical in shape with lecithin shell surrounded the polymer core in addition to the PEG corona from DSPE-PEG_{2000}. This confirm the formation of CS-LPHNs which surrounded by PEG.[37]

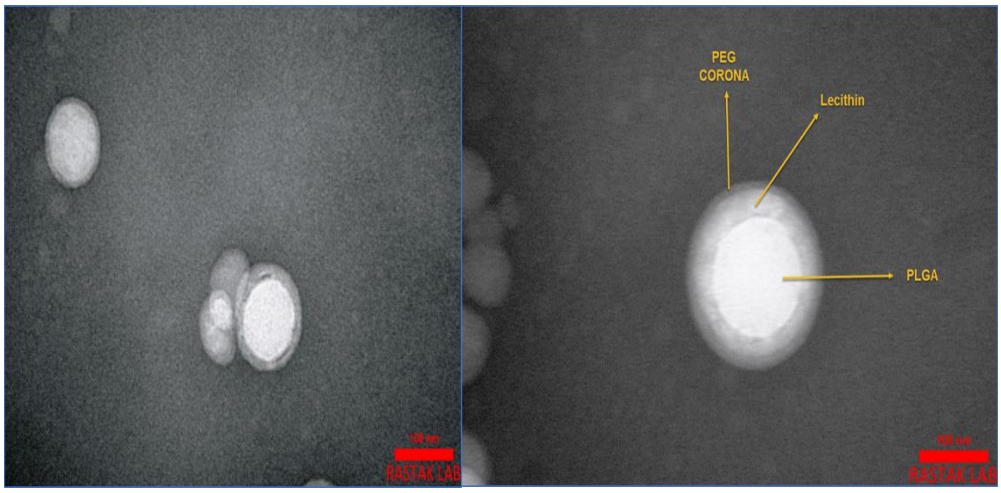

Fig.8: TEM images of TAC-CS-LPHNs

In-vitro cell culture studies

The Vero cell is the most common normal epithelial kidney cell used to assess the cell viability and accommodation of a newly formulated drug.

MTT cytotoxicity assay

The cytotoxicity study was performed by evaluating cell viability after treatment with free drug, drug loaded formula and blank formula. The MTT assay evaluate the cell viability through measuring the metabolic activity. Some metabolic activity may lead to a reduction in cell activity and apoptosis or necrosis.[38] The tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was reduced by mitochondrial dehydrogenase. The resulting intracellular purple formazan is dissolved in DMSO and measured by spectrophotometric means on a microtiter plate reader.

To initiate a basis for comparison, the free drug (TAC)loaded drug formula (TAC-CS-LPHNs) and blank formula (CS-LPHNs) are added in the same concentration. The IC_{50} of TAC, TAC-CS-LPHNs and CS-LPHNs are shown in figure (9).

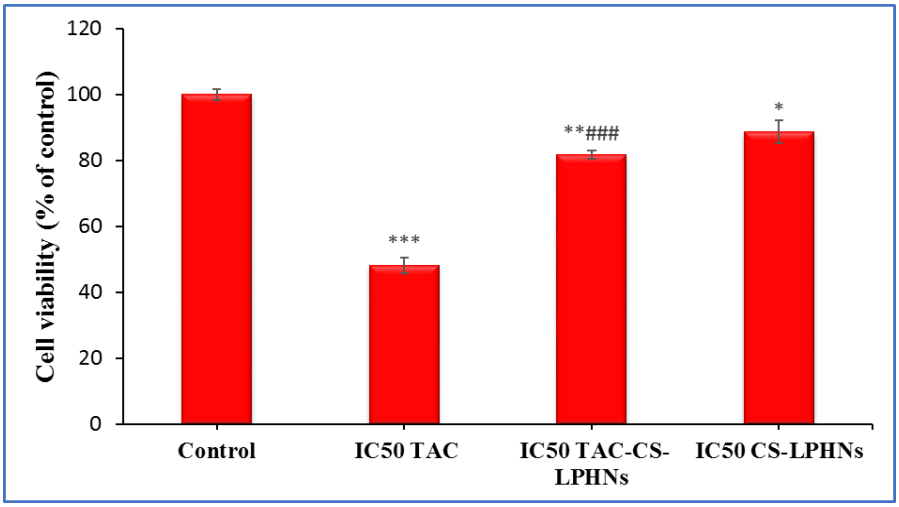

Fig.9:The cytotoxicity effect of IC_{50} of free TAC, TAC-CS-LPHNs and CS-LPHNs (without TAC) against Vero cells after 72 h of exposure. Data are represented as mean ± SEM (n=3). Difference between control and other groups is significant at *p<0.05, **p<0.01, and ***p<0.001. Difference between free drug and formulated drug groups is significant at ###p<0.001.

From this study, three facts have been proved (1), the CS-LPHNs shows a little increase in toxicity in compare to the control group which mean, it is biocompatible, (2) the free TAC was more cytotoxic than TAC-CS-LPHNs in, it directly act on receptor without releasing (3) incorporation of TAC inside CS-LPHNs provided a powerful tool for drug delivery of TAC which is biocompatible and biodegradable with less cytotoxicity on the normal Vero cell.

Uptake study

Qualitative study (Laser confocal microscopy analysis)

In order to verify the entrance of CS-PHNs into the Vero cell. CLSM had been used to visualize the nanoparticle intracellular and extracellular distribution.

After incubation of Vero cells for 2 hrs at 37˚C, a green dot seen in the cytoplasm and aggregate near the nucleus which prove internalization of the CS-LPHNs by Vero cells. According to this observation, we can conclude that CS-LPHNs can carry the TAC and enter the cell.

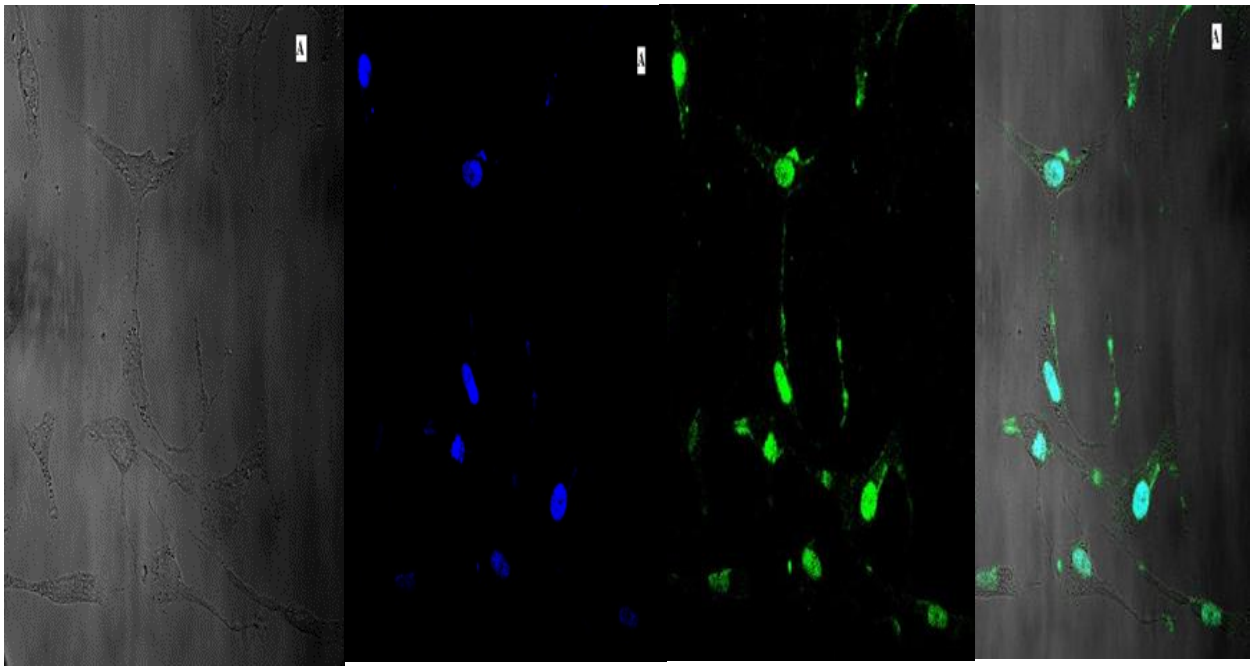

Fig.10: Confocal laser scanning microscope images of the Vero cell treated with 6-C-CS-LPHNs treated for 2hrs. at 37˚C.

Quantitative uptake study (Eliza analysis)

Quantitative uptake studies show that 6-C-CS-LPHNs are taken up by Vero cells. The untreated cells showed no fluorescence activity. Data in the table (1) and figure (10) reveals a direct and a significant increase ($p<0.01$) in cellular uptake with an increase in concentration of 6-C.

Table 1: The influence of concentration on the Cellular Uptake Efficiency

Concentration	Fluorescence intensity	
	6-C	6-C-CS-LPHNs
(100 µg/ml)	1332±13.17	1311±9.28
(200 µg/ml)	1397±4.05	1414±1.87

CONCLUSION

Tacrolimus monohydrate was successfully loaded in CS-LPHNs and evaluated for various physicochemical property. Data of particle size and entrapment efficiency shows a promising result of good entrapments and nanosized particles as shown in TEM and FESEM. Also, cytotoxicity and uptake study reveal safe drug delivery system which can be taken up by the cell in less than 2hrs at 37˚C.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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