University of South Florida
Digital Commons @ University of South Florida

DIGITAL COMMONS
@ UNIVERSITY OF SOUTH FLORIDA

USF Tampa Graduate Theses and Dissertations | USF Graduate Theses and Dissertations

June 2019

To compare Cyclosporine A Nanoformulations for their Effectiveness in Reducing Nephrotoxicity

Ilkin Nasirli
University of South Florida, ilkinnesirli92@gmail.com

Follow this and additional works at: https://digitalcommons.usf.edu/etd

 Part of the Medicinal Chemistry and Pharmaceutics Commons, Nanoscience and Nanotechnology Commons, and the Toxicology Commons

Scholar Commons Citation

Nasirli, Ilkin, "To compare Cyclosporine A Nanoformulations for their Effectiveness in Reducing Nephrotoxicity" (2019). *USF Tampa Graduate Theses and Dissertations.*
https://digitalcommons.usf.edu/etd/8394

This Thesis is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact digitalcommons@usf.edu.

To Compare Cyclosporine A Nanoformulations for their Effectiveness in Reducing Nephrotoxicity

by

Ilkin Nasirli

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Pharmaceutical Nanotechnology
Department of Pharmaceutical Sciences
College of Pharmacy
University of South Florida

Major Professor: Yashwant V. Pathak, Ph.D.
Vijaykumar Sutariya, Ph.D.
Daniel J. Denmark, Ph.D.

Date of Approval:
June 27 2019

Keywords: Nephrotoxicity, Cyclosporine A, PLGA nanoparticles.

Copyright © 2019, Ilkin Nasirli

ACKNOWLEDGEMENTS

It was a great honor being mentored by Dr. Yashwant Pathak. He supported me not only academically but also mentally.

I would like to thank to my thesis committee members Dr. Vijaykumar Sutariya and Dr. Denial Denmark for their support spending their valuable time on my thesis completion.

I also appreciate Dr. Priyanka and Gulimirerouzi Fnu who helped with my all studies during completing my thesis. Without their help, it is not possible for me to graduate.

At last, I would like to thank to Lisa Muma Weitz Laboratory for Advanced Microscopy & Cell Imaging and Department of Chemistry, College of Arts and Science.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Cyclosporine (CsA) is one of the main immune-suppressant agents which has been used widely in organ transplantation against graft rejection. However, the low oral bioavailability and the associated adverse effects such as nephrotoxicity are the main drawbacks of current usage of this drug. Thus, purpose of this research is to formulate PLGA nanoparticles of CsA to improve its effectiveness and to reduce the nephrotoxicity induced by the plain drug. CsA-loaded PLGA nanoparticles were prepared by the nanoprecipitation method. Particle size and zeta potential of the formulation was determined and percent drug entrapment were also determined. Quantitative estimation was carried out using validated UV spectroscopic method. The morphology of the nanoparticles was determined by Transmission Electron Microscopy. In vitro drug release profile was carried out utilizing dialysis method. In vitro cytotoxicity using MTT assay and in vivo toxicity studies are in progress and will be shared in the next presentations. The developed CsA loaded PLGA were developed and optimized having size around 80 nm with less than 0.2 PDI that shows narrow size distribution. The result from TEM studies also was in similar with DLS results. Zeta potential was found to be -29.32±2.7 mV, describing the stability of formulation. The optimized formulation could entrap 47.4±3.2 % of Cy-A in PLGA NPs. In vitro cytotoxicity using MTT assay shows fewer toxic effects in HEK-293 cells. CsA loaded

PLGA nanoparticles were successfully developed and characterized. The size of the particles was in the nanometer range with narrow size distribution. This nanoformulations may serve as an alternative formulation for CsA for effective drug delivery and to reduce the side effects.

CHAPTER ONE:

INTRODUCTION

Cyclosporine A (CsA) is one of the main immunosuppressants, that is widely used for organ transplantation against graft rejection (Fig. 1). Also, it is widely used for the treatment of autoimmune disease more than 30 years (Tedesco & Haragsim, 2012). The reason CsA has immunosuppressive activity is attributed to restraining the compound calcineurin phosphatase and according to this inhibits the outflow of cytokines, for example, IL2 and IFN ϒ and therefore T cell proliferation (Fig. 2) (Pradhan, Mohanty, & Sahoo, 2018). However, the low and variable oral bioavailability makes CsA toxic for kidneys (Tedesco & Haragsim, 2012). Nephrotoxicity is not the only side effects of CsA. There have also been reports of hypertension, anemia, paresthesia, hyperesthesia, hirsutism and gingival hyperplasia, altered coagulability, hepatotoxicity, neurotoxicity, lymphoproliferative neoplasms, and osteoporosis (Pradhan et al., 2018). In this work, nephrotoxicity resulting from CsA will be the primary focus.

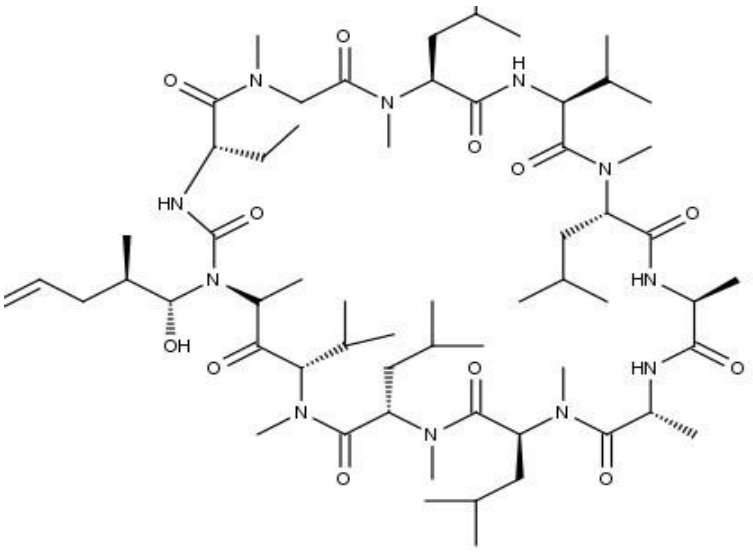

Figure 1. Chemical structure of CsA.

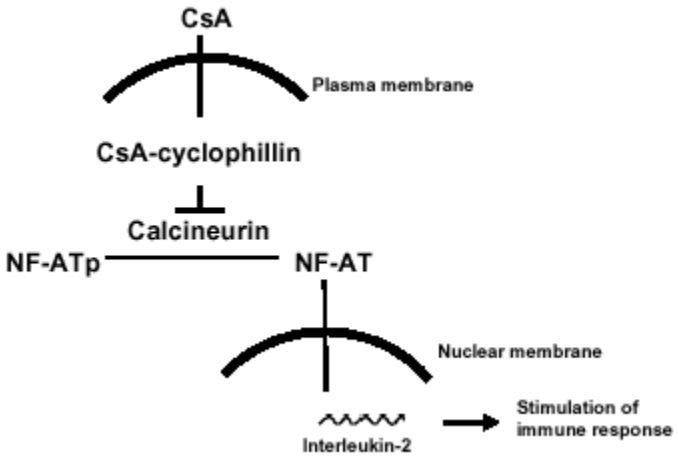

Figure 2. Immunosuppressive action mechanism of CsA. Adapted from Nabel (1999) (E. Mervaala et al., 1999)

The effects of Cyclosporine for transplant medicine were discovered in the lab of Sandoz in Switzerland in 1972, and its benefits were revolutionary for transplant medicine (Tedesco & Haragsim, 2012). The main reason of discovering CsA depends on the inhibition of both in vitro, cell-mediated lysis and lymphocyte sensitization using T

cells (Tedesco & Haragsim, 2012). Firstly when cyclosporine was used in clinical trials, it showed 94% survival rate, but after continuously using cyclosporine the nephrotoxic effects were discovered (Tedesco & Haragsim, 2012). The pathophysiology of the nephrotoxicity involves reducing renal blood flow and increasing renal vascular resistance (Tedesco & Haragsim, 2012). One of the reasons of this action depends to the renal sympathetic nervous system (Tedesco & Haragsim, 2012). Also, cyclosporine increases endothelin and thromboxane using renin-angiotensin system (RAS)(Tedesco & Haragsim, 2012). There are two key mediators as a macrophages and neutrophils for an anti-microbial immunity which their works are reducing depending to the CsA (Liddicoat et al 2019). CsA is an endecapeptide, it is extracted from Tolypocladium inflatium Gams (E. M. Mervaala et al., 1997). There is an increased risk of chronic kidney disease (CKD) associated with CsA (Chiu et al., 2015). According to the clinical trial provided by Chiu et al (2015), patients with rheumatoid arthritis (RA) have a 98% chance to experience CKD associated with nephrotoxicity (Chiu et al., 2015). That is why it is not advised to prescribe CsA by physicians for the elderly patients with diagnosed RA (Chiu et al., 2015).

As mentioned before Cyclosporine is widely used in organ transplantation. This includes kidney, liver, heart, lung and pancreases transplantation (Italia, Bhatt, Bhardwaj, Tikoo, & Kumar, 2007). It was described that cyclosporine has a low oral bioavailability, because of the cyclic structure and high molecular weight. Poor aqueous solubility is also one of the challenges of cyclosporine (Italia et al., 2007). Additionally, P-glycoprotein, pre-systemic metabolisms and liver reduces oral bioavailability of the

cyclosporine. That is not an all-inclusive list of the challenging factors regarding cyclosporine. Other than the low and variable oral bioavailability, cyclosporine has a critical dose in a narrow therapeutic window, and because of this commercial formulation of cyclosporine requires careful dosing protocol to prevent its nephrotoxicit. As mentioned before, cyclosporine can cause different forms of kidney dysfunction, which is why it is necessary to develop less toxic cyclosporine formulations. The main reason of this situation related to the narrow therapeutic window of CsA that is why the amount of CsA should be controlled by these formulations in the blood vesels (Italia et al., 2007).

From above, there is low oral bioavailability of cyclosporine and that is not the only issues which were investigated for CsA. Use of CsA in the ophthalmology field were investigated in 1981, and it was discovered that CsA has an application for corneal graft transplantation, in uveitis, corneal healing, vernal keratoconjunctivitis (Lallemand et al., 2017). CsA is able to reach different ocular tissues without showing sharp side effects as opposed to non-ocular administration (Lallemand et al., 2017). Due to these findings some CsA-loaded products have successfully reached to the pharmaceutical market place for the treatment of dry eye disease (DED) and other ocular diseases keratoconjunctivitis sicca (KCS) (Lallemand et al., 2017). Some of the CsA products are shown in the Table 1 according to the article written by Lallemand (2017) (Lallemand et al., 2017)

Table 1. CsA marketed ocular formulations in different years. Retrieved from (Lallemand et al., 2017).

Product name	Company	Dosage form	Indication	Marketed since
Restasis® (0.5 mg/mL)	Allergan	Anionic emulsion (UD)	DED	2003
Ikervis® (1.0 mg/mL)	Santen Pharmaceutical Co. Ltd.	Cationic emulsion (UD)	DED	2015
Papilock mini®	Santen Pharmaceutical Co. Ltd.	Solution (UD)	VKC - vernal keratoconjunctivitis	2005
Modusik-A Ofteno®	Laboratorios Sophia	Solution (MD)	KCS	2003
Lacrinmune®	Bausch & Lomb, Inc.	Emulsion (MD)	KCS	NA

As we can see from Table 1 Restasis® and Ikervis® actually effective for dry eye syndrome (DES) and Restasis® has an approval from US Food and Drug Administration (FDA), while Ikervis® is already used in European countries (Agarwal & Rupenthal, 2016) (Lallemand et al., 2017). However, these products are associated with ocular discomfort. In the article named "Modern approaches to the ocular delivery of cyclosporine A" written by Agarwal et al (2016) we learn information about different CsA Nanoformulations (Agarwal & Rupenthal, 2016). They collect information from

different references and describe advantages and limitations of ophthalmic dosage forms for CsA to treatment ocular diseases. These drug delivery systems include micelles, liposomes, proliposomes, nanospheres – nanocapsules, nanoemulsions, solid lipid nanoparticles, in situ gelling systems and hydrogels. Based on that report it is possible to develop CsA Nanoformulations for effectively decreasing nephrotoxicity. These CsA formulations improve bioavailability and they have target specific ocular tissues which increases side effects of cyclosporine (Agarwal & Rupenthal, 2016). CsA PLGA nanoparticles did not cause toxicities within dose range (Venkatpurwar et al 2015).

Objective

In this thesis, nanoformulations are used to decrease CsA nephrotoxicity. The focus is on comparing three Cyclosporine A nanoformulations. These nanoformulations include CsA PLGA nanoformulations with different concentration and weight ratio. Also, there are research articles demonstrating how antioxidants are helpful to decrease CsA nephrotoxicity (Sonaje et al., 2007). According to Sonaje et al (2007) ellagic acid (EA) improves oral bioavailability of CsA if it encapsulates the poly (lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) nanoparticles and in this case the suggesting dose decreases for three times (Sonaje et al., 2007).

Also, it is known that PLGA nanoparticles increases bioavailability of the drug comparing to their free form (Simon et al 2016). According to the Zhang et al (2015) CsA nanomicelle eye drops (CNED) shows better effects compared to the conventional CsA

eye drop (CCED) (Zhang, Wang, & Zhang, 2015). It was reported that CNED has better response by corneal transplantation (Zhang et al., 2015).

CsA oil-in-water nanoemulsion formulations also, can be effective in the brain for intranasal or intravenous administration, because it efficiently transports into the brain through the blood-brain barrier (Yadav, Gattacceca, Panicucci, & Amiji, 2015). The brain is not the only organ who showed effective response by CsA Nanoformulations on the animal studies. According to the Behr et al (2009) liposomal CsA PARI formulation shows increasing survival chances and there are no side effects related to the drug. (Behr et al., 2009). CsA loaded PLGA/polymeric liposomes also could be effective against spinal cord injury (SCI) (Gao et al 2017).

Nanotechnology increased the area of drug delivery field research, and different nano carrier increases poor oral bioavailability. There are few products on the market and clinical trials which uses nanotechnology-based oral formulations. Of course, there are two product of cyclosporine which uses nanotechnology: Sandummine Neoral ® and Gengraf ® (Desai et al 2012). Both these products use a system for spontaneously emulsifying and the dosage form of first of them is soft gelatin capsule, for the second one it is hard gelatin capsule. Sandummine Neoral ® is a product of Novartis Company and Gengraf ® is a product of Abbott Laboratories (Desai et al 2012).

Both these products have an immunosuppressant activity, increased bioavailability compared to cyclosporine and oily formulation of the previous product (Desai et al 2012). However, these formulations may increase the oral bioavailability, but they failed to decrease nephrotoxicity of CsA compared to cyclosporine loaded

PLGA nanoparticle (Italia et al., 2007). That is why in this research searching the appropriate cyclosporine loaded PLGA nanoparticle formulations is one of the goals for CsA.

Nanoemulsion formulations of CsA are also effective for reducing nephrotoxic side effects (Ganta et al 2014). According to the Ganta et al (2014), CsA nanoemulsion formulation using oil such as soybean oil and egg lecithin composition showed reducing nephrotoxic side effects which happened because of CsA drug compare to other nanoformulation - a Cremophor®EL/ethanol-based CsA(Sandimmune® Injection) (Ganta et al 2014). This example shows that even nanotechnology decreases the nephrotoxic side effects, but different nanoformulations reduces differently. In this research nanoformulations will be prepared using different research materials which were effective for decreasing nephrotoxicity according to the reviewed articles. Nanoformulations will be characterize using particle size and zeta potential methods and toxicity will be checked by cell line studies. In the end, it will be obvious which PLGA nano formulation is more effective for decreasing CsA nephrotoxicity.

CHAPTER TWO:

MATERIALS AND METHODS

Materials

CsA and PLGA, lactide/glycolide ratio of 50:50 was purchased from the Acros Organics (NJ, USA). Polyvinyl alcohol, Fetal Bovine Serum, penicillin-streptomycin and Trypsin (0.05%) were obtained from Fisher Scientific (USA). Dialysis membrane, MW cutoff 10000 was purchased from Sigma Aldrich (St. Louis, MO)., USA. MTT reagent [3-(4,5-dimethylthiazol-2-yr)-2,5-diphenyltetrazolium bromide salt] was outsourced from Tocris Bioscience (MN, USA). 293 [HEK-293], a human embryonic kidney cell line (ATCC® CRL1573™) and Eagle's minimum essential Medium (EMEM) was purchased from American Type Culture Collection (ATCC) (VA, USA). Phosphate Buffer Saline (1X) (PBS) was obtained from Corning Cellgro (Manassas, VA). All the analytical reagents were of analytical grade quality.

Preparation of CsA loaded PLGA NPs

CsA-NPs were prepared via the oil in water emulsion solvent evaporation method (Naeem et al 2018). The CsA drug and the PLGA were dissolved in a 1 mL of acetone as organic phase at a weight ratio of 1:7. The organic solution was slowly added into pH 9 deionized water with 23-gauge syringe while constantly stirring at 700 rpm at room temperature overnight for evaporation of solvent. The nanoparticulate suspension obtained was centrifuged at 5000 rpm during 5 mins then centrifuged at 18000 rpm at 4

°C 15 mins. After centrifugation, pelleted nanoparticles were collected washed thrice with distillated water. Blank nanoparticles were prepared in the same manner.

Particle Size and Zeta Potential

Particle size and polydispersity index (PDI) of CsA-NPs were measured by dynamic light scattering (DLS). Measurement of Size, PDI and Zeta Potential was performed triplicate at 25 °C temperature after proper dilution of prepared CsA-NPs formulation by using Zeta sizer (Nano ZS90, Malvern Instruments Ltd., UK, Zeta Sizer Software Ver. 7.10). The results were shown as Z-average and polydispersity index (PDI). This analysis was carried out using a detection angle of 90 ° at a temperature of approximately 25 °C for each sample, adequately diluted with filtered distilled water.

Transmission Electron Microscopy

Morphology and shape of CsA-NPs was obtained by transmission electrode microscope (TEM) (JEOL JEM 1400 electron microscope with Gaton camera, Peabody, MA, USA). Sample preparation included drop casting onto a formvar coated copper support film square grid having 200 mesh. Then it was allowed to air dry 5 to 10 minutes followed by treatment with 2% w/v phosphotungstic acid for negative staining. Voltage of about 120 kV with 40,000 magnification was used to conduct the imaging.

Calibration curve UV Method

2 mg of CsA was dissolved in 2 mL of methanol then diluted as appropriate for six different concentrations: 5 µg/mL, 20 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL and 80 µg/mL.

Then absorbance was determined using UV spectroscopy at 210 nm (λmax) wavelength. All concentrations were repeated three times for averaging in order to obtain the calibration curve.

Entrapment Efficiency

CsA-NPs was centrifuged after synthesis as mentioned in the preparation of formulation section, then 100 µl of product was taken and added into 2900 µl methanol. After sonication, absorbance of the formulation was analyzed by using UV spectrophotometer at a wavelength of 210 nm (λmax). The entrapment efficiency was calculated using the equation below.

$$\% \text{ Entrapment efficiency} = \frac{\text{Actual amount of drug loaded in nanoparticles}}{\text{Actual amount of drug used for preparation}} X\ 100$$

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to determine the thermal behavior of the CsA drug, PLGA, physical mixture of the CsAnd PLGA and CsA- NPs formulation respectively. This study conducted by using the DSC- Q 20 (TA Instruments, New Castle, DE USA, Q series Q20-2288-DSC software). Briefly, 5 mg of the sample was used while taking a blank aluminum pan as reference. Then altogether heated from 30 ^{o}C to 300 ^{o}C at a rate of 10°C/ min under the nitrogen purge (15).

Cytotoxicity Analysis

The MTT assay was performed to determine the level of cytotoxicity associated with the CsA-NPs comparing it with the CsAdrug solution, in 293 [HEK-293], a human embryonic kidney cell line (ATCC® CRL1573™). The cells were seeded at a suitable density of 5000 cells/well in a 96 well plate in 200 µl of EMEM supplemented with 10%

FBS. After 24 hours of incubation at 37 °C, 5% CO2 for 24 h till reaches cell confluency. After 24 hr incubation, the cells were treated with prepared EMEM and washed with sterile PBS then treated with both formulations and drug solutions in different concentrations. The treatment was kept for 4 hours then cells were washed once with sterile 1X PBS and added fresh complete EMEM medium containing 10% FBS for period of 24 to 48 h. After 24 hours, 100 µl of MTT reagent (1 mg/mL) solution replaced complete media followed by next 4 hours incubation. Then added 100 µl of dimethyl-sulfoxide. The intensity of the color of the formazan crystals was measured via microtiter plate reader at wavelength 595 nm. Cells treated with EMEM acted as the negative control while the cells were treated with Triton X were the positive control.

Statistical Analysis

All analyses were tested in triplicate. Data are represented as mean ± standard Deviation. NOVA, Student's t-test and GraphPad Prism (version 6, USA) was used for all analyses.

CHAPTER THREE:

RESULTS

Particle Size and Zeta Potential

The size of CsA-NPs was determined by using DLS technique and was found to be 79.64 ±1 nm with PDI of 0.09 ± 0.01. The mean zeta potential of CsA-NPs was found around -40.01±0.5 mV measured by Malvern zeta sizer Nano ZS90. Figure 3(A) shows Z-average for the particle size for CsA-NPs, (B) is the Zeta Potential for the formulation.

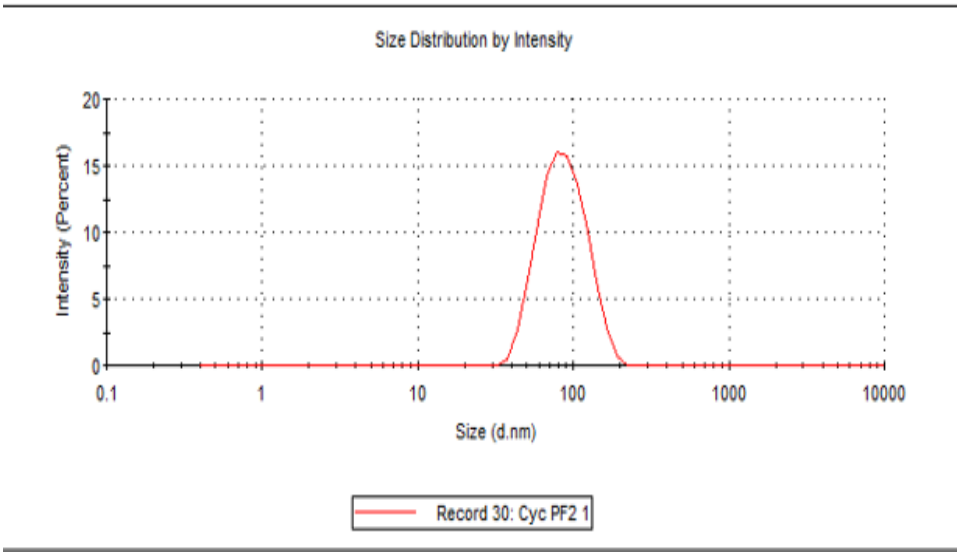

Figure 3: Average size of CsA-NPs

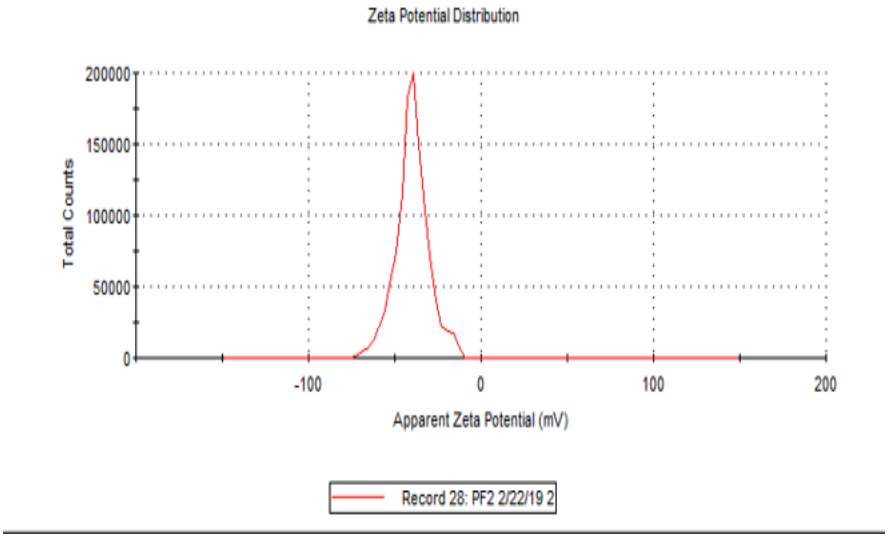

Figure 4: Zeta potential image of CsA-NPs

Transmission Electron Microscopy

The particle size of the CsA-NPs was found around 200 nm via TEM. Even though this particle size here is different from the size obtained from the DLS 80 nm, this difference can be explained by the different working property of the two studies. DLS gives the hydrodynamic diameter of the formulation while the TEM exhibits the size of the formulation fixed in grid. Figure 4 shows the uniform size and spherical surface.

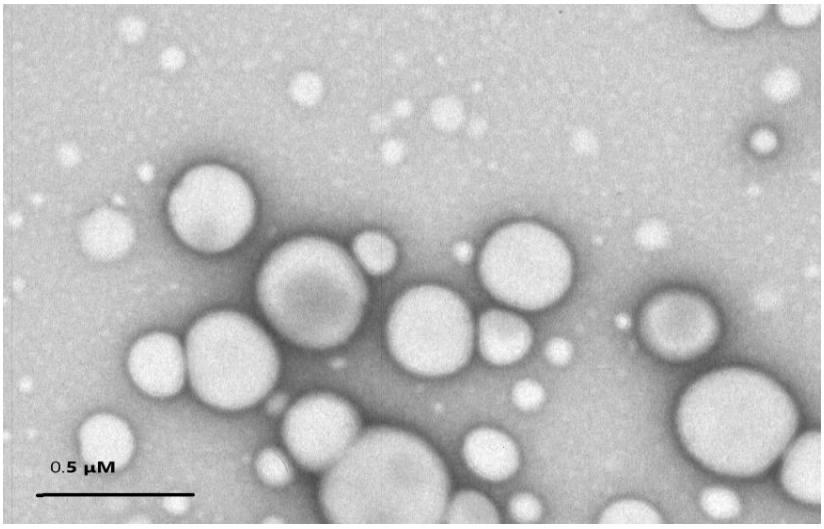

Figure 5: TEM image of CsA-NPs with 40,000X magnification ataccelerating voltage of 120 kV (scale bar 0.5 µM).

Entrapment Efficiency

Entrapment efficiency, was found 68% ± 3.5% by the equation given above, adding the CsA-NPs formulation into Methanol. Encapsulation efficiency was determined by using UV spectroscopy in methanol at 210 nm (λ max).

Calibration Curve UV Method

UV calibration curve of CsA was performed to help to calculate entrapment efficiency. Five different concentrations were checked by using UV spectroscopy at 210 nm triplicate. As figure 5 shown below R^2 =0.9995.

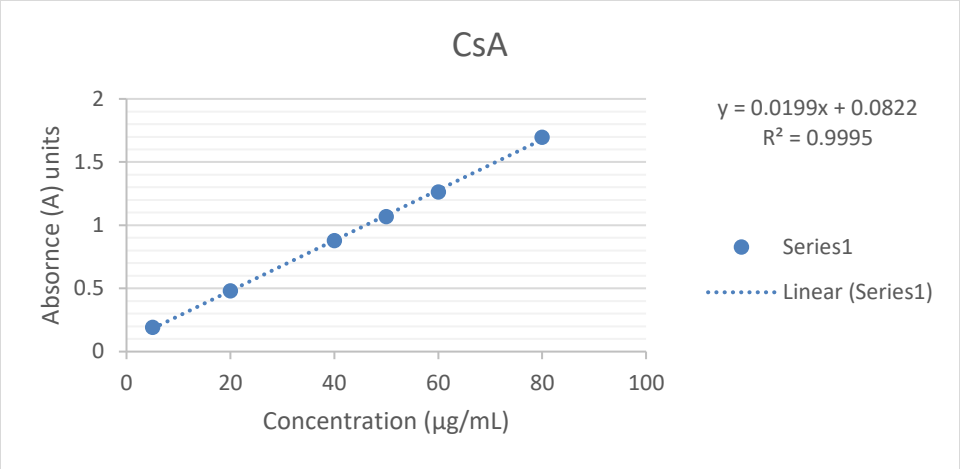

Figure 6: CsA calibration curve

Differential Scanning Calorimetry (DSC)

DSC was performed to help to know the compatibility of the CsAnd PLGA nanoparticles. As figure 6 shown below, the characteristic melting point of CsAis 208 ^{0}C. And this pick is showing in the physical mixture of CsAnd PLGA while not showing in the picture CsA-NPs formulation, indicating that the CsAis loaded in PLGA Nanoparticles.

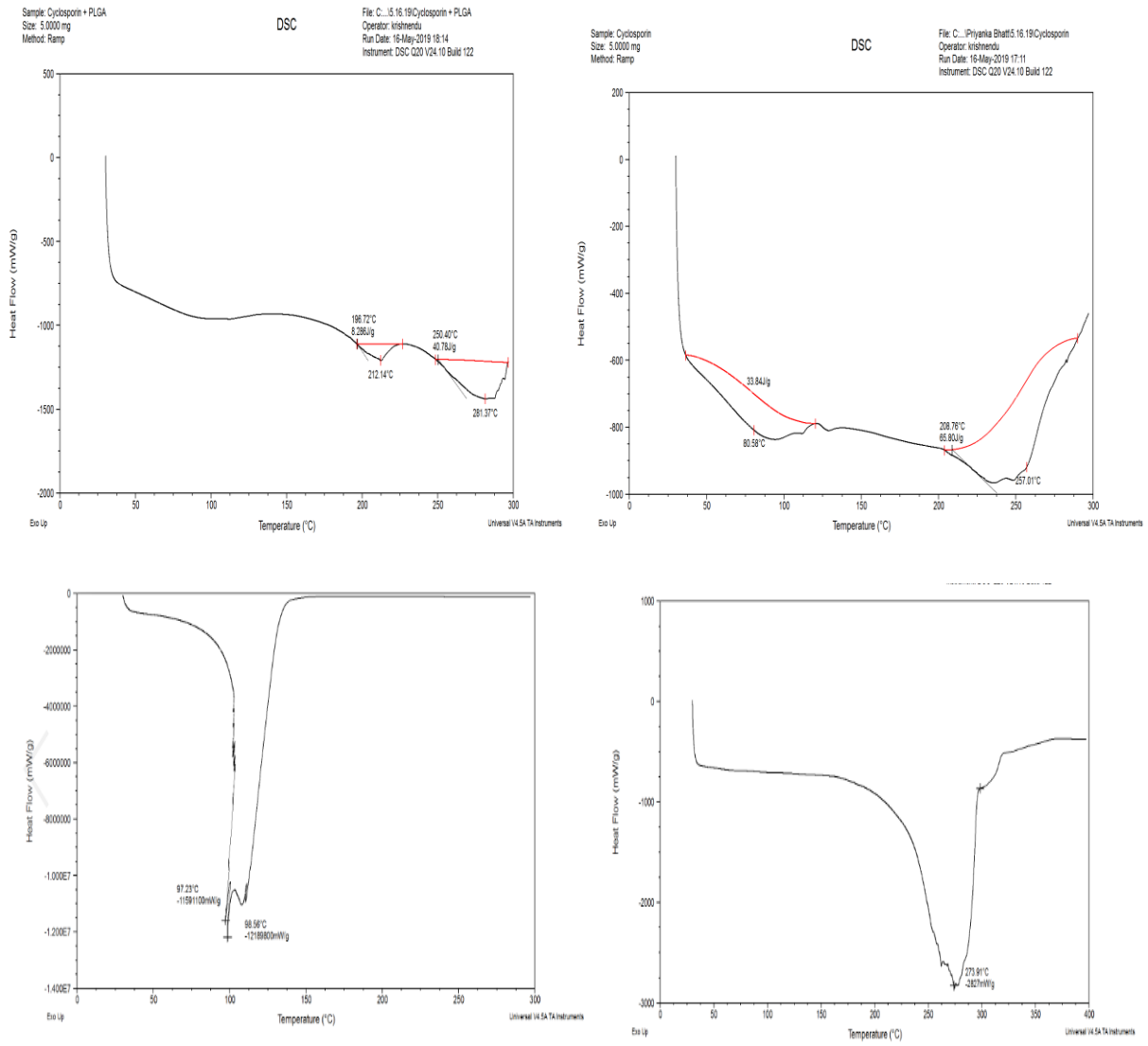

Figure 7: DSC spectra of CsA pure drug, PLGA, CsA and PLGA physical mixture and CsA-NPs formulation.

Cytotoxicity Study

The cytotoxicity of the CsA drug solution and CsA-NPs was studied by using the MTT assay in human kidney cells (HEK-293). Cells were treated with different concentrations (0, 0.001, 0.01, 0.1, 1, 10 and 20 µM) of CsA drug solution and CsA-NPs in triplicates for 24 and 48 h. As figure 7 shown below, results demonstrated the cell

viability for CsA-NPs formulation is higher than at almost all concentrations, while the cell viability for the drug solution was found 81% and 76% respectively at 10 μM and 20 μM concentrations. Therefore, showing the cell compatibility of the CsA-NPs formulation.

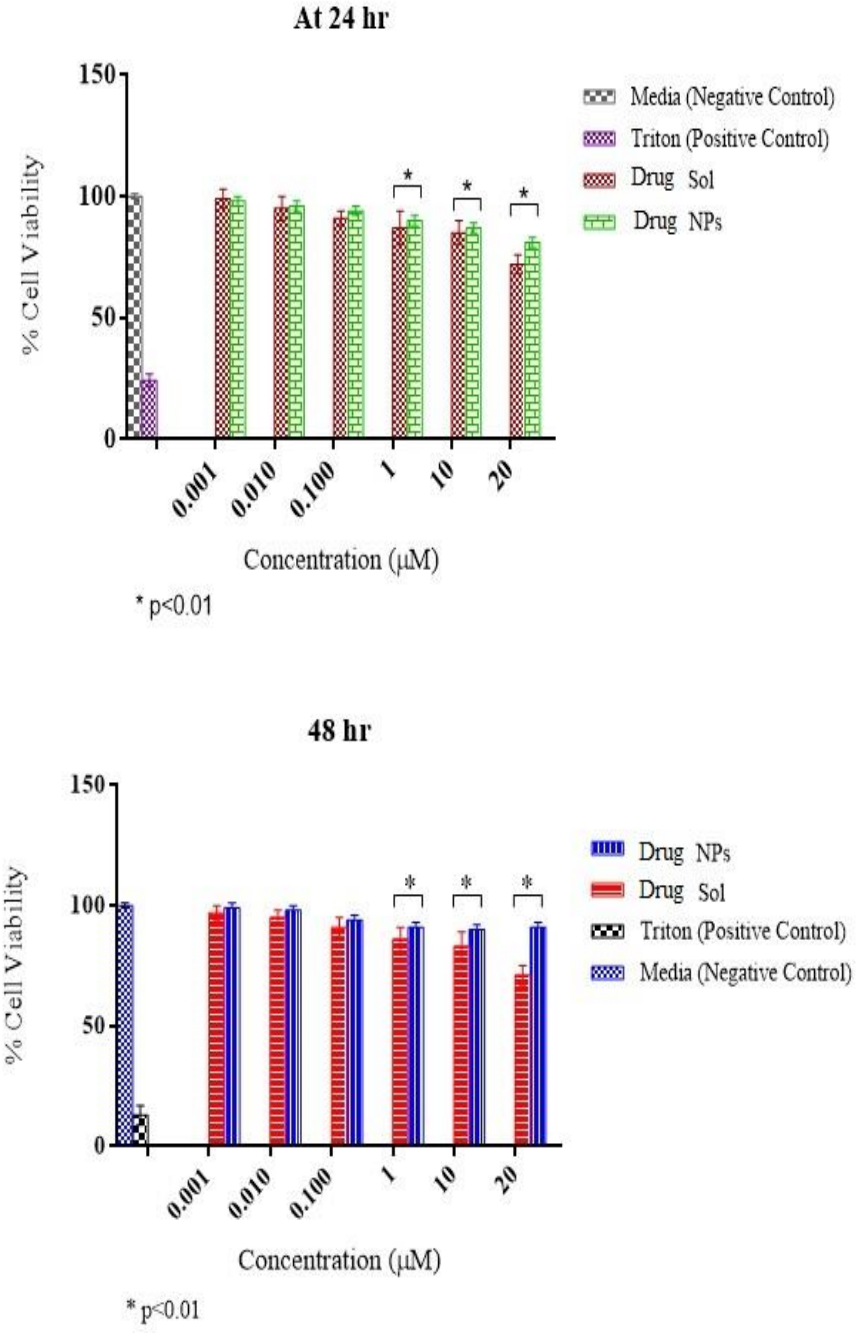

Figure 8: % cell viability at different concentrations of CsA drug solution and CsA-NPs at (A) 24 h and (B) 48 h in HEK-293 cell line, (mean ± *SD*, n =3).

CHAPTER FOUR:

CONCLUSIONS AND FUTURE PROSPECTS

CsA loaded PLGA nanoparticles were successfully developed and characterized. The size of the particles was in the nanometer range with narrow size distribution. CsA-NPs formulation is able to achieve sustained drug delivery from the drug release study. It is determined from the cytotoxicity study that the CsA-NPs is less toxic to the HEK- 293 cells. Thus, this nanoformulations may serve as an alternative formulation for CsA for effective drug delivery and to reduce the side effects. For future studies this CsA PLGA nanoformulation could be compared using MTT assay with other CsA nanoemulsion and liposomes.

REFERENCES:

1. Lamoureux, F., Mestre, E., Essig, M., Sauvage, F. L., Marquet, P., & Gastinel, L. N. (2011). Quantitative proteomic analysis of CsA-induced toxicity in a human kidney cell line and comparison with tacrolimus. *Journal of proteomics*, *75*(2), 677-694.
2. Servais, H., Ortiz, A., Devuyst, O., Denamur, S., Tulkens, P. M., & Mingeot-Leclercq, M. P. (2008). Renal cell apoptosis induced by nephrotoxic drugs: cellular and molecular mechanisms and potential approaches to modulation. *Apoptosis*, *13*(1), 11-32.
3. Jennings, P., Koppelstaetter, C., Aydin, S., Abberger, T., Wolf, A. M., Mayer, G., & Pfaller, W. (2007). CsA induces senescence in renal tubular epithelial cells. *American Journal of Physiology-Renal Physiology*, *293*(3), F831-F838.
4. Heidari-Soreshjani, S., Asadi-Samani, M., Yang, Q., & Saeedi-Boroujeni, A. (2017). Phytotherapy of nephrotoxicity-induced by cancer drugs: An updated review. *Journal of nephropathology*, *6*(3), 254.
5. Chiou, H., Chan, H. K., Heng, D., Prud'homme, R. K., & Raper, J. A. (2008). A novel production method for inhalable CsA powders by confined liquid impinging jet precipitation. *Journal of Aerosol Science*, *39*(6), 500-509.
6. Bennett, W. M., & Norman, D. J. (1986). Action and toxicity of CsA. *Annual review of medicine*, *37*(1), 215-224.
7. Desai, P. P., Date, A. A., & Patravale, V. B. (2012). Overcoming poor oral bioavailability using nanoparticle formulations–opportunities and limitations. *Drug Discovery Today: Technologies*, *9*(2), e87-e95.
8. Akhter, S., Anwar, M., Siddiqui, M. A., Ahmad, I., Ahmad, J., Ahmad, M. Z., ... & Ahmad, F. J. (2016). Improving the topical ocular pharmacokinetics of an immunosuppressant agent with mucoadhesive nanoemulsions: Formulation development, in-vitro and in-vivo studies. *Colloids and Surfaces B: Biointerfaces*, *148*, 19-29.
9. Ganta, S., Talekar, M., Singh, A., Coleman, T. P., & Amiji, M. M. (2014). Nanoemulsions in translational research—opportunities and challenges in targeted cancer therapy. *Aaps Pharmscitech*, *15*(3), 694-708.
10. Agarwal, P., & Rupenthal, I. D. (2016). Modern approaches to the ocular delivery of CsA. *Drug Discov Today, 21*(6), 977-988. doi: 10.1016/j.drudis.2016.04.002
11. Behr, J., Zimmermann, G., Baumgartner, R., Leuchte, H., Neurohr, C., Brand, P., . . . Keller, M. (2009). Lung deposition of a liposomal CsA inhalation solution in patients after lung transplantation. *J Aerosol Med Pulm Drug Deliv, 22*(2), 121-130. doi:10.1089/jamp.2008.0714

12. Chiu, H. Y., Huang, H. L., Li, C. H., Chen, H. A., Yeh, C. L., Chiu, S. H., . . . Ho, S. Y. (2015). Increased Risk of Chronic Kidney Disease in Rheumatoid Arthritis Associated with Cardiovascular Complications - A National Population-Based Cohort Study. *PLoS One, 10*(9), e0136508. doi: 10.1371/journal.pone.0136508
13. Italia, J. L., Bhatt, D. K., Bhardwaj, V., Tikoo, K., & Kumar, M. N. (2007). PLGA nanoparticles for oral delivery of CsA: nephrotoxicity and pharmacokinetic studies in comparison to Sandimmune Neoral. *J Control Release, 119*(2), 197-206. doi:10.1016/j.jconrel.2007.02.004
14. Lallemand, F., Schmitt, M., Bourges, J. L., Gurny, R., Benita, S., & Garrigue, J. S. (2017). CsA delivery to the eye: A comprehensive review of academic and industrial efforts. *Eur J Pharm Biopharm, 117*, 14-28. doi:10.1016/j.ejpb.2017.03.006
15. Mervaala, E., Lassila, M., Vaskonen, T., Krogerus, L., Lahteenmaki, T., Vapaatalo, H., & Karppanen, H. (1999). Effects of ACE inhibition on CsA-induced hypertension and nephrotoxicity in spontaneously hypertensive rats on a high-sodium diet. *Blood Press, 8*(1), 49-56.
16. Mervaala, E. M., Pere, A. K., Lindgren, L., Laakso, J., Teravainen, T. L., Karjala, K., . . . Karppanen, H. (1997). Effects of dietary sodium and magnesium on CsAlosporin A-induced hypertension and nephrotoxicity in spontaneously hypertensive rats. *Hypertension, 29*(3), 822-827.
17. Pradhan, J., Mohanty, C., & Sahoo, S. K. (2018). Protective efficacy of crocetin and its nanoformulation against CsA-mediated toxicity in human embryonic kidney cells. *Life Sci*. doi:10.1016/j.lfs.2018.11.027
18. Sonaje, K., Italia, J. L., Sharma, G., Bhardwaj, V., Tikoo, K., & Kumar, M. N. (2007). Development of biodegradable nanoparticles for oral delivery of ellagic acid and evaluation of their antioxidant efficacy against CsA-induced nephrotoxicity in rats. *Pharm Res, 24*(5), 899-908. doi:10.1007/s11095-006-9207-y
19. Tedesco, D., & Haragsim, L. (2012). CsA: a review. *J Transplant, 2012*, 230386. doi:10.1155/2012/230386
20. Yadav, S., Gattacceca, F., Panicucci, R., & Amiji, M. M. (2015). Comparative Biodistribution and Pharmacokinetic Analysis of CsA-A in the Brain upon Intranasal or Intravenous Administration in an Oil-in-Water Nanoemulsion Formulation. *Mol Pharm, 12*(5), 1523-1533. doi:10.1021/mp5008376
21. Zhang, H., Wang, L., & Zhang, L. (2015). CsAnanomicelle eye drop: a novel medication for corneal graft transplantation treatment. *Biol Pharm Bull, 38*(6), 893-900. doi:10.1248/bpb. b15-00111
22. Naeem, M., Bae, J., Oshi, M. A., Kim, M. S., Moon, H. R., Lee, B. L., ... & Yoo, J. W. (2018). Colon-targeted delivery of cyclosporine A using dual-functional Eudragit® FS30D/PLGA nanoparticles ameliorates murine experimental colitis. *International journal of nanomedicine*, *13*, 1225.
23. Simon, L. C., Stout, R. W., & Sabliov, C. (2016). Bioavailability of orally delivered alpha-tocopherol by poly (lactic-co-glycolic) acid (PLGA) nanoparticles and chitosan covered PLGA nanoparticles in F344 rats. *Nanobiomedicine*, *3*(Godište 2016), 3-8.

24. Liddicoat, A. M., & Lavelle, E. C. (2019). Modulation of innate immunity by Cyclosporine A. *Biochemical pharmacology*.
25. Venkatpurwar, V. P., Rhodes, S., Oien, K. A., Elliott, M. A., Tekwe, C. D., Jørgensen, H. G., & Kumar, M. R. (2015). Drug-not carrier-dependent haematological and biochemical changes in a repeated dose study of cyclosporine encapsulated polyester nano-and micro-particles: size does not matter. *Toxicology*, *330*, 9-18.
26. Gao, S. J., Liu, Y., Wang, H. J., Ban, D. X., Cheng, S. Z., Ning, G. Z., ... & Feng, S. Q. (2017). New approach to treating spinal cord injury using PEG-TAT-modified, cyclosporine-A-loaded PLGA/polymeric liposomes. *Journal of drug targeting*, *25*(1), 75-82.