Development of polymer-based nanoparticles in combination with Pheroid® technology to improve therapy for *Mycobacterium avium* complex

A Jakoet

orcid.org 0000-0002-3057-4035

Dissertation submitted in fulfilment of the requirements for the degree *Master of Science* in *Pharmaceutical Science* the Potchefstroom Campus of the North West University

Supervisor: Dr Y Lemmer

Co-supervisor: Mr L Kalombo

Assist supervisor: Prof AF Grobler

Graduation May 2018

Student Number: 24695009

Acknowledgements

In the name of God, the most Gracious, the most Merciful.

I would like to take this opportunity to thank all those who have played an integral and meaningful role in this journey that has now come to an end.

To my husband, Rushdi Abrahamse, thank you for all of your encouragement, love and patience. Thank you for taking care of our beautiful boys, Waseem and Mahir, when I needed it most, I love you.

To my mother, Naziera Jakoet, although you are far away, your guidance and faith in me has always pushed me to do better. Thank you for always believing in me and giving me that much needed tough love, when times seemed dim.

To my friend and partner in crime, Shakeela Sayed. Your shoulder was always free to cry on, thank you. You have always gone above and beyond to help me eventhough you had your own deadlines. Thank you.

To my collegue and friend Brendon Naicker, you were always a mentor to me, pushing and encouraging me to set my goals, high. Thank you for taking time to teach and train me, especially with the LCMS development work.

To Dr. Yolandy Lemmer, my supervisor, thank you for all of your valuable input and guidance your have given me throughout my studies. Thank you for all your positivity and encouragement.

To Prof. Anne Grobler, my assistant supervisor, thank you for granting me the opportunity to complete my studies at the PCDDP and be part of your supportive team.

To Mr. Lonji Kalombo, my co-superivor, thank you for your open door policy. Thank you for all your contributions and willingness to help.

I would also like to extend my utmost gratitude to the Council of Scientific and Industrial Research, Polymers and Composites for awarding me this opportunity to complete this degree. The studentships you have, is a great programme, it has definitely evolved me as a person. Thank you.

Last, and most definitely not least, I would like to thank the India-Brazil and South Africa tri-lateral for their financial assistance.

ABSTRACT

The difficulties associated with conventional therapy for *Mycobacterium avium* complex (MAC) treatment provide opportunities for drug delivery platforms. In this research investigation, the results obtained for the development of a hybrid system of poly (lactic-co-glycolic acid) (PLGA) nanoparticles encapsulated into a Pheroid® vesicle are reported. Ethambutol (ETB) and clarithromycin (CLR) together with mycolic acids (MA) were encapsulated into PLGA NPs by using a double emulsion solvent evaporation technique. The particles displayed an average size of 305–397 nm with an average zeta potential of -26.5 to -29.8 mV. Liquid chromatography mass spectrometry (LCMS) drug quantification revealed that PLGA/ETB/MA and PLGA/CLR/MA NPs had a drug encapsulation efficiency (EE) of 96.6 and 94.3%, respectively. The drug loaded particles were subjected to a cytotoxicity screening towards the HeLa cell line and THP-1 macrophages. The cytotoxicity evaluation revealed that PLGA, MA, and ETB displayed no cytotoxic effect after 24-hour exposure to the particles. PLGA-CLR NP's on the other hand had a much more prominent effect on the survival of the treated cells when compared to the DF and ETB NP's treated cells. *In vitro* tests indicated that the CLR incorporated in the PLGA NP's had a lower cytotoxic effect compare to the pure drug alone. Successful cellular uptake of all particles (Drug Free (DF), CLR and ETB, with and without MA) was observed into THP-1 macrophages thus suggesting that targeted delivery to the site of infection may be possible.

The nanoparticles containing the drug and/or the MA, were encapsulated into Pheroid® vesicles via a post mix approach. DF and ETB-loaded PLGA NPs were successfully encapsulated into Pheroid® vesicles, however the same fate was not observed for CLR-loaded PLGA-MA NPs. Furthermore, the cytotoxicity assay results indicated that Pheroid® vesicles were cytotoxic to HeLa cells at concentration ≥ 2% (v/v). Further evaluation indicated that Pheroid® vesicles were non-cytotoxic at low concentration when exposed to THP-1 macrophages after 24 hours of incubation. The in *vitro* uptake studies revealed that PLGA NPs were observed at a greater density in close proximity within THP-1 macrophages after 1 hour of incubation when compared to the control of PLGA NP formulations without Pheroid® vesicles, however further investigation is warranted for further conclusions to be drawn.

In summary, the PLGA NP-Pheroid® vesicle hybrid system may have potential to be considered as an attractive and promising approach to enhance the current conventional therapy for MAC.

Keywords: *poly (lactic-co-glycolic acid) (PLGA), Pheroid® vesicles, cellular uptake, cytotoxicity, hybrid*

OPSOMMING

Verskillende platforms vir die aflewering van aktiewe middels word genoodsaak deur die komplekse behandeling van *Mycobacterium avium* kompleks (MAC). Aanvanklike resultate van ‘n hibriede stelsel van poly (lactic-co-glycolic acid) (PLGA) nanopartiekels wat omhul word deur Pheroid® vesikels word bespreek. Ethambutol (ETB) en clarithromycin (CLR) saam met mikoolsure (MA), as teikenings middel, was saam gevoeg in PLGA NPs deur middel van ‘n dubbele emulsie verdampings proses. Die partiekels se gemiddelde grootte was ongeveer 305nm – 397nm met ‘n zeta potensiaal tussen -26.5 mV en – 29.8 mV.

PLGA/ETB/MA en PLGA/CLR/MA NPs het ‘n aktiewe middel enkapsulerings effektiwiteit van 96.9% en 94.3% elk gehad. Die medikasie vrye (DF) en PLGA-ETB NP’s met en sonder MA’s, toon na 24 uur se behandeling minimale toksisityd teenoor Hela en THP-1 makrofaag selle. PLGA-CLR NP’s aan die anderkant het n groter effek op die lewensvatbaarheid gehad wanneer dit vergelyk was met DF en ETB NP’s se formulasies. *In vitro* toetse het gewys dat die CLR in die PLGA NP’s ‘n laer sitotoksiese effek gehad het teenoor die CLR middel alleen. Fluoresseerend gemerkte PLGA NP’s wat gelaai was met ETB of CLR het indikasies getoon van opname in die THP-1 makrofaag selle.

Die nanopartiekels met en sonder die aktiewe middel en MA was gelaai in Pheroid vesikels met ‘n post-formulerings benadering. DF en ETB gelaaide PLGA NP’s wat geiinkorporeer was in Pheroid® vesikels, het nie dieselfe resultate getoon as die CLR gelaaide PLGA MA NP’s nie. Verder nog, het die Pheroid® vesikels sitotoksisityd gehad teenoor Hela selle met n konsentrasie groter as ≥ 2% (v/v). Die Pheroid® vesikels was wel nie sitotoksies na 24 uur in lae konsentrasies teenoor THP-1 makrofaag selle nie. Voorlopige *in vitro* opname studies het gewys dat die gekombineerde stelsel van PLGA NP’s in Pheroid® vesikels ‘n groter meerderheid interaksie met THP-1 makrofaag selle gehad het as net die partikels alleen. Verdere ondersoek word genood saak om die observasies te bevestig. In opsoming, die PLGA NP Pheroid® vesikels hibriede stelsel kan voordelige implikasies hê wanneer dit aangewend word vir behandeling in teenstelling met die huidige konvensionele terapie vir MAC

Sleutel Woorde: *poly (lactic-co-glycolic acid) (PLGA), Pheroid® vesikels, sellulêre opname, sitotoksisityd, hibriede sisteme.*

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ATD	anti-tubercular drugs
BODIPY	dipyromethane boron difluoride
CLR	clarithromycin
CLSM	confocal laser scanning microscopy
C_{max}	maximum plasma concentration
C6	coumarin-6
CV	Coefficient of variance
DCM	dichloromethane
DF	drug free
DL	drug loading
DMEM	Dulbecco's Modified Eagle's Medium
EA	ethyl acetate
EE	encapsulation efficiency
Em	emission
Ex	excitiation
ETB	ethambutol hydrochloride
FA	fatty acids
FCS	foetal calf serum
FDA	food and drug administration
FACS	fluorescence-activated cell sorting
FIA	flow injection analysis
GRAS	generally regarded as safe
HPESO	hydrolysed polymer of epoxidized soybean oil
HPLC	High pressure liquid chromatography
HIV	human immunodeficiency virus
INH	isoniazid
LCMS	Liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
LPN	lipid polymer hybrid nanoparticle
MA	mycolic acid
MAC	*Mycobacterium avium* complex
MIC	minimum inhibitory concentration
MS	mass spectrometry

M.tb	*Mycobacterium tuberculosis*
NPs	nanoparticles
NTM	nontuberculous mycobacteria
NWU	North-West University
PDI	poly-dispersity index
PEG	polyethylene glycol
PFA	paraformaldehyde
PLGA	poly (lactic-co-glycolic acid)
PMA	phorbol 12 myristate 13-acetate
PVA	polyvinyl alcohol
PYZ	pyrazinamide
RIF	rifampicin
MRM	multiple reaction monitoring
SEM	scanning electron microscopy
SLN	solid lipid nanoparticles
SRM	selective reaction monitoring
T_{max}	maximum time
5-BMF MA	5-bromomethyl fluorescein mycolic acid
DF-PLGA NP	drug free poly (lactic-co-glycolic acid) nanoparticles
DF-PLGA-MA NP	drug free poly (lactic-co-glycolic acid) nanoparticles with mycolic acid
PLGA-ETB NP	ethambutol loaded poly (lactic-co-glycolic acid) nanoparticles
PLGA-ETB-MA NP	ethambutol loaded poly (lactic-co-glycolic acid) nanoparticles with mycolic acid
PLGA-CLR- NP	clarithromycin loaded poly (lactic-co-glycolic acid) nanoparticles
PLGA-CLR-MA NP	clarithromycin loaded poly (lactic-co-glycolic acid) nanoparticles with mycolic acid
w/v	weight per volume
v/v	volume per volume

Chapter 1: Study Rationale, aim and objectives

1.1 Introduction

MAC is a group of opportunistic non-tuberculous mycobacterium (NTM). MAC consists of two species namely, *Mycobacterium avium* and *Mycobacterium intracellulare*. The two species are phenotypically difficult to distinguish hence are frequently identified as a complex (Reed *et al*., 2006; Scholar, 2007). These bacteria are commonly found in soil, water, food and dairy products (Nishiuchi *et al*., 2017). They cause symptoms which are indistinguishable from tuberculosis, as they also infect the lungs, lymph nodes, bones and intestines (Karakousis *et al*., 2004).

MAC can cause infection amongst the general population; however, the most stricken population is amongst patients with acquired immune deficiency syndrome (AIDS) (Whiley *et al.,* 2012). At least 10 – 30% of AIDS patients are affected by MAC owing to their low $CD4^+$ lymphocyte cell count which is less than 0.05×10^9 cells/mL (Han *et al*., 2005). Before the implementation of anti-retroviral therapy, a high mortality rate with patients with a co-infection of AIDS and MAC was observed (Wu *et al.,* 2009). The development of different treatment strategies and combined regimens during the last two decades has resulted in a suppression of the mycobacterial colony in MAC-affected patients thus leading to significant improvement in the survival of patients (Karakousis *et al*., 2004).

1.2 Current treatment

It is well established that MAC bacteria reside and multiply in macrophages (Cosma *et al*., 2003). To kill the bacteria effectively, the active bactericidal compound needs to be sufficiently taken up by the macrophages followed by its penetration/diffusion through the MAC cell wall.

MAC is commonly treated with a combination therapy consisting of two or more of the following drugs: rifabutin, rifampin, clofazimine, ethionamide, ethambutol, azithromycin and clarithromycin. These actives can reach inhibitory levels in the plasma when administered 10-fold their respective minimum inhibitory concentration (MIC), hence leading to severe toxic side effects that limit their clinical use (Clemens *et al*., 2012).

Thus, a treatment regimen that could selectively deliver the drug to the MAC-infected macrophage, should result in an increased therapeutic index by achieving a higher drug concentration at the site of infection with a lower dosage administered (Clemens *et al*., 2012). Subsequently, the MAC bacteria would be inhibited within a shorter period resulting in shortened treatment duration.

1.3 Brief introduction to nanomedicine

Nanomedicine is the application of nano-sized agents for diagnosis and therapy of various ailments and diseases (Chraavi & Duraisami, 2011). These systems may be designed to include a combination of hydrophilic and lipophilic phases. They exhibit relatively high solubility in aqueous environments and allow transportation across cellular membranes resulting in a rapid distribution throughout the body (Garnett & Kallinteri, 2006). Nanomedicine offers an advantage over conventional therapy as the active is protected from drug degradation, elimination or modification before it is delivered to the infected sites (Clemens *et al*., 2012).

A library of different drug delivery platforms exists for the treatment of various diseases, such as tuberculosis, cancer, HIV/AIDS and diabetes. These drug delivery systems include solid lipid nanoparticles, liposomes, polymeric nanoparticles, Pheroid® and emulsion systems (Park, 2008; Grobler, 2009). For the scope of this research project polymeric nanoparticles and Pheroid® delivery systems will be discussed.

1.4 Polymeric drug delivery systems

Poly (lactic-co-glycolic acid) (PLGA) is extensively researched as a potential drug delivery system owing to its biodegradability and biocompatibility. This polymer can be synthesised by means of ring-opening co-polymerization of lactic acid and glycolic acid. PLGA has shown to have favourable degradation properties and possess the potential for controlled drug release (Hirenkumar *et al*., 2011).

Pandey and co-workers (2006) have investigated the application of PLGA nanoparticles (NPs) for the nano-encapsulation of anti-tubercular drugs (rifampicin, isoniazid, pyrazinamide and ethambutol). The PLGA delivery system showed an increase in bioavailability of the anti-tubercular drugs (ATD) when compared to the free drugs. Drug concentrations were detectable and maintained above the MIC in the plasma for 5 days and in the organs (lungs, liver, spleen) for 7–9 days whereas the free drug was only detected until 24 to 48 hours post oral administration into mice (Pandey *et al*., 2006).

Semete and co-workers (2012) have also investigated the application of PLGA with the encapsulation of the same four anti-tubercular drugs. PLGA nanoparticles were prepared using a patented technology by Kalombo in 2011 that includes the addition of surfactants and additives to potentially modify the polymer matrix, thereby increasing the blood circulation time. This technology has shown a sustained drug release profile which was in agreement with Pandey *et al*., (2006) together with an added increase in residence circulation time (Semete *et al*., 2012).

1.5 Pheroid® delivery system

Pheroid® is a lipid-based drug delivery system that comprises of three phases including: an aqueous phase, an oil phase as well as a gas phase. It has been shown to increase absorption and improve the overall efficacy of oral therapeutics *in vivo* (Grobler, 2009). The outer layer of the Pheroid® is composed of essential fatty acids which are advantageous, as it allows for non-immunogenic responses and in turn enhances cellular uptake (Grobler, 2009).

A pilot study in mice was conducted at the North-West University (NWU) to investigate the effect of the Pheroid® delivery system for ATDs. A preliminary investigation by Mathee (2007), showed that the time taken for ATDs to reach the maximum plasma concentration (C_{max}) significantly decreased after administration in mice and could possibly be explained by the rapid movement of encapsulated drugs across physiological barriers (Grobler, 2009).

A similar finding of enhanced absorption was confirmed for an anti-malarial drug, artemisone, loaded into the Pheroid® formulations (Steyn *et al.,* 2011). This study showed that the half-life of artemisone was delayed and the time taken (T_{max}) to reach C_{max} was improved which could potentially allow for therapeutic drug concentrations at a decreased dose (Steyn *et al.*, 2011).

Additional advantages can be obtained when targeting ligands are incorporated into drug delivery systems. This would allow for targeted delivery of the active to the site of infection i.e, infected macrophage cells, thus inhibiting the bacteria without the need for high drug dosages leading ultimately to increased unwanted side-effects (Natarajan & Meyyanathan, 2012).

1.6 Targeted drug delivery

Targeted drug delivery assists in delivering therapeutics to a specific site of interest. The goal of a targeted drug delivery system is to prolong, localise, target and have a protected drug interaction at the site of infection (Muller & Keck, 2004). There is a vast range of different ligands that can be utilised for targeted delivery. These include small molecules, carbohydrates, peptides, proteins or antibodies each with their own affinity or mechanisms to varying receptors or sites (Nicholas *et al*., 2012).

Lemmer and co-workers (2015), have shown that mycolic acids (MA), which is a long chain fatty acid found in *Mycobacterium tuberculosis (M.tb)* cell walls may be used as a possible targeting ligand to TB-infected macrophages owing to its cholesteroid nature (Lemmer *et al*., 2015). This lipid molecule will be included in this project as a potential targeting molecule to macrophage cells.

1.7 Problem statement

A treatment regimen for Mycobacterium avium complex (MAC) exists, however, the current treatment is inefficient. One of the reasons is the inadequate therapeutic levels at the targeted site of infection, where the drugs should be able to enter the macrophage cells and penetrate the cell wall of the bacteria (Jacobson & Aberg, 2006). Therefore, there is a need to improve the current treatment regimen with new chemotherapeutics or novel drug delivery systems. This investigation utilises nano-drug delivery systems, a branch of Nanomedicine. The aim is to improve the current treatment by implementing a combined carrier vehicle system that includes a targeting agent i.e. mycolic acids, incorporated into a polymeric nanoparticle for sustained drug release together with a Pheroid® vesicle coating to assist uptake in the intestines.

1.8 Aim

The purpose of this study was to investigate a delivery system that could potentially assist in decreasing MAC drugs dosages with enhanced uptake and limited toxicity.

1.8.1 Objectives

The objectives of this study were to:

- Synthesise mycolic acid (MA)-labelled PLGA nanoparticles (NPs) encapsulating MAC drugs; specifically, clarithromycin (CLR) and ethambutol dihydrochloride (ETB),
- Synthesise Pheroid® vesicles,
- Combine the NPs and Pheroid® delivery system,
- Evaluate the uptake of Pheroid®, PLGA and PLGA-MA-Pheroid® combined formulations, into macrophages and
- Test the cytotoxicity of the Pheroid® and PLGA-MA formulations by means of a WST-1 cell proliferation assay.

1.9 Significance of study

Semete and co-workers (2012) have shown that PLGA NPs have a sustained drug release profile and Lemmer (2015) has effectively demonstrated the use of MA as a targeting ligand with enhanced uptake of ATD into the macrophage cells. On the other hand, Grobler (2009) and Steyn (2011) have shown a drastic change in T_{max} and C_{max} for various drugs that were loaded into Pheroid® formulations. It was hypothesised that a synergistic therapeutic effect may derive from the combination of the two systems, whereby mycolic acids containing PLGA

NPs are encapsulated into Pheroid®. This combination could potentially result in the availability of actives at a high concentration at the site of interest, shortly after administration which may lead to a decrease in the drug dosage and dose frequency which may ultimately result in minimal toxic side effects as well as increased patient compliance.

1.10 Layout of the dissertation

Chapter 1 provides a brief introduction to the dissertation thus highlighting the purpose, aim and objectives of the study. Chapter 2 is the literature review focusing on a basic overview of *Mycobacterium avium* complex, different drug delivery systems, i.e. polymeric drug delivery, Pheroid® technology and hybrid systems, and the important physiochemical properties of nanoparticles. Chapter 3 is a full-length article focusing on the development, cytotoxicity and uptake ability of the prepared polymeric particles. Chapter 4 highlights the results of the polymeric and Pheroid® hybrid delivery system. These results are also prepared in a full-length article. The 2 full length articles reference list, will be in line to that listed by the author guidelines for the respective journals. Chapter 5 consists of the LCMS method development that was used to quantify the EE of the CLR and ETB drug in the PLGA NPs. It will also provide a detailed discussion of the results. A summary of the all the work, conclusion and recommendations are presented in Chapter 6.

References

Chraavi, J.C.B. & Duraisami K.S.N. 2011. Review on nanoparticle based therapeutics and drug delivery systems. *Asian Journal of Biochemical and Pharmaceutical Research*, 1(2):162-170.

Clemens, D.L., Lee, B.Y., Xue, M., Thomas, C.R., Meng, H., Ferris, D., Nel, A.E., Zink, J.I. & Horwitz, M.A. 2012.Targeted intracellular delivery of antituberculosis drugs to Mycobacterium tuberculosis-infected macrophages *via* functionalized mesoporous silica nanoparticles. *American Society for Microbiology*, 56(5):2535-2545.

Cosma, C.L., Sherman, D.R. & Ramakrishnan, L. 2003. The secret lives of the pathogenic mycobacteria. *Annual Review of Microbiology*, 57:641-676.

Garnett, M.C. & Kallinteri, P. 2006. Nanomedicine and nanotoxicology: some physiological principles. *Occupational Medicine*, 56:307-311.

Grobler, A.F. 2009.Pharmaceutical applications of Pheroid technology.Potchefstroom: NWU. (Thesis-PHD).

Han, X.Y., Tarrand, J.J., Infante, R., Jacobson. K.L. & Truong, M. 2005. Clinical significance and epidemiologic analyses of *Mycobacterium* avium and *Mycobacterium* intracellulare among patients without AIDS. *Journal of Clinical Microbiology*, 43(9):4407-4412.

Hirenkumar, K., Siegal, M. & Siegal, S.J. 2011. Poly lactic-co-glycolic acid (PLGA) nanoparticles as biodegradable controlled drug delivery carrier. *Polymers (Basel),* 3(3):1377-1397.

Jacobson, M.A. & Aberg, J.A. 2006. Mycobacterium avium Complex and Atypical Mycobacterial Infections in the Setting of HIV Infection. http://hivinsite.ucsf.edu/InSite?page=kb-00&doc=kb-05-01-05 Date of access: 28 September 2017.

Kalombo, L., 2011. Nanoparticle carriers for drug administration and process for producing the same. (Patent: US 0033550A1). http://www.google.com/patents/US20110033550 Date of access: 28 September 2017.

Karakousis, P.C., Moore, R.D. & Chaisson, R.E. 2004. Review Mycobacterium avium complex in patients with HIV infection in the era of highly active antiretroviral therapy. *The LANCET Infectious Diseases*, 4(9):557-565.

Lemmer, Y., Kalombo, L., Pietersen, R.D., Jones, A.T., Semete-Makokotlela, B., Van Wyngaardt, S., Ramalapa, B., Stoltz, A.C., Baker, B., Verschoor, J.A., Swai, H.S. & De Chastellier, C. 2015. Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis. *Journal of Controlled Release*, 211:94-104.

Mathee, L. 2007. A preclinical evaluation of the possible enhancement of the efficacy of antituberculosis drugs by Pheroid technology.Potchefstroom: NWU. (Dissertation-MSc).

Muller, R. & Keck, C. 2004. Challenges and solutions for the delivery of biotech drugs -a review of drug nanocrystal technology and lipid nanoparticles. *Journal of Biotechnology*, 113(1):151-170.

Nicolas, J., Mura, S., Brambilla, D., Mackiewicz, N. & Couvreur, P. 2013. Design, functionalization strategies and biomedical applications of targeted biodegradable/biocompatible polymer-based nanocarriers for drug delivery. *Chemical Society Review,* 42:1147-1235.

Nischiuchi, Y., Iwamoto, T. & Maruyama, F. 2017. Infection Sources of a Common Non-tuberculosis Mycobacterial Pathogen, Mycobacterium avium Complex. *Front Med (Lausanne)*, 4(27):1-17.

Natarajan, J. & Meyyanathan, S.N. 2012. Polymeric nanoparticles for drug delivery and targeting: A comprehensive review. *International Journal of Health and Allied Sciences*, 1(4):17-22.

Park, K. 2008. Nanotechnology: What it can do for drug delivery. *Journal of Controlled Release*, 120(2):1-3.

Pandey, R., Sharma, S. & Khuller, G.K. 2006. Chemotherapeutic efficacy of nanoparticle encapsulated anti-tubercular drugs. *Drug Delivery*, 13(4):287-294.

Reed, C., von Reyn, F., Chamblee, S., Ellerbrock, T.V., Johnson, J.W., March, B.J., Johnson, L.S., Trenschel, R.J. & Horsburgh, C.R. 2006. Environmental risk factors for infection with Mycobacterium avium complex. *American Journal of Epidemiology*, 164(1):32-40.

Scholar, E. 2007. Mycobacterium Avium-Intracellular Infections. *Reference Module in Biomedical Sciences*, 1-5.

Semete, B., Booysen, L., Kalombo, K., Ramalapa, B., Hayeshi, R. & Swai, H. 2012. Effect of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration. *International Journal of Pharmaceutics,* 424(1-2):115-120.

Steyn, J.D., Wiesner, l., du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W, Haynes, R.K. & Kotze, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. *International Journal of Pharmaceutics*, 414(1-2):260-266.

Whiley, H., Keegan, A., Giglio, S. & Bentham, R. 2012. Mycobacterium avium complex - the role of potable water in disease transmission. *Journal of Applied Microbiology*, 113(2):223-32.

Wu, U.I., Chen, M.Y., Hu, R.H. Hsieh, S.M. Sheng, W.H., Lo, Y.C. & Chang, S.C. 2009. Peritonitis due to Mycobacterium avium Complex in patients with AIDS: report of five cases and review of the literature. *International Journal of Infectious Diseases*, 13:285-290.

Chapter 2: Literature Review

2.1 Introduction

Mycobacterium avium complex (MAC) is a group of slow-growing non-tuberculous mycobacteria (NTM) that is part of 150 species and more, which are ubiquitous to the environment (Tortoli, 2014). They are believed to be contracted by ingestion, inhalation or physical contact and often lead to lymphadenitis, pulmonary and disseminated infections (Nishiuchi *et al.,* 2017). The number of NTM infections are on the rise, owing to various factors which could potentially include, an increase in environmental sources, and increase in the susceptible individuals (poverty stricken and immunocompromised individuals, new developments in laboratory detection as well as overall awareness (Shah *et al.,* 2016). Although there has been an increase in NTM infections, effective treatment regimens have not been established to date. When an individual is infected with MAC, the eradication of the bacilli is very difficult and requires prolonged therapy with the possibility of reinfection (Lee *et al.,* 2015).

2.2 Epidemiology of *Mycobacterium avium* Complex

Unfortunately comparing the prevalence of NTM and MAC infection worldwide is difficult as reporting of these incidences are not required by most countries (Nishiuchi *et al.,* 2017) therefore, no correlation can be drawn. Despite this being the case there have been reports indicating a rise of NTM infections since the 1950s (Prevots & Marras, 2015).

2.3 Treatment of MAC

MAC infection was initially treated with anti-tuberculosis drugs (ATDs) alone. This course of treatment was deemed unsatisfactory which prompted the need for newer drugs such as macrolides to use in combination with ATDs. This combination therapy includes clarithromycin or azithromycin, and rifampin or rifabutin, ethambutol and streptomycin, or amikacin. The combination therapy has shown a great improvement in the outcomes of these regimes despite being associated with adverse effects and prolonged treatment. Unfortunately to date, no optimal regimen has been established, as numerous amounts of trials has shown inconsistent efficacy (Sim *et al.,* 2010).

Monotherapy with a macrolide often result in drug resistance therefore combination therapy with two or more drugs are commonly recommended to delay or prevent the resistance. Clarithromycin (CLR) together with ethambutol (ETB) is the preferred initial treatment. CLR is commonly used as it has shown great initial clinical and bacteriologic improvement (Kim *et al.,* 2011) but has an absolute bioavailability of 50-55% (Chu, *et al.,* 1992) with side effects that include nausea, vomiting, diarrhoea and abdominal pain (Kim, *et al.,* 2011) whereas ETB has

been shown to reduce the circulating load of MAC but with a bioavailability of at least 50% (Antimicrobe, n.d). This relatively low bioavailability necessitates the need for high dosages to achieve a therapeutic effect.

A preliminary study by Miwa and colleagues (2014) compared the effect of a double drug regimen with ETB and CLR against a triple drug regimen with ethambutol, clarithromycin and rifampicin showing a similar effect. Previous reports have shown that the inclusion of rifampicin affects the concentration of CLR in serum levels (van Ingen, *et al.,* 2012). To improve the treatment outcomes a higher dose of CLR was introduced which contradicted the research by Miwa *et al*. (2014) that found that the double regimen CLR-ETB was not inferior to that of triple drug regimen as there was no significant difference in treatment outcomes.

A systematic review of treatment outcomes was conducted by Xu *et al.* (2014) that pooled the different treatments for MAC over the past 30 years. It was evident that there was and is still no standard guideline for the treatment of MAC and that treatment was mainly based on experts' opinion and physician's experience (Griffith *et al.,* 2007). All studies had different treatment regimens and durations as well as different outcomes. Most of the treatment regimens involved the utilisation of ETB, CLR in combination with other drugs. From the review, the treatment outcomes have improved over the past 10 years, but the success rate remains unsatisfactory (Xu *et al.,* 2014). Owing to the frequent combination of the CLR and ETB used for most of the investigations, this study will only focus on these two drugs.

Considering the above, MAC treatment appears complex and tedious thus promoting low patient compliance which further leads to a high relapse rate and subsequent mortality. Therefore, a treatment with a shorter duration and greater efficacy is urgently needed. Steenwinkel and co-workers (2007) together with other investigators had suggested the application of targeted drug delivery systems that will assist in the rapid clearance and elimination of mycobacteria which could in turn result in a higher curing rate, prevention of relapse and reduced treatment duration, hence improving patient compliance. Targeted drug delivery systems are a branch of nanomedicine which has emerged as an innovative and promising alternative over conventional therapy. Targeted drug delivery systems are specifically designed to target the site of infection and deliver the therapeutic payload with the capabilities of accumulating at the site of interest by active or passive targeting without being eliminated by the body (Shapira *et al.,* 2011).

2.4 A brief introduction to nanomedicine

Nanomedicine is the medical application of nanotechnology. The application of Nanomedicine involves the modification of biodegradable material such as lipids, polymers, macromolecules

and metals into therapeutic systems. These systems are capable of targeted drug delivery or non-invasive imaging agents that result in diagnosis, prevention and/or treatment of diseases (Ventola, 2012). The small sizes (10 – 1000 nm) possess the flexibility to be tailored to potentially assist in:

- intercellular drug delivery and target specificity,
- sustained release over a desired time period,
- reduction in toxicity while maintaining therapeutic effects and
- faster development of new safe medicines (De Jong & Borm, 2008).

There are various functional considerations that are considered in the development of new nano-delivery systems. The overall design is foremost dependent on the desired functionality of the drug delivery system and is governed by the formulation parameters to obtain an adequate system featuring:

- an appropriate drug loading, release profile and
- overall fate of the systems in terms of its biocompatibility, bio-distribution and targeted specificity.

In addition to these functional considerations, applied pharmaceutical considerations are also evaluated. These include storage, stability, administration route, re-dispersibility, limiting aggregation and overall impurities. The overall considerations are dependent on the final purpose of the delivery system and its ability to maintain its chemical integrity (Krishna *et al.,* 2006; Jawahar & Meyyanathan, 2012).

Nanomedicine in MAC treatment offers an improved method of treatment by designing drug delivery systems which are specific for the treatment of these bacteria. With specific targeting, the toxicity, dosage frequency and amount can potentially be lowered. In addition, it could potentially enhance the efficacy of the chosen drugs at the molecular level thus potentially improving the bioavailability of the drug and in turn lowering the adverse drug effects and ultimately improving patient compliance (Nasiruddin et al., 2017).

Nasiruddin *et al.,* (2017) reviewed the potential of nanomedicine for the treatment of tuberculosis with the implementation of liposomes, polymeric NPs, solid lipid NPs and nanosuspensions. All ATDs were reviewed, one example specifically relevant to this study was documented by Zahoor *et al.,* (2006) which displayed the detection of ETB from ETB-loaded PLGA NPs in blood plasma levels after 7 days and was still detectable in the tissues after 14 days.

Clarithromycin the second drug of choice for this study has been previously utilised by Mohammadi *et al.* (2011) which showed that CLR entrapped in a colloidal drug delivery system displayed enhanced anti-bacterial activity at an eighth of the concentration when compared to the free drug.

These therapeutics when coupled with nano-drug delivery systems show great potential for the treatment of MAC and opens a range of different possibilities in the implementation of different treatment regimes.

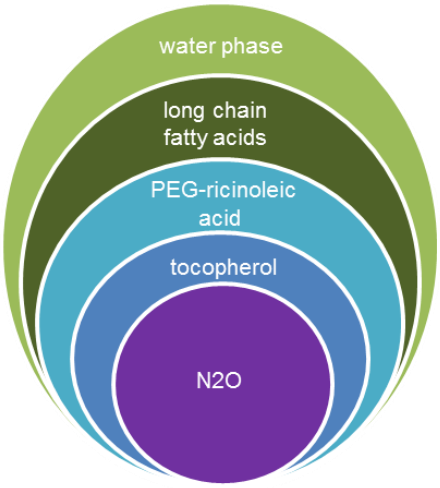
2.5 Types of delivery systems

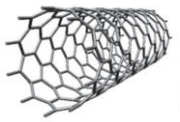
There is a vast amount of delivery systems that can be adapted to treat different diseases, modify active ingredients and alter modes of administration.

Table 2.1 was adapted and modified from Faraji and Wipf (2009) and displays a brief overview and description of a few of the different types of delivery systems that are commonly investigated.

Table 2.1: An overview of nano-carrier types and their respective description

Nanoparticle Type	Description
 Polymeric	Polymeric NPs are composed of biodegradable and biocompatible polymers. Drug can be entrapped, encapsulated or adsorbed onto the surface. They are easily modified to provide good pharmacokinetic control. A wide range of therapeutic agents; hydrophilic or hydrophobic are easily encapsulated (Safari & Zarnegar, 2014).
 Solid Lipid	Solid lipid nanoparticles (SLN) are lipid-based submicron delivery systems. It is a rigid structure with a thin layer of surfactants which includes a hydrophobic lipid core that is solid at ambient and body temperature. The lipid core provides protection against drug degradation as well as gives the benefit of sustained drug release. SLNs can be manufactured on a large production scale and

	provide prolonged product stability (de Jesus & Zuhorn, 2015).
 Liposome	Liposomes are concentric bilayered vesicles with aqueous compartments existing in the core or between the bilayers surrounded by a phospholipid membrane. They consist of a hydrophilic head and hydrophobic tails. They are easily modified owing to their amphiphilic nature and can encapsulate both hydrophilic and hydrophobic drugs (Agarwal *et al*., 2016).
 Pheroid®	The Pheroid® delivery system is composed of essential fatty acids (FA), i.e, ethyl esters of oleic, linolenic and linoleic acids. These FA are emulsified in water saturated with nitrous oxide. Grobler (2009) reported that this delivery system is capable of increasing permeation owing to its kinked structure. It is hypothesised that this kinked structure may disrupt the formation of intracellular lipids (Grobler, 2009).
 Nanocrystal	Nanocrystals are formed by the combination of therapeutic aggregates which lead to the formation of its crystalline structure. They are composed of 100% drug and stabilised with surfactants or steric stabilizers that prevent quick dissolution. These system is mainly utilised for poorly soluble drugs which may further lead to possible delivery of high dosages (Junyaprasert & Morakul, 2015).

 Nanotube	Nanotubes can be organic or inorganic self-assembly sheets of atoms that are arranged into tubes. Most nanotubes are synthesised using carbon owing to its bonding capabilities to yield completely different properties (Eatemadi *et al.,* 2014). The large internal volume and external surface, allow for easy modification.
 Dendrimer	Dendrimers are macromolecules that are composed of monomeric or oligomeric units such that the layer of branching units doubles or triples the number of peripheral groups. Owing to their structure they have similar properties to that of micelles and liposomes. They allow for greater pharmacokinetic control, as they have attractive structural features i.e. monodispersity, nano size, easy surface modification and functionalization and water solubility (Najwande *et al.,* 2009)

Each of the delivery systems listed, possess unique characteristics that could potentially undergo modification for an intended application. For the scope of this study, only polymeric nanoparticles and the Pheroid® system will be further elaborated on.

2.5.1 Polymeric nanoparticles

The use of biodegradable and biocompatible polymers in targeted and sustained drug delivery alleviates most of the limitations presented by conventional therapy utilising non-encapsulated drugs (Natarajan & Meyyanathan, 2012). These limitations include high dosages, high dose frequency, and low patient compliance and increased side effects.

Polymeric NPs are solid colloidal particles with a diameter in the size range of 10 – 1000 nm. The term NPs is a collective term used for two types of particles; nanospheres and nanocapsules. Nanospheres are particles wherein the active ingredient is dissolved, embedded, encapsulated or chemically bound to the polymer matrix. Nanocapsules on the other hand are vesicular reservoir systems with a hydrophobic or hydrophilic cavity surrounded by a polymer coating (Malodia *et al.,* 2012).

2.5.2 Common polymers used for drug delivery

Polymers are macromolecules. They are large chain molecules with a varying degree of different functional groups giving rise to different chemical and physical characteristics. Polymers are a versatile class of materials that can be divided and modified in high and low molecular weights, natural and synthetic and can be further classified into biodegradable and non-degradable polymers.

2.5.2.1 Natural polymers

Polymers that are derived from plants and animals are called natural polymers. These polymers are essential for life and can be grouped as starch, cellulose, proteins, nucleic acids (Akash *et al*., 2015).

A few natural polymers include:

- Protein and protein-based polymers: They are biocompatible and non-toxic. Typically, elastic materials that are used as implants in tissue engineering (Parveen *et al.,* 2012).
- Collagen: Is widely found in the extracellular matrix. Owing to its carboxyl and secondary amines groups, the formation of crosslinking to form hydrogels is possible. They are easily modified in terms of size, surface area and dispersion ability in water, collagen NPs can exhibit sustained release profiles for various actives (Nitta & Numata, 2013).
- Albumin: Is the most abundant blood plasma protein. It is versatile and used in cell and drug microencapsulation. It is robust in various conditions and possesses advantageous characteristics such as non-toxicity, biodegradability and immunogenicity (Kratz, 2008; Maham *et al.,* 2009).
- Carboxymethyl cellulose: Is a biocompatible macromolecule that has been used for various investigations for controlled release. Owing to its adhesive nature, it may also be used as a bio-adhesive material (Butun *et al.,* 2011).
- Alginates: Are a group of anionic polysaccharides that are hemo-compatible and have not been found to accumulate in organs as there is evidence available of *in vivo* degradation (Motwani *et al.,* 2007).

2.5.2.2 Synthetic polymers

These polymers are manmade polymers that are synthetically modified and manufactured in laboratories. They can be grouped as:

- Polyester: Poly (lactic acid), poly (glycolic acid) and their copolymers: biodegradable and easily modified to achieve desired release profiles. Commonly used in drug delivery and tissue engineering (Gavasane & Pawar, 2014).
- Polyanhydride: biodegradable and used for bio active release. They are easily cleared *in vivo* owing to the degradation into their diacid groups (Vilar *et al.,* 2012)
- Polyamides: Have repeated units of amine groups with the possibility of high hydrogen bonding ability. Owing to their polar behaviour and good mechanical properties they are primarily used to deliver low molecular weight drugs (Vilar *et al.,* 2012)
- Others: Poly cyanoacrylates, Polyurethanes, Polyorthoester, Polyacetals etc.

Each class of polymer offers different advantages. Although natural polymers are non-toxic, biocompatible, and naturally available, synthetic polymers are most frequently used owing to its reliability and reproducibility. Natural polymers are prone to microbial contamination and are dependent on environmental and seasonal factors (Kotke & Edwards, 2002). There is also a chance of batch to batch variation as the materials are dependent on region, species and climate. Therefore, synthetic polymers are more feasible as manufacturing is a controlled procedure with fix quantities and sources of ingredients (Gavasane & Pawar, 2014).

The choice of polymer is challenging owing to its current diversity and structure. Therefore, careful consideration should be taken into account such that the chosen polymer is capable of fulfilling the desired chemical, interfacial, mechanical and biological properties required.

For the scope of this study a polyester polymer i.e PLGA will be investigated for the delivery of MAC therapeutics to the target site.

2.6 Poly (D,L-lactic-co-glycolic acid) nanoparticles

PLGA is a copolymer of poly (lactic acid) (PLA) and poly (glycolic acid) (PGA) (Makadia & Segel, 2012). It is commonly used owing to its Food and Drug Administration (FDA) approval, its biodegradability and biocompatibility properties (Mirakabad *et al.,* 2014). This polymer presents an advantage of being commercially available and easily modified in terms of its monomer ratios.

Owing to its design and performance it has been widely applied to various areas of research including tissue engineering and controlled drug release systems. Numerous pharmaceutical ingredients have already been encapsulated in PLGA-based drug delivery systems with proven *in vivo* therapeutic effect (Kerimoğlu & Alarçin, 2012). These include the successful encapsulation of antibiotics (Toti *et al.,* 2011), vaccines (Zhao *et al.,* 2014), anti-cancer

molecules (Rafiei & Haddadi, 2017), anti-tuberculosis agents (Semete *et al.,* 2012) and numerous others for various ailments and diseases. PLGA has shown to be versatile as it has successfully encapsulated various active ingredients. These ingredients have their own chemical and physical properties and therefore encapsulation may be of a different process and design.

2.7 Preparation methods of polymeric nanoparticles

PLGA has been investigated by various research groups by means of various techniques such as multiple emulsion solvent evaporation, nanoprecipitation, salting out and much more, to produce microparticles or nanoparticles aiming at efficient delivery of active compounds.

2.7.1 Solvent evaporation

Nanoparticles can be formed by means of a single or a multiple emulsion. This method is used to encapsulate both hydrophilic and hydrophobic compounds. The polymer, owing to its hydrophobic nature, is first dissolved in an oil phase which is usually an organic solvent that is thereafter emulsified with an aqueous phase containing adequate surfactant or stabiliser. Hydrophobic drugs are added to the oil phase together with the polymer whereas the hydrophilic drugs are frequently added to the initial water phase. In case of a double emulsion, the aqueous phase containing the hydrophilic active agent is emulsified into the polymer organic phase by utilising high shear homogenisation. This first emulsion obtained is thereafter re-dispersed into an aqueous phase of a stabiliser and other desired additives, resulting in a double emulsion with very fine droplets size (Mirakabad *et al.,* 2014). The organic solvent of the single/double emulsion is thereafter evaporated after several hours of stirring and hardened nanoparticles are collected and washed by means of centrifugation followed by lyophilisation. Alternative technique consists of immediately spray drying the emulsion after it is formed and equally resulting in a free-flowing powder with the addition of drying additives (Kalombo, 2011).

2.7.2 Nanoprecipitation

Nanoprecipitation also known as interfacial precipitation is a low energy input process used for the preparation of polymeric nanoparticles. In this case, it is required that the organic solvent containing both the encapsulating polymer and the active compound, be partially soluble in an aqueous solution and highly volatile for the anti-solvent effect to occur. Nanoparticles are formed when droplets of the organic phase are injected into the aqueous phase with a stabiliser in solution. Spontaneous precipitation occurs owing to the rapid diffusion and evaporation of the solvent out of the aqueous phase and subsequent saturation

of the hydrophobic polymer. The active agent is therefore incorporated into the polymeric matrix either by co-precipitation or solid solution formation. This technique is so far designed only for both hydrophobic matrices and hydrophobic active compounds (Nagavarma *et al.,* 2012).

2.7.3 Salting out

This method involves the separation of a water miscible solvent from an aqueous solution achieved by a salting out effect. Both the polymer and active are dissolved in acetone followed by the emulsification into an aqueous gel containing a salting-out agent (electrolytes) and a stabiliser. The formation of nanospheres is induced by the further addition of aqueous solution which enhances the diffusion. The main disadvantage of this technique is the extensive washing step and its specificity to lipophilic drugs (Nagavarma *et al.,* 2012).

2.8 Advantages of polymeric nanoparticles

The utilisation of polymers for drug delivery offers the following advantages:

- Easy physical characteristic modification i.e. changes in size and surface charge to assist in passive or active drug targeting (Singh *et al.,* 2010).
- A sustained drug release profile is possible from the matrix which enhances bioavailability thus leading to lesser dose frequency, side effects and improved patient compliance (Parveen *et al.,* 2012).
- The preservation of drug integrity and activity with encapsulation (Dadwal, 2014).
- Increased lymphatic residence time and specific tissue and cell targeting from surface functionalised NPs with surface targeting ligands (Moghimi, 2006).
- Various routes of administration, i.e. oral, nasal, parenteral and intra-ocular and so forth (Natarajan & Meyyanathan, 2012).

Despite these advantages, the small sizes and large surface areas achieved with polymeric NPs can occasionally result in an insufficient drug loading and induce initial burst release (Nagal & Singla, 2013).

2.9 Disadvantages of polymeric nanoparticles

- Owing to its smaller size and larger surface area, particle-particle aggregation makes physical handling of nanoparticles difficult in liquid or dry form (Natarajan & Meyyanathan, 2012). The stability and storage issues are of concern owing to its size.
- This highly sophisticated technology requires a certain degree of expertise.

- Dosage adjustment is difficult.

The advantages and disadvantages of polymeric nanoparticles are greatly influenced by its physical characteristics; its particle size, surface charge, surface modification and hydrophobicity.

Therefore, various nano-scale materials are utilised to modify the pharmacokinetic and pharmacodynamic properties of the active ingredient such that an improvement in its bioavailability, specificity and controlled release profile is achieved (Ina, 2011).

2.10 Pheroid® delivery system

The lipid-based Pheroid® delivery system is multidimensional and is capable of entrapment of various actives with different physiochemical properties. It is a colloidal system comprising of essential fatty acids that are formed in an aqueous medium upon mechanical agitation. Owing to the plausible characteristics; increased efficacy, lower cytotoxicity, reduced immunological response as well as enhanced cellular uptake; the Pheroid® system is deemed to be ideal for drug delivery. This technology has been extensively investigated for the treatment of malaria as well as the delivery of actives that are transdermally administered for treating various skin diseases. Furthermore, this system is versatile and easily prepared with ingredients that are considered as GRAS (generally regarded as safe).

2.10.1 Historical perspective of the Pheroid® delivery system

Pheroid® is derived from Emzaloid™ technology. This technology was formulated by MeyerZall (Pty) Ltd Laboratories to treat psoriasis. The product formulated was proven to be more effective with reduced side effects. Further conclusions led to the hypothesis that was later proven correct; that the enhanced absorption and resulting efficacy was due to the encapsulation of the active into micro-vesicles (Fuhrmann *et al.* 2015).

In 2003, The North-West University (NWU) obtained the intellectual rights of this system. A Pheroid® is not an Emzaloid™, there are several differences in their manufacturing protocol. Emzaloid™ possess essentially the same components but with the following exceptions:

- Emzaloid™ is gassed with nitrous oxide at 80kPa for 4 hours whereas Pheroid® are gassed at 200 kPa overnight,
- A difference in components ratios exist and
- All Pheroid® formulations contain α-tocopherol (Grobler, 2009).

The components of the Pheroid® technology will be discussed in the subsequent section.

2.10.2 Pheroid® components

2.10.2.1 Essential fatty acid component

Pheroid® is a unique colloidal system consisting primarily of ethylated and pegylated polyunsaturated or esters of essential fatty acids (Grobler, 2009); dl-α-tocopherol, vitamin F ethyl ester and Kolliphor® EL. Essential fatty acids are generally grouped as vitamin F; consisting of oleic, linoleic and alpha-linolenic acid. These fatty acids are not synthesised by the human body and are essential for various cell functions (Grobler, 2014). They serve as the lipid building blocks for the Pheroid®. Kolliphor® EL is a registered trade name for BASF Corp and is a version of polyethoxylated castor oil. It is utilised as a non-ionic surfactant that stabilises the lipid vesicles in the aqueous phase Furthermore, dl, α-tocopherol is an antioxidant which assists in the prevention of peroxyl radicals *in vivo* which may cause damage to DNA, lipids, proteins and other bio-molecules (Halliwell, 1996), thus giving the Pheroid® membrane an additional function as a peroxy radical scavenger.

2.10.2.2 Nitrous oxide component

Sterile water is saturated with nitrous oxide (N_2O). For Pheroid® formation, N_2O contributes to fatty acid dissolution, self-assembly of vesicles and overall stability. Furthermore, molecular modelling has shown possible interactions of N_2O and fatty acids to form a functional model that assists in the transportation of hydrophilic and hydrophobic active ingredients (Grobler, 2009).

2.10.2.3 Pheroid® types

Owing to its simple preparation and dynamic constituents, the Pheroid® can be easily manipulated in terms of its size, structure, morphology and overall nature. This manipulation depends on the intended application and the following factors of delivery including absorption, distribution, release mechanism, metabolism and clearance. Manipulation is achieved by; alteration in fatty acid composition, addition of charge inducing agents as well as changing the aqueous medium (pH, ionic strength, etc). There are three classifications of Pheroid®; vesicles, microsponges and pro-Pheroid®.The pro-Pheroid® constitutes the ‘oil’ phase of the system and upon the addition of the ‘water phase’ (added externally or bodily fluids) a Pheroid® is formed. Depending on the ratio of the oil constituents, either vesicles (a lipid bilayer) or sponges (a depot of active sites) are formed. Figure 2.1.A depicts vesicles with fluorescent actives whereas 2.1.B depicts sponges with many depots for drug encapsulation (Grobler, 2009).

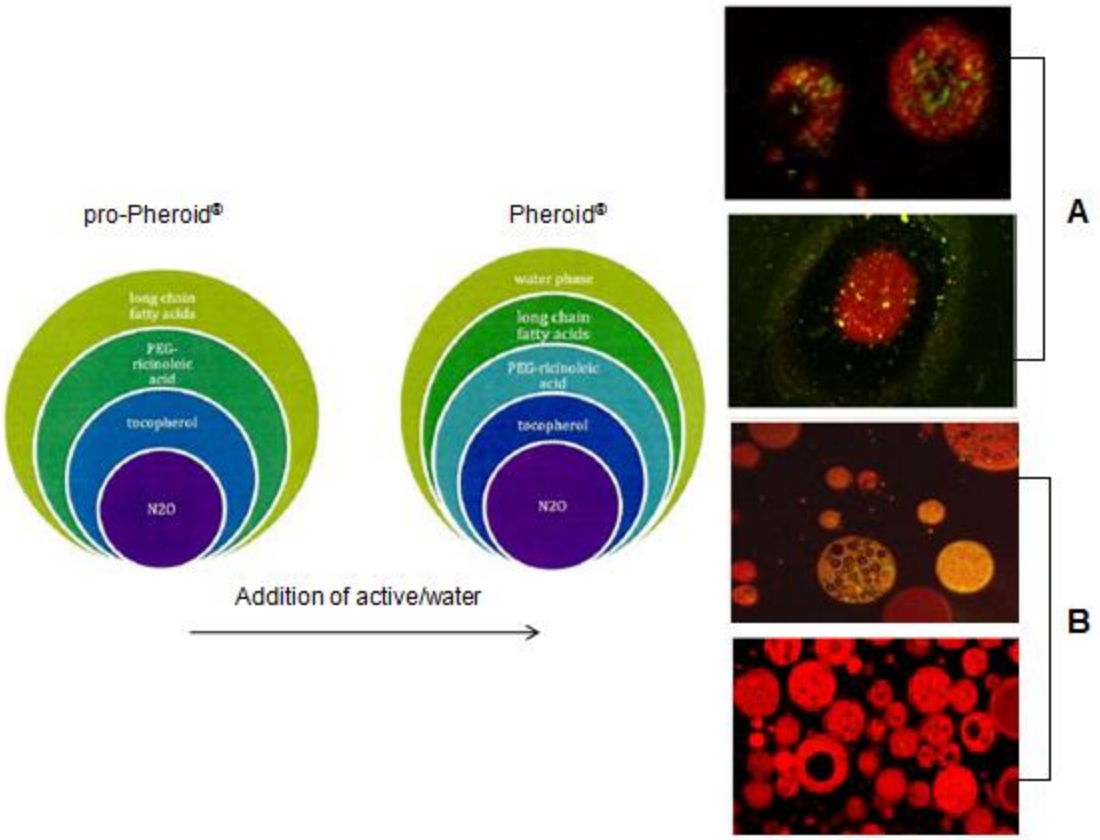


Figure 2.1: Proposed schematic of Pheroid® vesicle layers and confocal images of vesicles (A) and sponges (B) (reprinted from permission from the author), (Grobler, 2009).

2.10.3 Cellular uptake of Pheroid®

The mechanisms for cellular uptake of Pheroid® are still speculative. However, literature suggests that a similarity between the uptake of essential fatty acids (EFA) and Pheroid® exists. The hydrophobic nature of fatty acids contributes to its insolubility in aqueous environments, thus more specific trafficking mechanisms are required for its delivery. Preliminary evidence suggests that Pheroid® uptake is facilitated by acid membrane binding proteins generally present within lipid rafts in the cell membrane and this uptake is a function of the shape, size, geometry and fatty acid ratios of Pheroid® (Grobler, 2009).

2.10.4 Similarities and difference between Pheroid® and lipid-based delivery systems

Table 2.3 below depicts the similarities and differences between Pheroid® and other delivery systems. The Pheroid® delivery system provides more advantages to that of other lipid based systems. The essential fatty acid composition makes this delivery system unique and ideal. A delivery system that displays enhanced membrane penetration, low cytotoxicity, enhanced

bioavailability and high encapsulation efficiency could alleviate all the problems posed by conventional therapy.

Table 2.2: Similarities and difference between Pheroid® and lipid-based delivery systems (Adapted from Grobler, 2009; Uys, 2006).

Pheroid®	**Lipid-based delivery systems**
Consists mainly of essential fatty acids that are needed by the body.	Contains a proportion of foreign substances to the body e.g. artificial polymers
An affinity exists between the Pheroid® and cell membrane to ensure penetration and delivery.	Binding and uptake mechanisms have not been described for most lipid-based delivery systems.
Low cytotoxicity is observed, since the essential fatty acids are part of the natural biochemical pathways.	Cytotoxicity and impaired cell integrity are common problems with foreign substances in the body.
Pheroid® is polyphilic in nature and is capable of entrapping actives which of hydrophilic or hydrophobic in nature.	Most delivery systems are either lipophilic or hydrophilic.
Pheroid® enhances the bioavailability of orally or topically administered entrapped actives.	Liposomal delivery systems have shown to enhance absorption across biological barriers.
Entrapment efficiency has shown to be in the range of 85-100%.	Charge and steric hindrance of delivery systems may cause low entrapment efficiency.
Pheroid® may exist in micro-sponges which are ideal for combination therapy as some actives may entrap in the interior volume and others in the sponge spaces.	Combination therapy has been problematic for most delivery systems.

When the Pheroid® system is compared to other delivery systems they appear to be the superior delivery system.

To date, there is little to none literature available exploiting the advantages of both polymeric nanoparticles and Pheroid® delivery systems. Owing to the vast amount of similarities between Pheroid® and lipid based systems a brief overview of lipid-polymer based hybrid nanoparticles will be discussed, as this forms the basis of the combined delivery system we aimed to achieve.

2.11 Lipid-polymer hybrid nanoparticles

To address and overcome the limitations presented by liposome and polymeric carriers as well as to exploit the advantages of these single delivery systems, recent efforts are being expanded to merge the two delivery systems; giving rise to a dual delivery vehicle termed lipid-polymer hybrid nanoparticles (LPNs) (Zhang *et al.,* 2008).

LPNs would possess a dual advantage, combining the advantages of both polymeric NPs and liposomes. A hybrid delivery system could essentially lead to a system with high biocompatibility, stability and favourable pharmacokinetic profiles (Hadinoto *et al.,* 2013). An increase in drug loading and encapsulation is easily achievable with the encapsulation of hydrophilic and hydrophobic therapeutic actives in the polymeric core/matrix or in between the lipid layers of the liposome (Fang *et al.,* 2014).

There are three main characteristics that contribute to this new generation of delivery systems as shown in Figure 2.4; 1) a polymeric core that can encapsulate hydrophilic drugs and release it at a sustained rate, 2) a lipid shell coating the polymeric core which assists in the prevention of drug diffusion and water penetration and 3) a stealth coating to assist in the evasion of the LPN by the immunogenic response (Zhang & Zhang, 2010).

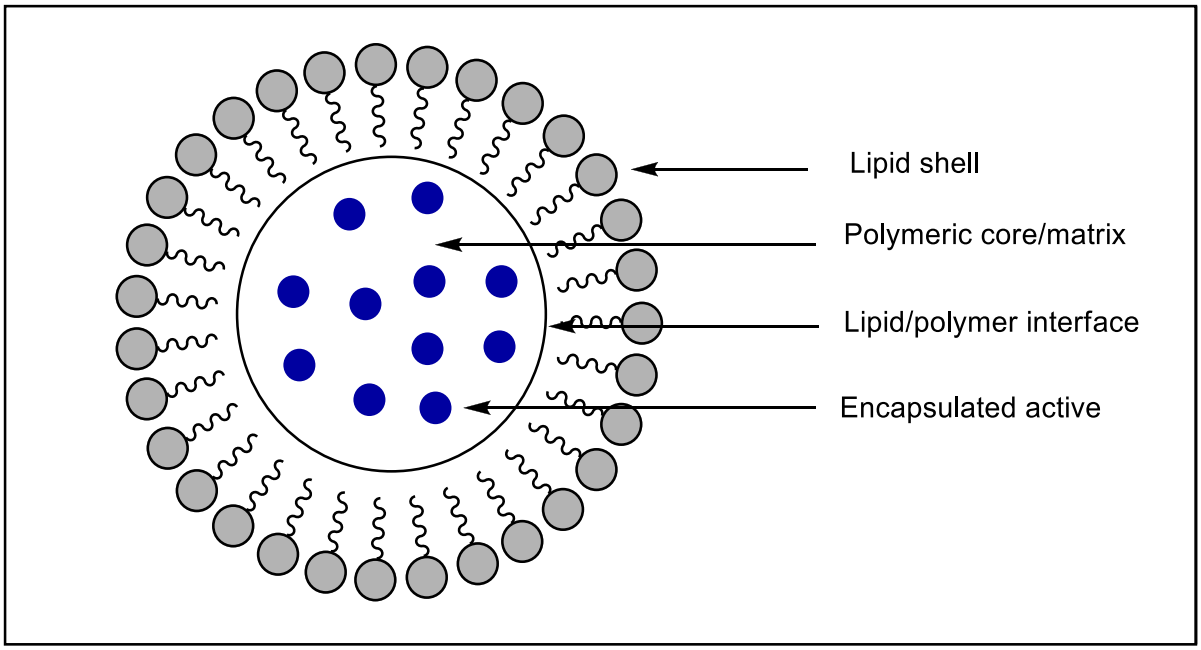


Figure 2.2: Proposed structure of lipid-polymer nanoparticle with an inner polymeric core and an outer lipid shell (Adapted from Zhang and Zhang, 2010; Image drawn with ChemBioDraw 11.0 Software)

The polymer core and lipid shell are associated with each other by either hydrophobic interactions, non-covalent interactions, van der Waals forces or electrostatic interactions (Zhang & Zhang 2010).

2.11.1 Conditions for the formation of the hybrid vesicle

The stability of the hybrid suspensions is important as it directly affects the physiological behaviour of the particles and in turn will determine its application (Carolina *et al.,* 2012). The stability of these hybrid systems can be achieved by controlling the chemical composition and size of the hydrophobic segments between the polymers and lipids as well as the chemical composition of the nanoparticles (Le Meins *et al.,* 2013; Hall *et al.,* 2007). The thermodynamic incompatibility owing to the entropic and enthalpic differences between the polymer and lipid blocks may result in a phase separation in the hybrid suspension (Le Meins *et al.,* 2013).

2.11.2 Preparation of lipid-polymer hybrids

There are two methods in which hybrid nanoparticles can be prepared. The first method (Figure 2.3, a) involves separate preparations of polymeric nanoparticles and lipid vesicles followed by their co-incubation with desirable molar ratios to achieve hybrid nanoparticles by needle extrusion, high-speed homogenization or vortexing (Zhang *et al.,* 2008).

The lipid layer is formed on the polymeric surface by favourable electrostatic interaction between the two systems (Cheow *et al.,* 2011).

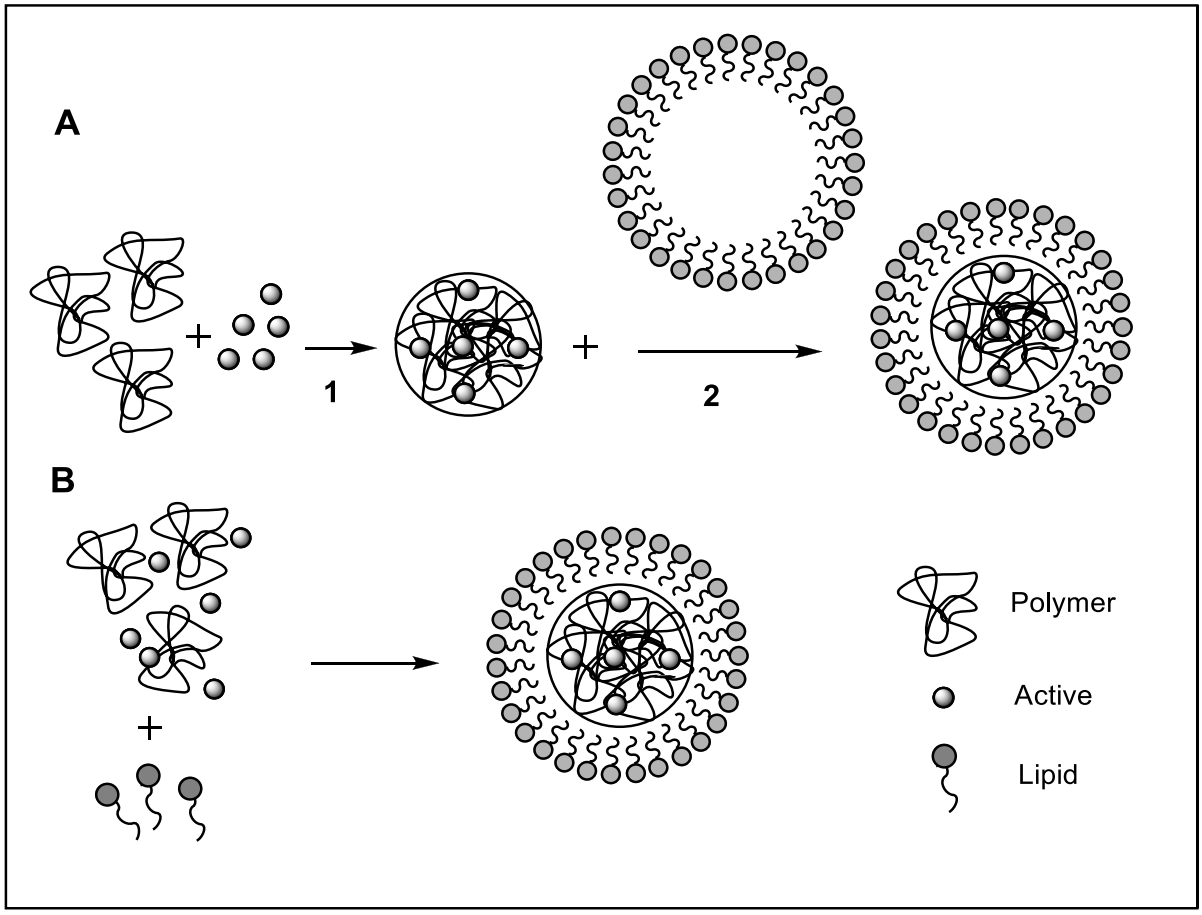


Figure 2.3: Schematic illustrations of lipid-polymer hybrid nanoparticles, a) two-step synthesis for LPN and b) one-step synthesis (Adapted from Zhang & Zhang, 2010)

The second method (Figure 2.3, b) is a single method, which involves the dissolution of the free polymer and hydrophobic drug into a water-miscible solvent and addition to a lipid aqueous medium under agitation. The formation of the NP is achieved by the diffusion of the water-miscible solvent into the lipid aqueous phase resulting in the co-precipitation of the polymer and drug into NPs onto which the lipids self-assemble owing to hydrophobic interactions (Fang *et al.,* 2014). This method is limited as it is only possible for hydrophobic drugs that solubilise in water-miscible solvents.

2.11.3 Applications of lipid-polymer hybrid delivery systems

Wong and co-workers (2006) have designed a LPN system of doxorubin to assist in the treatment of multidrug resistant human breast cancer. The team utilised hydrolysed polymer of epoxidized soybean oil (HPESO) as the polymer component with stearic acid as the lipid

component. The results revealed that the hybrid system resulted in an 8-fold increase in cytotoxicity when compared to free doxorubin. Despite the high cytotoxicity the cellular uptake and retention of doxorubin by multidrug resistant cells was enhanced significantly. It was further concluded that the polymer-lipid hybrid system was essential for the effective delivery of doxorubin (Wong *et al.,* 2006).

Zhang and co-workers (2008) have reported a novel lipid LPN as a robust drug delivery platform. PLGA was utilised as a model hydrophobic polymer to form the polymeric core and polyethylene glycol (PEG) covalently bonded to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) was used as a stealth coat. The lipid monolayer at the interface of the PLGA shell was achieved with lecithin. The results indicated that a hybrid NP with high drug loading, sustained drug release, good serum stability and good cellular targeting ability was developed (Zhang *et al.,* 2008).

Owing to the essential fatty acid component of the Pheroid® and all its advantages listed above (Table 2.2), i.e. enhanced membrane penetration, low cytotoxicity, enhanced bioavailability and high encapsulation efficiency, it was easily proposed that Pheroid® could be utilised as the lipid component in lipid-polymer hybrid systems. These can be prepared similarly to the method above in 2.5.2 with separate preparation of polymeric and lipid systems followed by co-incubation.

2.12 Important physico-chemical characteristics of nanoparticles

2.12.1 Particle size, size distribution and shape

Particle size, size distribution and shape are important characteristics as they may influence the *in vivo* fate of the particles, i.e. circulation time, cellular response and transportation through the blood capillaries. The opsonisation and cellular response from macrophages is strongly influenced by size and surface properties (He *et al.,* 2010).

There are three probable main uptake mechanisms of particles through gastrointestinal and physiological barriers that have been identified:

- Paracellular uptake (particles < 50 nm) – particles ‘kneading’ between epithelial cells owing to their extremely small sizes,
- Endocytotic uptake (50 nm < NP size < 500 nm) - particles absorbed through endocytosis by intestinal enterocytes and
- Lymphatic uptake (500 nm < NP size < 5 µm) – particles are absorbed by M cells of the Peyer’s patches (Florence *et al.,* 1995; Win & Feng, 2005).

Nanoparticles loaded with drugs can be administered intravenously or through oral administration. It was reported that polymeric NPs of more than 500 nm can cross the M cells of the Peyer's patches and easily taken by the lymphatic system thus resulting in an improvement in bioavailability (Kulkarni & Feng, 2013).

Apart from influencing cellular uptake of particulates, the size of the NPs plays a key role in the overall stability of the system, with smaller particles presenting a greater risk for aggregation during storage, transportation and dispersion (Singh & Lillard, 2009).

It is equally important to evaluate the effects of the size and shape of the NPs on cells simultaneously as it may have direct implications on the cytotoxicity of the nanomaterials, their particle transport characteristics and cell-particle interactions, which may alter drug release kinetics (Nel *et al.,* 2009). The shape influences the *in vivo* membrane uptake during endocytosis or phagocytosis (Verma & Stellaci, 2009). Champion and Mitragotri (2006) have shown that spherical particles had a greater cellular uptake when compared to that of rod-shaped particles and less toxic irrespective of their homo/heterogenicity (Lee *et al.,* 2007). This may be explained by the difference in particle curvature, with a greater membrane contact time required for the elongated particles (Verma & Stellaci, 2009).

In addition to the size and shape a small and narrow size distribution is crucial for *in vitro* and *in vivo* investigations. These characteristics determine the biological fate, toxicity, target ability and distribution of particles. In addition, physico-chemical properties such as drug loading, drug release profile and stability are affected to a greater extent (Panyam & Labhasetwar, 2003).

2.12.2 Surface properties

Surface charge and surface functionality of NPs are important physicochemical characteristics that determine the cellular uptake of particles by cells. Previous research has shown that polystyrene microparticles with a primary amine on the surface underwent a greater deal of phagocytosis compared to particles with sulphate, hydroxyl and carboxyl groups. It therefore suggests that neutral and positively charged particles have a higher cellular uptake rate but with a short blood circulation half-life. The design of surface functionalisation is a crucial factor to be considered when developing short or long circulatory NPs, depending on the application (Alexis *et al.,* 2008).

A study by Kim *et al*, (2005) demonstrated that the *in vivo* pharmacokinetics and bio-distribution profile of a colloidal delivery system is on their physiochemical properties which include size and surface. The majority of investigations evaluate size and shape concurrently to determine its effect on cytotoxicity, particle transport, cell-particle interactions and drug release kinetics of the NPs (Dunne *et al.,* 2000; Nel *et al.,* 2009).

Positively and neutral charged particles have demonstrated a higher cellular uptake rate however, this is at the cost of a short blood circulation time. To counter this, emphasis is added to surface modification since it is a crucial parameter in drug delivery design which can enable the achievement of a desired circulation time (Alexis *et al.,* 2008).

2.12.3 Targeting ligand surface functionalisation

Conventional drug delivery is achieved through absorption across barriers, whereas targeted delivery is the delivery of a drug load at a specific site that is diseased. This type of delivery assists with the maintenance of drug concentrations in blood plasma and tissue levels such that healthy cells are unharmed. Targeted drug delivery is preferred over conventional therapy as it prohibits the damage to healthy cells. Furthermore, targeted delivery can be further classified into active or passive delivery (Rani & Paliwal, 2014).

Active targeting is achieved with a cell-specific ligand conjugated to the surface of a delivery system which enables the localisation of an active at a specific target site. Passive targeting involves the incorporation of the agent/ligand into the particle with accumulation of the active at the site of infection based in the delivery systems' physicochemical properties as well as the pharmacological factors of the disease (Singh & Lillard, 2009; Rani & Paliwal, 2014).

There is a vast number of different ligands that can be used for smart targeting of nanoparticles. A few of these ligands include small molecules, antibodies, peptide domains and aptamers (Friedman *et al.,* 2013).

2.12.4 Targeting ligands used for treatment of Tuberculosis

There are various targeting strategies employed to enhance the treatment of Tuberculosis with Nanomedicine.

A few of these strategies include:

- Nanostructured lipid carriers in an optimal aerodynamic diameter range and decorated with mannose to selectively target macrophages. This was developed on the

propensity of macrophages to engulf NPs as well as the receptors for mannose which are highly expressed on the macrophages (Vieira *et al*., 2017).

- Reduction of mycobacteria in macrophages by the interfering with the acquisition of essential nutrients needed for growth. Iron (Fe) is required for the survival of mycobacteria residing macrophages. The ability of Iron to undergo redox cycles between ferrous (Fe2+) and ferric (Fe3+) oxidation states allows it to function as an electron transporter in many enzymatic systems, including those involved in DNA replication. Thus, blocking Fe acquisition by mycobacteria tuberculosis is a potential way reduce the growth of mycobacteria tuberculosis within macrophages. Gallium (GA) is a trivalent cationic element with many features that are similar to Fe, making it largely indistinguishable from Fe to many biologic systems. A study by Narayanasamy and co-workers showed that Ga nanoformulations inhibited the growth of mycobacteria within monocyte-derived macrophages by releasing Ga(III) over 15 days (Narayanasamy *et al.,* 2015).

- The coating of solid lipid nanoparticles with chitosan to improve mucoadhesion and delivery of NPs to pulmonary mucosa and also to improve drug delivery to the alveolar macrophages. Vieira and co-workers, has utilised chitosan to avoid mucociliary clearance from the airways. The chitosan coated SLN has shown higher permeability in alveolar macrophages than uncoated and was shown to be a promising carrier for the management of TB (Vieira *et al*., 2017).

These targeting strategies provide a brief introduction to the various methods employed to assist in the treatment for Tuberculosis.

Nanoparticles can be formulated, modified and functionalised to deliver across biological barriers or target diseased cells directly (Blanco *et al.,* 2015). This study encompasses the use of active targeted drug delivery of CLR and ETB to macrophages with the assistance of mycolic acid (a long chain fatty acid) as a direct targeting ligand. To allow a quick uptake of the nanocarriers through gastro-intestinal track following oral administration, it appeared convenient to incorporate them into Pheroid® vesicles that have demonstrated rapid absorption across physiological barriers (Grobler, 2009).

2.12.5 The use of mycolic acid as a targeting ligand

Previously a study was conducted by Lemmer (2015), which had exploited the cholestroid nature of mycolic acids and implemented it as a targeting ligand in a nano-drug delivery

system for the treatment of tuberculosis. Mycolic acids are long chain fatty acids found in the mycobacterial cell wall envelope and the most dominant lipid in the outer cell wall (Minnikin, 1982). These highly hydrophobic molecules not only play a physical role of protection for the bacteria it has also been shown to play an immunological role towards the host (Dubnau *et al*., 2000; Korf, *et al.,* 2005). Some of these attributes could be taken advantage of to be used as a targeting molecule. The cholesterol mimicking properties of these mycolic acids could be used to target the encapsulated drugs towards the site of infection in infected macrophages by being attracted to cholesterol present in the plasma membrane (Benadie, *et al*., 2008; Beukes *et al*., 2010). Alternatively, because antibodies to mycolic acids are found in infected individuals the mycolic acids containing capsules could form complexes with anti-MA antibodies in the vicinity of the sites of infection to cause a localized immune complex that may enhance uptake of the encapsulated drugs (Pan *et al*., 1999). Since MAC also colonize macrophages similar to the pathogenic mycobacteria the assumption is made that the same targeting principles can also be applied in this case (Inderlied, *et al*., 1993). Therefore, this study applied the same logic and exploitation of mycolic acid for the treatment of MAC.

2.12.6 Drug determination in delivery systems

There are two terms that are commonly used in drug delivery in relation to the drug content present in the NP, i.e. the encapsulation efficiency (EE) and the drug loading (DL). The EE refers to the percentage of drug encapsulated into the polymer shell or embedded in the polymer matrix.

$$Encapsulation\ Efficiency\ EE\ \% = \frac{Mass\ of\ total\ drug - Mass\ of\ free\ drug}{Mass\ of\ total\ drug}\ x\ 100$$

The EE can further be calculated using a direct and indirect method where the direct method is the quantification of a drug encapsulated into the particle and the indirect is the quantification of the drug that was not encapsulated into the particle. A direct method encompasses the dissolution of the NP with the encapsulated drug in an appropriate solvent system determined by the nature of the particle as well as the chemical nature of the drug. The concentration of the drug is measured via an analytical method most suited for the system. Whereas with an indirect method, the free drug remaining in supernatant after the particle collection step (centrifugation) will be measured. In both cases the EE is expressed as a percentage of the drug in the formed NP with respect to the initial amount of drug used in the preparation of the particles.

A high drug loading (DL) is preferable for the successful administration of the nano-drug delivery system. The DL is the ability of the NP system to encapsulate a certain active and is expressed as the weight of the drug in the NP in relation to the weight of the NPs expressed as a percentage.

$$DL = \frac{Mass\ of\ drug\ bound}{Total\ mass\ of\ formulation}$$

Unfortunately, most drug delivery systems have a low drug loading. This is mainly attributed to the chemical nature of the actives as well as their possible interactions with the carrier vehicle (Shenoy & Amiji, 2005). Therefore, a large quantity of the drug delivery systems needs to be administrated to ensure the deliverance of a relevant dose. Thus, it is important to design a system that can offer the potential of high DL to ensure that a lower dose of the drug delivery system can be administered to ensure efficient delivery of active as well as in turn lowering the cost of treatment (Rocca *et al.,* 2012).

For this current study, only the EE was determined by means of the indirect method with the use of Liquid Chromatography Mass Spectrometry (LCMS) as the analytic quantification method.

2.12.7 Drug release profile of drug delivery systems

The drug release profile is important for the proposed application. Understanding of the release mechanism of these systems assists in the smart manipulation of the formulation parameters such that desirable release profiles are achieved. The release mechanisms and profile are dependent on the physical properties of the NPs and furthermore chemical properties of the materials used. The rate of release of drug from the delivery system is usually determined by the solubility of the drug itself, the desorption of the drug on the bound surface, the diffusion process through the polymer matrix/wall of nano-capsule and the erosion of the NP matrix (Mahapatro & Singh, 2011).

The drug release kinetics depends on the size and DL. Larger particles with a higher DL have shown a small initial burst and release rate. When the drug is uniformly distributed through the polymer, the release occurs through erosion or diffusion (Kumari *et al.,* 2010) which assists in a sustained release profile. Therefore, it is of utmost importance to understand the chemical properties of the system as well as the initial components in order to achieve the desired release characteristics.

2.12.8 Cytotoxicity profile of drug delivery systems

It is important to understand the behaviour and reaction of an active in the body. To do this, cell culture studies are recommended before the commencement of animal studies. It is essential for cell culture studies to be well monitored as cells are sensitive to minute changes in the environment. Therefore, closely monitored studies would ensure that any cell death can be attributed to the tested compound.

It is crucial that a cytotoxicity assay is applicable to the application. The most common representation of cytotoxicity investigations is the measure of cell death through colorimetric methods which measure plasma membrane integrity and/or mitochondrial activity. There are several ways in determining cell viability after exposure to test compounds, which include:

- Neutral red: It is a spectrophotometric test in which cell cultures are incubated with neutral red (toluylene red). Live cells metabolise this dye, therefore a higher cellular uptake is representative of a higher cellular viability (Lewinski *et al.,* 2008).
- Trypan blue assay: Optical microscopy is used to quantify cellular viability. Trypan blue is a diazo dye that is permeable to damaged cellular membranes, therefore this type of dye is representative of the dead cells (Lewinski *et al.,* 2008).
- Tetrazolium reduction assay: There are variety of compounds that assist in the detection of viable cells which include MTT, MTS, XTT, and WST-1 which can be further classified into two categories of positively and negatively charged that influences cellular penetration (Lewinski *et al.,* 2008). This test assesses the cell viability by means of the measurement of mitochondrial activity, achieved by mitochondrial dehydrogenase enzymes which are only found in living cells and are capable of cleavage of the tetrazolium ring from tetrazolium salts (Lewinski *et al.,* 2008).
- Lactate dehydrogenase (LDH) release monitoring: LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into the extracellular space when the plasma membrane is compromised (Chan *et al.,* 2013). This method utilizes standard spectroscopy to quantify the formation a tetrazolium salt which is converted to a coloured formazan product by nicotinamide adenine dinucleotide (NADH) after the oxidation of lactate and NAD+ from pyruvate in the presence of the LDH enzyme (Fornaguera & Solans, 2017).
- A live/dead viability test: Is the measure of the cell membrane integrity and permeability which use of a two-colour fluorescence assay. This includes

calceinacetoxymethyl (Calcein AM) and ethidium homodimer which assist in quantification of interrupted membrane activity (Wang *et al.,* 2013).

A cytotoxicity profile for any investigation is of utmost importance as it can shed light to the potential behaviour of delivery systems *in vivo* and whether the carrier vehicle could hold any advantage in reducing the cytotoxicity of the therapeutic molecule. Therefore, to determine the cytotoxicity of the NP delivery systems developed in this study, a WST-1 tetrazolium based assay was conducted. There are some shortfalls that are associated with this assay, which include the lack of sensitivity owing to the absorbance detection method but can be enhanced by determining the optimum reagent concentration and incubation time. However, there is a degree of toxicity that the reagent exhibits onto the cells. Despite these disadvantages, this assay was chosen as it is widely used in literature, quick and inexpensive (Riss, 2017). This assay will provide a general idea of the cytotoxicity of the particles developed in this study, however to draw solid conclusions it will be best to couple this assay with a cell apoptosis assay which was out of the scope of this preliminary study.

2.13 Conclusion

A treatment regime exists for MAC but not without its own disadvantages. To date no optimum and uniform treatment exist, however CLR together with ETB in combination has shown to be effective (Miwa *et al.,* 2014).

Nanomedicine with specifically drug delivery offer great advantages over conventional treatment, where there is intracellular delivery, sustained drug release and the possible toxicity reduction while maintaining therapeutic effects (De Jong & Borm, 2008). There are many different types of delivery systems that can offer these advantages, but for this investigation, polymeric nanoparticles, particularly PLGA together with liposomes and Pheroid® delivery systems were reviewed in hope to develop a polymeric-Pheroid® hybrid delivery system that offers the same enhanced advantages of LPN systems.

PLGA NPs offer advantages such as easy surface modification (Singh *et al.,* 2010), drug integrity preservation (Dadwal, 2014) and a sustained release profile (Parveen *et al.,* 2012). The system we propose to develop will be easily modified, by the expression of a novel targeting ligand, MA, expressed on its surface to potentially enhance PLGA phagocytic uptake by macrophages.

With the development of a hybrid system in mind, the Pheroid® delivery system together with its differences between conventional lipids systems were reviewed, as little to none literature exist on the development of a hybrid system with Pheroid® technology. The Pheroid® delivery

system is mainly composed of essential fatty acids and offers advantages such as; enhanced membrane penetration and low cytotoxicity (Grobler, 2009). With this in mind, we hope to observe these advantages together the advantages of PLGA NPs in the proposed hybrid system and in turn improve the treatment for MAC.

References

Agarwal, R., Iezhitsa, I., Agarwal, P., Nasir, N.A.A., N., Alyautdin, R. & Ismail, N.M. 2016. Liposomes in topical ophthalmic delivery: an update. *Drug Delivery*, 4:1075-1091.

Alexis, F., Pridgen, E., Molnar, L.K., & Farokhzad, O.C., 2008. Factors affecting the clearance & biodistribution of polymeric nanoparticles. *Molecular Pharmaceutics*, 5(4):505-515.

Akash, M.S.H., Rehman, K. & Chen, S. 2015. Natural and Synthetic polymers as drug carriers for delivery of therapeutic proteins. *Polymer Reviews,* 55(3):371-406.

Antimicrobe n.d. *Ethambutol (EMB. Available from:* Http://www.antimicrobe.org/drugpopup/Ethambutol.htm Date of access: 04 September 2017].

Blanco, E., Shen, H. & Ferrari, M. 2015. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nature Biotechnology,* 33:941-951.

Benadie, Y., Deysel, M., Siko, DGR., Robert, V., van Wyngaardt, S.H., Thanyani, S.T., Sekanka Gm Ten Bokum, A.M.C., Collett, L.A., Baird, M.S. & Verschoor, J.A., 2008. Cholestroid nature of free mycolic acids from M. *Tuberculosis*. *Chemistry and Physics of Lipids,* 152:94-103.

Beukes, M., Lemmer, Y., Deysel, M., Al Dulayymi, J.R., Baird, M.S., Koza, G., Iglesias, M., Rowles, R.R., Theunissen, C., Grooten, J., Toschi, G., Roberts, V.V., Pilcher, L., Van Wyngaardt, S., Mathebula, N., Bolgun, M., Stoltz, A.C.& Vershoor, J.A. 2010. Structure-function relationships of the antigenicity of mycolic acids in tuberculosis patients. *Chemistry and Physics of Lipids,* 163(8):800-808.

Chan, F., Moriwaki, K. & De Rose, M.J. 2014. Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) activity. *Methods in Molecular Biology,* 979:65-70.

Butun, S., Ince, F.G., Erdugan, H. & Sahiner, N. 2011. One-step fabrication of biocompatible carboxymethyl cellulose polymeric particles for drug delivery systems. *Carbohydrate Polymers,* 86:636-643.

Carolina, S.M., Pedro, M.V., Thakkar, A.B., Swanson, E. & Langer, R. 2012. Recent developments in multifunctional hybrid nanoparticles: opportunities and challenges in cancer therapy. *Frontiers in Bioscience*, 4:529-545.

Champion, A. & Mitragotri, S. 2006. Role of target geometry in phagocytosis*. Proceedings of National Academy Sciences of the United States of America,* 103(13): 4930-4934.

Cheow, W.S.& Hadinoto, K. 2011. Factors affecting drug encapsulation and stability of lipid-polymer hybrid nanoparticles. *Colloids and Surfaces B: Biointerfaces,* 85:214-220.

Chu, S.Y. Deaton, R., Cavanaugh, J. 1992. Absolute bioavailability of clarithromycin after oral administration in humans. Antomicrob. Agents. Chemother. 36(5): 1147-1150.

de Jesus, M.B.& Zuhorn, I.S. 2015. Solid Lipid Nanoparticles as a nucleic delivery system: Properties and molecular mechanisms. Journal of Controlled Release, 201:1-13.

Dubnau, E., Chan, J., Raynaud, C., Mohan, V.P., Laneelle, M.A., Yu, A.,Quemard, A., Smith, I. & Daffe, M. 2000. Oxygenated mycolic acids are necessary for virulence of Mycobacterium tuberculosis in mice, *Molecular Microbiology*, 36:630-637.

Dadwal, M. 2014. Polymeric nanoparticles as promising novel carriers for drug delivery: An Overview. *Journal of Advanced Pharmacy Education and Research,* 4(1):20-30.

De Jong, W.H. & Borm, P.J.A. 2008. Drug delivery and nanoparticles: Applications and hazards. *International Journal of Nanomedicine,* 3(2):133-149.

Dunne, M., Fessel, J.& Kumar, P. 2000. A randomized, doubleblind trial comparing azithromycin and clarithromycinin the treatment of disseminated Mycobacterium avium infection in patients with HIV. *Clinics of Infectious Diseases,* 31:1245–52.

Eatemadi, A., Dararee, H., Karimkhanloo, H., Kouhi, M., Zarghami, N., Akbarzadeh, A., Abasi, M., Hanifehpour, Y. & Joo, S.W. 2014. Carbon nanotubes: properties, synthesis, purification, and medical applications. *Nanoscale Research Letters,* 9(1):393.

Fang, D.L., Chen, Y., Xu, B., Ren, K., He, Z.Y., He, L.L., Lei, Y., Fan, C.M. & Song, X.R. 2014. Development of the lipid-shell and polymer core nanoparticles in water-soluble salidroside for anti-cancer therapy. *International Journal of Molecular Science,* 15:3373-3388.

Faraji, A.H. & Wipf, P. 2009. Nanoparticles in cellular drug delivery. *Bioorganic & Medicinal Chemistry*, 17(8):2950-2962.

Florence, A.T., Hillery, A.M., Hussain, N. & Jani, P.U. 1995. Nanoparticles as carriers for oral peptide absorption: studies on particle uptake and fate. Journal of *Controlled Release,* 6:39–46.

Formaguera, C. & Solans, C. 2017. Methods for the In Vitro Characterization of Nanomedicines—Biological Component Interaction. *Journal of Personalized Medicine, 7*(1).

Friedman, A.D., Claypool, S.E. & Lui, R. 2013. The Smart Targeting of Nanoparticles. *Current Pharmaceutical Design,* 19(35):6315-6329.

Fuhrmann, G., Serio, A., Mazo, M., Nair, R. & Stevens, M. 2015. Active loading of extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *Journal of Controlled Release,* 205: 35-44.

Gatfield, J., & Pieters, J. 2000. Essential role for cholesterol in entry of mycobacteria into macrophages, Science, 288(5471):1647-1650.

Gavasane, J. & Pawar, H.A. 2014. Synthetic Biodegradable Polymers Used in Controlled Drug Delivery System: An Overview. *Clinical Pharmacology & Biopharmaceutics*, 3(2):1-7.

Griffith, D.E., Aksamit, T., Brwon-Elliot, B.A., Catanzaro, A., Daley, C., Gordin, F., Holland, S.M. Horsburgh, R., Huitt, G. & Lademarco, M.F. 2007. Mycobacterial Diseases Subcommittee; American Thoracic Society; Infectious Diseases Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *American Journal of Respiratory and Critical Care Medicine,* 175: 744-745.

Grobler, A.F. 2009. Pharmaceutical applications of pheroid™ technology. Potchefstroom: NWU. (Thesis – PhD).

Grobler, L., Grobler, A., Haynes, R., Masimireembwa, C., Thelingwani, R., Steenkamp, P. & Steyn, H.S. 2014. The effect of the Pheroid delivery system on the in vitro metabolism and in vivo pharmacokinetics of artemisone. *Expert Opinion on Drug Metabolism & Toxicology*, 10(3): 313-325.

Hadinoto, K., Sundaresan, A. & Cheow, W.S. 2013. Lipid-polymer hybrid nanoparticles as a new generation therapeutic delivery platform: A review*. European Journal of Pharmaceutics and Biopharmaceutics,* 85:427-443.

Hall, J.B., Dobrovolskaia, M.A., Patri, A.K. & McNeil, S.E. 2007. Characterisation of nanoparticles for therapeutics. *Nanomedicine*, 2(6):789-803.

Halliwell B. 1996. Antioxidants in human health and disease. *Annual Review of Nutrition,* 16:33–50.

He, C., Hu, Y., Yin, L., Tang, C.& Yin, C. 2010. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials.* 31:3657-3666.

Ina M. 2011. Dendrimer: A Novel drug delivery system. *Journal of Drug Delivery & Therapeutics,* 1(2):70-74.

Inderlied, C.B., Kemper, C.A. & Bermudez, L.E.M. 1993. The Mycobacterium avium complex. *Clinical Microbiology Reviews*, 266-310.

Jawahar, N. & Meyyanathan, S.N. 2012. Polymeric nanoparticles for drug delivery and targeting: A comprehensive Review. *International Journal of Health & Allied Sciences*, 1(4): 217-223.

Junyaprasert, V.B. & Morakul, B. 2015. Nanocrystals for enhancement of oral bioavailability of poorly water-soluble drugs. *Asian Journal of Pharmaceutical Sciences*, 10(1):13-23.

Kalombo, L., 2011. Nanoparticle carriers for drug administration and process for producing the same. (Patent: US 0033550A1). http://www.google.com/patents/US20110033550 Date of access: 28 September 2017.

Kerimoğlu, O. & Alarçin, E. 2012. Poly(lactic-co-glycolic acid) based drug delivery devices for tissue engineering and regenerative medicine. *Ankem Derg*, 26(2):86-98.

Kim, S.H., Jeong, J.H., Chun, K.W. & Park, T.G. 2005. Target-specific cellular uptake of PLGA nanoparticles coated with poly (l-lysine) poly (ethylene glycol)-folate conjugate. *Langmuir*, 21(19):8852-8857.

Kim, E.Y., Chi, S.Y., Oh, I.J., Kim, K.S., Kim, Y. Lim, S.C., Kim, Y.C. & Kwon, Y.S. 2011. Treatment outcome of Combination Therapy including Clarithromycin for Mycobacterium avium Complex Pulmonary Disease. Korean. *Journal of Internal Medicine,* 26(1): 54-59.

Kotke, M.J. & Edward, M.R. 2002. Tablet Dosage Forms IN: Banker GS. Rhodes CT Modern Pharmaceutics. Marcel Dekker, Inc, New York. pp:287-333.

Kratz, J. 2008. Albumin as a drug carrier: Design of prodrugs, drug conjugates and nanoparticles*. Journal of Controlled Release,* 132 (3):171-183.

Krishna, R.S.M., Shivakumar, H.G., Gowda, D.V. & Banerjee, S. 2006. Nanoparticles-a novel colloidal drug delivery system*. Indian Journal of Pharmaceutical Education and Research*, 40:15-21.

Korf, J., Stoltz, A., Verschoor, P. & De Baetselier, J. 2005. Grooten, The Mycobacterium tuberculosis cell wall component mycolic acid elicits pathogen-associated host innate immune responses, *European Journal of Immunology,* 35:890-900.

Kulkami, S.A. & Feng, S.S. 2013. Effects of Particle Size and Surface Modification on Cellular Uptake and Biodistribution of Polymeric Nanoparticles for Drug Delivery. *Pharmaceutical Research*, 30:2512-2522.

Kumari, A., Yadev, S.K. & Yadav, SC. 2010. Biodegradable polymeric nanoparticles based deliver systems. *Colloids and Surfaces B: Biointerfaces,* 75:1-18.

Lee, B.Y., Kim, S., Hong, Y., Lee, S.D., Kim, W.S. & Kim, D.S. 2015. Risk factors for recurrence after successful treatment of *Mycobacterium avium* complex lung disease. *Antimicrobial Agents & Chemotherapy,* 59:2972–7.10.

Lee, M., Lim, S. & Kim, C. 2007. Preparation, characterization and in vitro cytotoxicity of paclitaxel-loaded sterically stabilized solid lipid nanoparticles. *Biomaterials.* 28(12):2137-2146.

Le Meins, J.F., Schatz, C., Lecommandoux, S. & Sabdre, O. 2013. Hybrid polymer/lipid vesicles: State of the art and future prospectives. *Materials Today,* 16:397.

Lemmer, Y., Kalombo, L., Pietersen, R.D., Jones, A.T., Semete-Makokotlela, B., Van Wyngaardt, S., Ramalapa, B., Stoltz, A.C., Baker, B., Verschoor, J.A., Swai, H.S. & De Chastellier, C. 2015. Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis. *Journal of Controlled Release*, 211:94-104.

Lewinski, N.; Colvin, V. & Drezek, R. 2008. Cytotoxicity of nanoparticles. *Small*. *4*: 26–49.

Maham, A., Tang, Z., Wu, H., Wang, J. & Lin, Y. 2009. Protein-based Nanomedicine Platforms for Drug Delivery. *Small,* 5(15):1706 – 21.

Mahaptro, A. & Singh, D.K. 2011. Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines. *Journal of Nanobiotechnology,* 9-55.

Malodia, K., Singh, S.K., Mishra, D.N. & Shrivastava, B. 2012. Nanoparticles: An advance technique for drug delivery. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 3(3):1186-1208.

Miner, M.D., Chang, J.C., Pandey, A.K., Sassetti, C.M., Sherman, D.R. 2009. Role of cholesterol in *Mycobacterium tuberculosis* infection, Indian Journal of Experimental Biology, 47:407-411.

Minnikin, D.E. 1982. Lipids: Complex Lipids, their Chemistry, Biosynthesis and Role, Academic Press, London, UK.

Mirakabad, F.S.T., Nejati-Koshki, K., Akbarzadeh, A., Yamchi, M.R., Milani, M., Zarghami, N., Zeighamian, V., Rahimzadeh, A., Alimohammadi, S., Hanifehpour, Y. & Joo, S.W. 2014. PLGA-based nanoparticles as cancer drug delivery systems. *Asian Pacific Journal of Cancer Prevention,* 15(2):517-35.

Miwa, S., Shira, M., Toyoshima, M., Shirai, T., Yasuda, K., Yokomura, K., Yamada, T., Masuda, M., Inui, N., Chida, K., Suda, T. & Hayakawa, H. 2014. Efficacy of Clarithromycin and Ethambutol for Mycobacterium avium Complex Pulmonary Disease. A preliminary Study. *Annals of the American Thoracic Society*, 11(1):23-30.

Moghimi, SM. 2006. Recent developments in polymeric nanoparticle engineering and their applications in experimental and clinical oncology. *Anti-Cancer Agents Medicinal Chemistry*, 6:553-561.

Mohammadi, G., Nokhoschi A, Barzegar-Jalali, Lotipour, F., Adibkia, K., Ehyaei, N. & Valizadeh, H. 2011. Physiochemical and anti-bacterial performance characterization of Clarithromycin nanoparticles as colloidal drug delivery system. *Colloid and Surfaces B: Biointerfaces,* 88(1):39-44.

Motwani, S.K, Chopra, S., Talegaonkar, S., Kanchan, K., Ahmad, F.J. & Khar, R.K. 2008. Chitosan-sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: Formulation, optimisation and in vitro characterisation. *European Journal of Pharmaceutics and Biopharmaceutics*, 68:513-525.

Nanjwade, B., Bechra, H.M., Derkar, G.K., Manvi, F.V. & Nanjwade, V.K. 2009. Dendrimers: Emerging polymers for drug delivery systems. *European Journal of Pharmaceutical Sciences, 38(3): 185-196.*

Nagal, A. & Singla, R.K. 2013. Nanoparticles in Different Delivery Systems: A Brief Review. *Indo Global Journal of Pharmaceutical Sciences*, 3(2): 96-106.

Nagavarma, B.V.N., Yadav, H.K.S., Ayaz, A., Vashudha, L.S. & Shivakumar, H.G. 2012. Different techniques for preparation of polymeric nanoparticls- A Review, A*sian Journal of Pharmaceutical and Clinical Research,* 5(3):16-23.

Narayanasamy, P., Switzer, B.L. & Britigan, B.E. 2015. Prolonged-acting, Multitargeting Gallium Nanoparticles Potently Inhibit Growth of Both HIV and Mycobacteria in co-infected Human Macrophages. *Science Report*, 5:8824.

Nasiruddin, M., Neyaz, K. & Das, S. 2017.Nanotechnology-Based Approach in Tuberculosis Treatment. *Tuberculosis Research and Treatment*, 1-12.

Natarajan, J. & Meyyanathan, S.N. 2012. Polymeric nanoparticles for drug delivery and targeting: A comprehensive review. *International Journal of Health and Allied Sciences,* 1(4): 217-22.

Nel, A.E., Madler, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., Klaessig, F., Castranova, V. & Thompsom, M. 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials*, 8:543-557.

Nischiuchi, Y., Iwamoto, T. & Maruyama, F. 2017. Infection Sources of a Common Non-tuberculosis Mycobacterial Pathogen, Mycobacterium avium Complex. *Front Med (Lausanne)*, 4(27):1-17.

Nitta, S.K. & Numata, K. 2013. Biopolymer-Based Nanoparticles for Drug/Gene delivery and Tissue Engineering. *International Journal of Molecular Sciences,* 14:1629–1654.

Pan, J., Fujuwara, N., Oka, S., Maekura, R., Ogura, T., & Yano, L. 1999. Anti-Cord Factor (Trehalose 6,6'-Dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses, *Microbiology and Immunology*, 43:863–869.

Panyam, J. & Labhasetwar, V. 2003.Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Advanced drug delivery Reviews,* 55:329-347.

Parveen, S., Misra, r. & Sahoo, S.K. 2012. Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine: Nanotechnology, Biology and Medicine,* 8(2):147-166.

Prevots, D. & Marras, T.K. 2015. Epidemiology of human pulmonary infection with nontuberculous mycobacteria: a review. *Clinics in Chest Medicine*, 36:13–34.

Rafiei, P. & Haddadi, A. 2017. Docetaxel-loaded PLGA and PLGA-PEG nanoparticles for intravenous application: pharmacokinetics and biodistribution profile. *International Journal of Nanomedicine,* 13:935-947

Rani, K., Paliwal, S. 2014. A Review of Targeted Drug Delivery: its Entire Focus on Advanced Therapeutics and Diagnostics. *Scholars Journal of Applied Medical Sciences,* 2(1C):328-331.

Riss, T. 2017. Is Your MTT Assay Really the Best Choice? Available from: http://worldwide.promega.com/resources/pubhub/is-your-mtt-assay-really-the-best-choice/. [Date accessed: 10 May 2018].

Rocca, J.D., Liu, D.& Lin, W. 2012. Are high drug loading nanoparticles the next step forward for chemotherapy. *Nanomedicine,* 7(3):303-305.

Safari, J. & Zarnegar, Z. 2014. Advanced Drug Delivery Systems: Nanotechnology of health design A review. Journal of Saudi Chemical Society, 18(2):85-99.

Semete, B., Booysen, L., Kalombo, K., Ramalapa, B., Hayeshi, R. & Swai, H. 2012. Effect of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration. *International Journal of Pharmaceutics,* 424(1-2):115-120.

Shah, N.M., Davidson, J.A., Anderson, L.F., Lalor, M.K., Kim, J. & Thomas, H.L. 2016. Pulmonary *Mycobacterium avium-intracellulare* is the main driver of the rise in non-tuberculous mycobacteria incidence in England, Wales and Northern Ireland, 2007-2012. *BMC Infectious Diseases*, 16:195.

Shapira, A., Livney, Y.D., Broxterman, H.J. & Assaraf, Y.G. 2011. Nanomedicine for targeted cancer therapy: towards the overcoming of drug resistance. *Drug Resistance Update*, 14(3):150-163.

Shenoy, D.B. & Amjii, M. 2005. (Poly)ethylene-modified poly(caprolactone) nanoparticles for targeted delivery of tamoxifen in breast cancer*. International Journal of Pharmaceutics*, 293(1-2):261-270.

Sim, Y.S., Park, H.Y., Jeon, K., Suh, G.Y., Kwon, O.J. & Koh, W.J. 2010. Standardized Combination Antibiotic Treatment of Mycobacterium avium complex Lung Disease. *Yonsei Medical Journal*, 51(6): 888-894.

Singh, R. & Lillard, J.W. 2009. Nanoparticle-based targeted drug delivery. *Experimental and Molecular Pathology*, 86(3):215-223.

Singh, A., Garg, G. & Sharma, P.K. 2010. Nanospheres: a novel approach for targeted drug delivery system. *International Journal of Pharmaceutical Reviews,* 5:84-88.

Steenwinkel, J.E.M., Van Vianen, W., Kate, M.T., Verbrugh, H.A., Agtmael, M.A., Schiffelers, R.M. & Bakker-Woudenberg, I.A.J.M. 2007. Targeted drug delivery to enhance efficacy and shorten treatment duration in disseminated *Mycobacterium avium* infection in mice. *Journal of Antimicrobial Chemotherapy,* 60(5): 1064-1073.

Tortoli E. 2014. Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. *Clinical Microbiology Reviews,* 27:727–752.

Toti, U.S., Guru, B.R., Hali, M., McPharlin, C.M., Wykes, S.M., Panyam, J. & Whittum-Hudson, J.A. 2011. Targeted delivery of antibiotics to intracellular chlamydial infections using PLGA nanoparticles. *Biomaterials,* 32(27): 6606-6613.

Uys, C. 2006. Preparation and characterisation of pheroid vesicles. Potchefstroom: NWU. (Dissertation – MSc).

van Ingen, J., Egelund, E.F., Levin, A., Totten, S.E., Boeree, M.J., Mouton, J.W., Aarnoutse, R.E., Heifets, L.B., Peloquin, C.A. & Daley, C.L. 2012. The pharmacokinetics and pharmacodynamics of pulmonary Mycobacterium avium complex disease treatment. *American Journal of Respiratory and Critical Care Medicine,* 186:559–565.

Ventola, C.L. 2012. The Nanomedicine Revolution. Part 1: *Emerging Concepts.* PT, 37(9): 512-517.

Verma, A. & Stellaci, F. 2010.Effect of surface properties on nanoparticle-cell interactions. *Small,* 6(1):12-21.

Vieira, A.C.C., Chaves, L.L., Pinheiro, S., Pinheiro, M., Lima, S.C., Ferreira, D., Sarmento, B. & Reis, S. 2017. Mucoadhesive chitosan-coated solid lipid nanoparticles for better management of tuberculosis. 536(1):478-485.

Vieira, A.C.C., Magalhaes, J., Rocha, S., Cardoso, M.S., Santos, S.G., Borges, M., Pinheiro, M. & Reis, S. 2017. Targeted macrophages delivery of rifampicin-loaded lipid nanoparticles to improve tuberculosis treatment. Nanomedicine. (12)24:2721-2736.

Vilar, G., Tulla-Puche, J. & Albercio, F. 2012. Polymers and Drug Delivery Systems. *Current Drug Delivery,* 9:367-394.

Wang, C., Zhou, Y., Li, S., Li, H., Tian, L., Wang, H. & Shang, D. 2013. Anticancer mechanisms of temporin-1CEa, an amphipathic α-helical antimicrobial peptide, in Bcap-37 human breast cancer cells. Life Sciences, 92: 1004-1014.

Win, K.Y. & Feng, S.S. 2005. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials,* 26:2713-2722.

Wong, H.L., Rauth, A.M., Bendayan, R.M., Manias, J.L., Ramaswamy, M., Lu, Z., Erhan, S.Z. & Wu. X.Y. 2006. A New Polymer–Lipid Hybrid Nanoparticle System Increases Cytotoxicity of Doxorubicin Against Multidrug-Resistant Human Breast Cancer Cells. *Pharmaceutical Research*, 23:1574-1585.

Xu, H.B., Jiang, R.H & Li, L. 2014. Treatment outcomes for Mycobacterium avium complex: a systematic review and meta-analysis. *European Journal of Clinical Microbiology Infectious Disease,* 33: 347-358.

Zahoor, A., Pandey, R., Sharma, S., Khuller, G.K. 2006. Pharmacokinetic and pharmacodynamics behaviour of antitubercular drugs encapsulated in alginate nanoparticles at two doses. International Journal of Antimicrobial Agents, 27: 409-416.

Zhang, L., Chan, J.M., Gu, F.X., Rhee, J.W., Wang, A.Z., Raovic-Moreno, A.F., Alexis, F., Langer, R. & Farokhzad, O.C. 2008. Self-assembled lipid-polymer hybrid nanoparticles: a robust drug delivery platform. *American Chemical Society Nano,* 2:1696-1702.

Zhang, L. & Zhang, L. 2010. Lipid-polymer hybrid nanoparticles: synthesis and characterization and applications. Nano Life, 1-2:163-173.

Zhao, L., Seth, A., Wibowo, N., Zhao, C.X., Mitter, N., Chengzhong, Y. & Middleberg, A.P.J. 2014. Nanoparticle vaccines. *Vaccine*, 32(3):327-337.

Chapter 3: The preparation and in vitro evaluation of clarithromycin and ethambutol loaded-poly (D,L-lactic-co-glycolic acid) nanoparticles for the treatment of Mycobacterium avium complex

Aeysha Jakoet[a]

Yolandy Lemmer[a],

Lonji Kalombo[a]

Anne F. Grobler[b]

a) CSIR Materials Science and Manufacturing, Polymers and Composites, Pretoria, 0001, South Africa

b) DST/NWU Preclinical Drug Development Platform, Faculty of Health Sciences, North-West University, Potchefstroom, 2531, South Africa

3.1 Introduction to the chapter

This chapter was written for the International Journal of Pharmaceutics (see Annexure A for author guidelines). This journal focuses on the physical, chemical and biological properties of drug delivery systems for drugs, vaccines and biological actives. These properties include the evaluation of drugs, surfactants, polymers and novel materials. This manuscript, which is intended for submission to the journal, focuses on the preparation and characterisation of PLGA NPs loaded with anti-MAC therapeutic actives, i.e. CLR and ETB together with MA as a targeting ligand. A preliminary cytotoxicity and uptake assessment of these particles will be evaluated. This work will serve as a screening to whether PLGA together MA as a ligand, serves as a promising drug delivery candidate for CLR and ETB for the treatment of MAC.

Abstract

Combination therapy of Clarithromycin (CLR) and Ethambutol (ETB) is prescribed for the treatment of *Mycobacterium avium complex* (MAC) infection in immunocompromised HIV infected patients. With a view to develop a targeted and sustained release system with the intention to improve bioavailability, CLR and ETB was encapsulated into poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) labelled with mycolic acid (MA). The NPs were prepared by a double emulsion, solvent evaporation method. The CLR and ETB PLGA MA NPs had a size of 350 ± 70 nm and 305 ± 10 nm with a polydispersity index (PDI) of 0.18 ± 0.02 and 0.25 ± 0.01 respectively. The prepared particles were subjected to cytotoxicity screening towards the HeLa cell line and THP-1 macrophages. Cytotoxicity results revealed that PLGA, MA, CLR and ETB displayed no cytotoxic effect after 24 hours, however after 48 hours exposure a slight cytotoxic effect was observed. Successful cellular uptake of all particles was observed into THP-1 macrophages thus suggesting that targeted delivery to the site of infection may be possible. The objective of using the PLGA carriers with targeted delivery was to primarily enhance the oral bioavailability of CLR and ETB which may in turn decrease the dose and dose frequency for the successful management of MAC infection.

Keywords: *Mycobacterium avium* complex, Poly (D,L -lactic-co-glycolic acid), nanoparticles, uptake, cytotoxicity

3.2 Introduction

Mycobacterium avium complex (MAC) is an opportunistic infection that is primarily responsible for the difficulties experienced by immunocompromised patients such as those who has acquired immunodeficiency syndrome (AIDS) (Han et al., 2005). The treatment for MAC infection is lifelong, laborious and toxic despite the availability of an effective therapeutic regimen. High dosages and dose frequency are required to maintain its therapeutic effect thus increasing the risk of adverse effects and eventually increasing the likelihood of low patience compliance (Mwandumba et al., 2004; Vyas et al., 2004; Kilinc et al., 2002). This warrants the search for an optimal treatment regimen i.e. a targeted drug delivery system that facilitates the delivery of the drugs to the site of infection thus negating the need for prolonged and frequent dosing (Salouti and Ahangari., 2014).

There are multiple conventional strategies for the treatment of MAC. Combination therapy with clarithromycin or azithromycin, ethambutol and rifamycin (rifampicin or rifabutin) is commonly recommended (Griffith et al., 2007; Kadota et al., 2016). An investigation by Miwa and co-workers (2014) have shown that a two-drug regimen with clarithromycin (CLR) and ethambutol (ETB) had a comparable outcome to a three-drug regime with rifamycin making it a favourable combination of choice to use (Miwa et al., 2014).

Despite ETB and CLR being effective in combination, these actives have limitations that could be addressed with encapsulation and targeting strategies. ETB is a bacteriostatic agent with a mechanism of action that has been suggested to occur by inhibition of mycobacterial cell wall synthesis with a reported bioavailability of 80% (Palomino and Martin, 2014; Jönnson et al., 2011). Despite the high bioavailability, ETB has a low plasma binding ability whereby 70% of oral doses are recovered unchanged in urine (Jönnson et al., 2011). CLR has an oral bioavailability of approximately 55% (Rae et al., 2017) and is one of the few antimicrobial agents for which a relationship between *in vitro* susceptibility and clinical response for MAC was shown (Tanaka et al., 1999; Kadota et al., 2015).

The leading investigations of drug delivery systems in the treatment of mycobacterial infections are lipid- or polymer-based nanoparticulate systems which have extensively been studied in experimental models of MAC and TB infection by many investigators who have encapsulated a variety of antibacterial agents (Pinheiro et al., 2011; Kaur et al., 2016). For the treatment of MAC infection these lipid-based delivery systems include the encapsulation of macrolides (azithromycin or clarithromycin), a rifamycin (rifampin or rifabutin), an aminoglycoside (amikacin) and ethambutol (Ladavière and Gref, 2015). A team of investigators has recently shown that the encapsulation of rifabutin in solid lipid nanoparticles

has increased the relative bioavailability by five-fold when compared to free rifabutin (Nirbhavane et al., 2016). Other groups have shown that the encapsulation of amikacin in sterically stabilised liposomes as a site-specific delivery system. The liposomal treatment resulted in rapid and complete elimination of the mycobacteria in all infected organs in half the treatment duration of the non-liposomal treatment which unfortunately had a considerable number of persistent mycobacteria remaining after treatment (Steenwinkel et al., 2007). Although the utilisation of polymer based delivery systems for the specific treatment of MAC is limited, a vast amount of experimental research exists for its use in enhancing efficacy of actives for TB infection (Kaur et al., 2016).

Poly (D,L-lactic-co-glycolic acid) (PLGA) is a commonly used synthetic polymer in drug delivery owing to its biodegradability, biocompatibility and favourable degradation properties (Hirenkumar et al., 2011). PLGA NPs are versatile as a result of its easy surface modification that allows for potential targeting ligands or molecules which aid in prolonged systemic residence time and drug delivery at the site of infection (Muller and Keck, 2004). PLGA NPs in combination with the first line anti-tuberculosis drugs (ATD); rifampicin (RIF), isoniazid (INH) pyrazinamide (PYZ) and ethambutol (ETB) have demonstrated an increase in bioavailability when compared to free drug (Pandey et al., 2006). Alternative method preparation of PLGA NPs with ATDs with additional surfactants and additives to modify the polymer matrix have shown to prolong the circulation time (Semete et al., 2012). Various amount of research exists on the utilisation of PLGA NPs with several modifications to improve the treatment of TB. These may include the use of targeting ligands, where the delivery of the drug is at the site of infection. Previous research published by Lemmer and co-workers (2015) have exploited the fact the MA are found in the mycobacterial cell wall and have hypothesised that the MA on the surface of the NP may interact with anti-MA antibodies in the area of infection and promote an enhanced uptake of NPs of infected and uninfected macrophages. They've shown that with the addition of MA to PLGA NPs loaded with INH, a significant increase on phagocytic uptake was observed (Lemmer et al., 2015).

It is therefore safe to assume, that with the same approach for MAC therapeutics, we could potentially improve the treatment for MAC by enhancing the overall efficacy of CLR and ETB by increase phagocytic uptake.

In this preliminary work, PLGA NPs with the addition of MA as a targeting agent to TB-infected sites was prepared with a double emulsion solvent evaporation process. The collected particles were investigated in terms of its physiochemical properties such as particle size, zeta potential and encapsulation efficiency. Furthermore, its cytotoxicity was evaluated against

HeLa and THP-1in vitro models. Lastly, the effect of MA on the potential enhancement of cellular uptake of NPs by the macrophages was viewed with confocal microscopy.

3.3 Materials and Methods

3.3.1 Materials

Unless stated otherwise all reagents were purchased from Sigma-Aldrich Chemical Co., (St Louis, MO, USA). The 5-bromomethyl fluorescein mycolic acid was generously donated by Prof. J.A.Verschoor from the University of Pretoria. Clarithromycin and ethambutol dihydrochloride was purchased from DB fine chemicals, South Africa. For the cytotoxicity and uptake investigation, Foetal calf serum (FCS), penicillin/streptomycin, RMPI – 1640 medium with L-glutamine and DMEM was purchased from Life technologies, (California, USA). Paraformaldehyde for cell fixation was purchased from Merck, Darmstadt, Germany.

3.3.2 Methods

3.3.2.1 Preparation of NPs

NPs were prepared via a double emulsion solvent evaporation technique, followed by freeze-drying (Lemmer et al., 2015; Lamprecht et al., 1999). Briefly, 100 mg of PLGA 50:50 (Mw: 30 000-60 000) was dissolved in dichloromethane (DCM) (6 mL) with or without the MA pre-dissolved in dichloromethane. To this solution, 1% (w/v) PVA aqueous phase (2 mL) containing ETB (100 mg) was added and homogenised by means of a Silverson high speed homogeniser (Silverson L4R Buckinghamshire, UK) at 8000 rpm for 3 minutes.

The resulting water-in-oil emulsion (w/o) was added to 40 mL of 2% w/v PVA aqueous solution. This mixture was further emulsified for 7 minutes at 8000 rpm resulting in the final double emulsion (water-in-oil-in-water, w/o/w). The final w/o/w emulsion was stirred overnight at room temperature to allow solvent evaporation and subsequent precipitation of NPs.

For CLR NPs:
CLR (100 mg) was dissolved in the 6 mL DCM with or without pre-dissolved MA. The emulsification was followed exactly to that described for ETB NPs.

For particle collection, the evaporated emulsion underwent a double centrifugation process that assisted in the separation of particles of different sizes at 845 and 33 902 rcf for 10 and 15 minutes respectively. The supernatant was collected and further analysed for free drug. The resulting particles were dried by lyophilization in a Virtis Benchtop freeze dryer (SP Industries, Gardiner, New York, USA).

For Fluorescent labelled NPs:
Two sets of fluorescent labels coumarin-6 (C6) and 50% 5-bromomethyl fluorescein labelled mycolic acid (5BMF-MA) were prepared for cellular uptake evaluation.

C6 particles were prepared to that described by Ma et al., (2012). A dye solution (50 µg of C6 in 50 µL chloroform) was added to the polymer solution prior to the described emulsification process.

5BMF-MA NPs were prepared similarly to the method earlier described for ETB and CLR-loaded PLGA NPs, with a direct substitution of MA with the 50% labelled MA (5BMF).

3.3.2.2 Characterisation: Size distribution and zeta potential

Approximately 1 mg of sample was suspended in 10 mL of distilled water and sonicated for 5 minutes. Size and zeta potential was measured on the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) instrument at ambient temperature, viscosity of 0.8872 cP, refractive index (RI) of 1,330, measurement angle of 173° Backscatter and a dielectric constant of 78.5.

3.3.2.3 Characterisation: Surface morphology

The surface morphology was examined using a Carl Zeiss scanning electron microscope, (Oberkocken, Germany). A small amount of the sample was mounted onto aluminium stubs with double-sided adhesive carbon tape. The samples were sputtered with carbon (Emitech K550 Super Coated; Emitech Ltd, South Stour Avenue Ashford, Kent, UK) under an argon atmosphere. Samples were observed for morphology at a voltage of 2.00 kV and imaged at 10 000X magnification.

3.3.2.4 Drug Encapsulation Efficiency

The encapsulation efficiency (EE) was achieved by means of an indirect drug quantification method. The supernatant after particle collection was analysed for free drug by means of a Shimadzu ultra-performance liquid chromatography (UPLC) interfaced with ABSciex 3200 Q–Trap triple quadrupole mass spectrometer. Chromatographic separations were performed using a Gemini 5 µm C18 110 Å column, 250 x 4.6 mm. The oven was set at room temperature and the mobile phase consisted of 10 mM ammonium acetate in methanol and was isocratically eluted at a flow rate of 1 min/mL with an injection volume of 20 µL.

The EE was calculated from the difference between the total drug used in preparation and the amount of non-encapsulated drug (free drug) present in the supernatant after isolation of particles, after washing.

The amount of drug loss in the centrifugation process was calculated with equation 1.

Equation (Eq) 1: Free drug = a ($\frac{\mu g}{mL}$) x b (mL) x c

where a is: the mean calculated concentration, b: volume of supernatant collected and c: dilution factor

The amount of drug remaining in the pellet was calculated using Eq 2:

Equation (Eq) 2: Encapsulated drug (mg) = Initial drug added (mg) – free drug (mg)

Encapsulation Efficiency (EE) was calculated using Eq.3.

Equation (Eq) 3: $\mathrm{EE} = \frac{\text{Mass of Encapsulated drug}}{\text{Inital drug mass}}\ x\ 100$

3.3.2.5 Cytotoxicity of PLGA NPs with THP-1 macrophages and HeLa cells

THP-1 and HeLa cells were purchased from ATCC, USA. The cells were grown and maintained at a confluency of 0.5 – 5 x 10^6 cells/mL, in RPMI-1640 for THP-1 and DMEM media for HeLa cells. Additives in the media included penicillin (50 µg/ml), streptomycin (50 µg/ml) and 10% heat inactivated foetal calf serum (FCS). The cells were maintained at 37 ºC in a 5% CO_2 (g) humidified incubator according to general cell culture practices. THP-1 monocytes were differentiated into macrophages with 50 nM Phorbol 12-myristate 13-acetate (PMA) for 48 hours as previously described by Kisich et al 2007. Cell viability after exposure to the different nanoparticle formulations for 24 or 48-hour incubation periods, was determined using a WST assay (Quick Cell Proliferation Assay Kit II, Biovision, USA) and was utilised in accordance to the manufacturer's instructions. Briefly, this product utilises the tetrazolium salt WST-1, which is reduced to water-soluble orange formazan by cellular mitochondrial dehydrogenase present in viable cells. The absorbance reading at 450 nm with a reference wavelength at 630 nm was used to measure the dye which was further related to the amount of viable cells.

3.3.2.6 Statistical analysis

The cytotoxicity measurements were collected from a single set of data of (n=16) per test formulation. Owing to the assay not repeated, as this was a provisional screening, statistical significance was not determined. However, these initial studies provide good indications on the protective effect the particles have towards the cells. The error bars in the graphs indicate standard deviation.

3.3.2.7 Uptake of fluorescent labelled particles into THP-1 macrophages

The uptake of the particles into the macrophages cells was achieved by the addition of C6 and 5-BMF fluorescently labelled particles (0.1 mg/mL) to newly chemically differentiated THP-1 macrophages. This was followed by a 1-hour incubation period and a subsequent triplicate washing step consisting of phosphate buffer saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

The macrophages were fixated onto glass slides by the addition of paraformaldehyde (PFA, 3%) in PBS followed by a 20-minute incubation period and thereafter rinsed with distilled H_2O. The cover slip was drip-dried and mounted face down with fluoroshield (fluorescent mounting medium solution). The cover-slip was sealed onto the glass slide with clear nail polish. The prepared slides were viewed with confocal laser scanning microscopy (CLSM, a Nikon Eclipse TE-3000, wavelengths: excitation (Ex) 488 nm, emission (Em) 515 nm) to determine potential cellular uptake of NPs.

3.4 Results

3.4.1 Size and Zeta Potential

During the manufacturing of the various particles a bimodal size distribution was obtained. The different sizes of the particles were separated by utilising two different centrifugal forces, i.e., the lower centrifugal force was used to collect the larger sized particles with an average particle size range of 616 – 1171 nm depending on the formulation and the higher force to collect particles in the mean average size range of 305 – 498 nm. The size and zeta potential of the different particles produced are represented in Table 3.1. The average particle size distribution was greater for the larger sized particles compared to the smaller sized particles. The smaller sized particles were chosen for subsequent studies as smaller sizes were previously shown to have greater cellular uptake ability (Panyam and Labhasetwar, 2003).

Table 3.1: Size and zeta potential of PLGA NPs (DF:drug free, ETB and CLR loaded) labelled with and without mycolic acids (n=3)

	DF PLGA	DF PLGA MA	PLGA ETB	PLGA ETB MA	PLGA CLR	PLGA CLR MA
Centrifugation Speed: 845 rcf						
Size (nm)	1617 ± 125*	1567 ± 53	1171 ± 60	916 ± 37	615 ± 28	1005 ± 59
PDI	0.76 ± 0.03	0.57 ± 0.14	0.86 ± 0.05	0.41 ± 0.05	0.27 ± 0.05	0.48 ± 0.41
Zeta Potential (mV)	- 8.6 ± 0.3	- 9.2 ± 0.2	- 29.4 ± 0.4	- 28.8 ± 0.2	- 23.4 ± 1.8	- 23.4 ± 0.5
Centrifugation Speed: 33 902 rcf						
Size (nm)	413 ± 12	498 ± 42	397 ± 70	305 ± 10	372 ± 15	350 ± 70
PDI	0.38 ± 0.05	0.53 ± 0.13	0.23 ± 0.01	0.25 ± 0.01	0.28 ± 0.02	0.18 ± 0.02
Zeta Potential (mV)	- 8.5 ± 0.3	- 8.2 ± 1.0	- 27.2 ± 1.9	- 26.5 ± 0.7	- 27.0 ± 0.8	- 29.8 ± 0.5

The larger sized particles obtained during preparation was potentially attributed to the use of DCM as a solvent in the formulation owing to its high interfacial tension, vapour pressure and poor aqueous solubility (Naik et al., 2012). These factors contribute to droplet instability present in the primary emulsion and low diffusion of DCM into the aqueous phase thus resulting in possible droplet coalescence (Vinneeth et al., 2014). The replacement of DCM with ethyl acetate (EA) was trial and errored as it has shown properties of lower interfacial tension and fast aqueous diffusion (Meng et al., 2003). EA was incorporated into the formulation to assist in the prevention of coalescence of droplets. Various ratios of DCM to EA were investigated but due to the limited solubility of the highly hydrophobic MA molecule, the formation of nanoparticles was unachievable (results not shown). Therefore, DCM was used despite its contribution to the higher poly-dispersity index (PDI).

The zeta potential was strongly influenced by the addition of the drugs. The values ranged from -8.2 to -29.8 mV (Table 3.1). The DF NPs showed a low zeta potential range from -8.56 to -9.22 mV. With the addition of CLR and ETB, the negative zeta potential increased to an average of -28 mV. The increased potential can be explained by the contribution of the hydroxyl groups present in the CLR and ETB structures, if they are exposed on the surface of the particles.

3.4.2 Surface Morphology

Scanning electron microscopic images of the PLGA NPs (Figure 3.1), regardless of the antibiotic content and whether MA were included or not, revealed a spherical shape with a smooth surface without any noticeable pinholes or cracks which is ideal for uniformity in NP degradation and drug release in *in vitro* and *in vivo* assays. The diameters of the particles ranged from 300-500 nm as confirmed by the laser light scattering measurement. In addition, no aggregation or agglomeration was observed conferring their stability and suitability for the drug delivery.

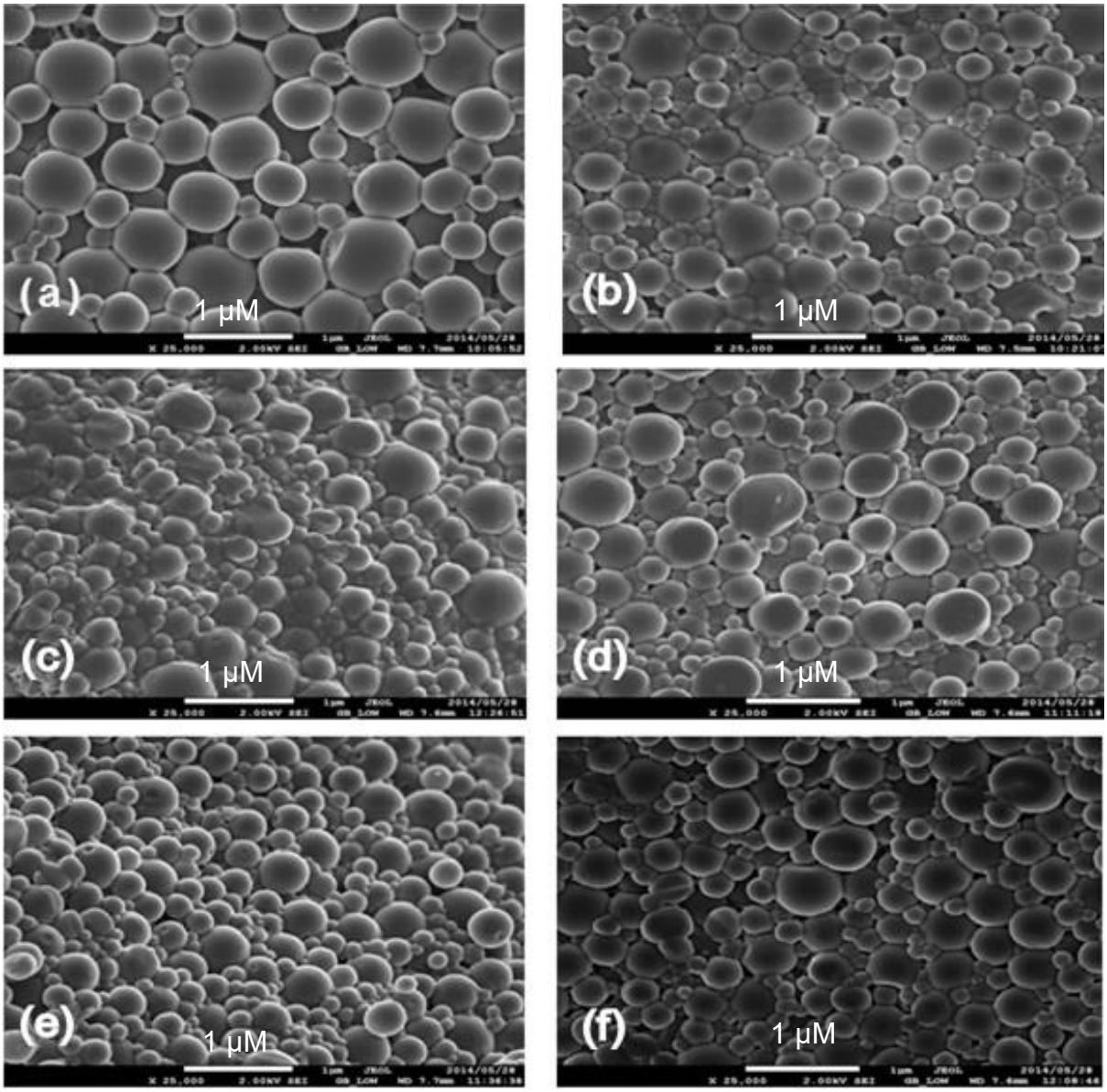


Figure 3.1: SEM images of (a) DF-PLGA NPs, (b) DF-PLGA-MA NPs, (c) PLGA-ETB NPs, (d) PLGA-ETB-MA NPs, (e) PLGA-CLR NPs and (f) PLGA-CLR-MA NPs (Scale bar: 1 µm, Magnification = 40 000X).

3.4.3 Drug loss and EE determination

The indirect drug encapsulation determination method was utilised to calculate EE. The results in Table 3.2 are representative of the drug loss during production, the remaining drug in the sample and furthermore the EE. Drug loss during production of PLGA CLR NP formulations was calculated to an average of 5 mg with an average EE of 94.4% for formulations with and without MA. Drug loss during production of PLGA ETB NP formulations was slightly less than CLR and was calculated to an average of 3 mg with an average EE of 96.6% for ETB formulations with and without MA.

Table 3.2: Tabulated results of the mean calculated concentration, EE and the amount of CLR and ETB lost during sample preparation, where the mean calculated concentration was generated by the LCMS analyte software 1.6.1 package, where n = 3.

Analyte	Mean calculated concentration	Calculated drug loss[1]	Drug remaining in collected sample[2]	
	(µg/mL)		Initial mass: 100 mg	EE[3]
		(mg)	(mg)	(%)
PLGA-CLR	75.5 ± 7.31	5,4	94,6	94,6
PLGA-CLR-MA	73.1 ± 3.32	5,3	94,3	94,3
PLGA-ETB	47.1 ± 1.97	3,4	96,6	96,6
PLGA-ETB-MA	47.6 ± 1.04	3,4	96,6	96,6

where 1,2,3 is calculated using equations 1 (free drug) 2 (encapsulated drug) and 3 (encapsulation efficiency)

The difference in EE may be a result of the slight difference in particle preparation in the manufacturing process. CLR was embedded into the polymer matrix owing to is hydrophobicity, opposed to ETB, a hydrophilic molecule, which was encapsulated into the aqueous core. From results presented in Table 3.2 it was apparent that incorporation of MA did not affect the ability of the formulation design to embed or encapsulate the drug. It was further assumed that the remainder of the drug was present in the particles collected.

3.4.4 Cytotoxicity of PLGA NP test formulations

To elucidate the possible cytotoxic effects attributed to the polymer, antibiotic or the MA used to prepare the NPs, the *in vitro* cell viability was evaluated by means of the WST assay, which measures the mitochondrial dehydrogenase activity as an indicator for cell proliferation, for 24 and 48 hours on HeLa (cervical epithelioid carcinoma) and THP-1 (monocyte leukemic) cells. After exposure to PMA, the THP-1 cells differentiate into a mature macrophage phenotype with a lower level of cell proliferation and a higher rate of phagocytosis which provided a good macrophage model (Qin, 2012) and therefore provided a realistic model for our particles. WST results showed that drug free NPs with and without MA did not induce cell toxicity in the range of the tested concentrations (Figure 3.2) for 24 and 48 hours for HeLa cells. The calculated viability values were similar to the control (100%) showing no significant differences.

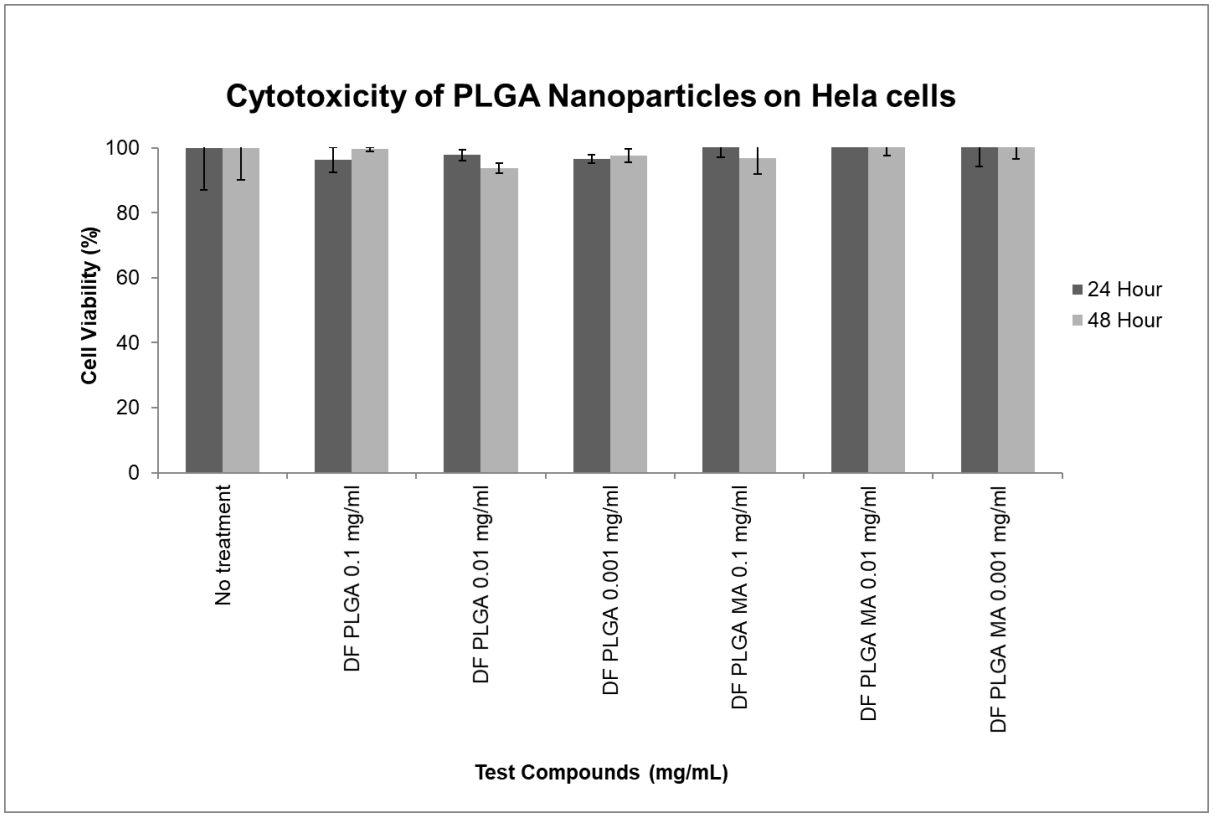


Figure 3.2: The cytotoxic evaluation of HeLa cells after the treatment of DF PLGA formulations with and without MA at different concentrations; no treatment = cells only, DF PLGA = drug free PLGA NPS, DF PLGA MA = drug free PLGA NPs with mycolic acid. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation.

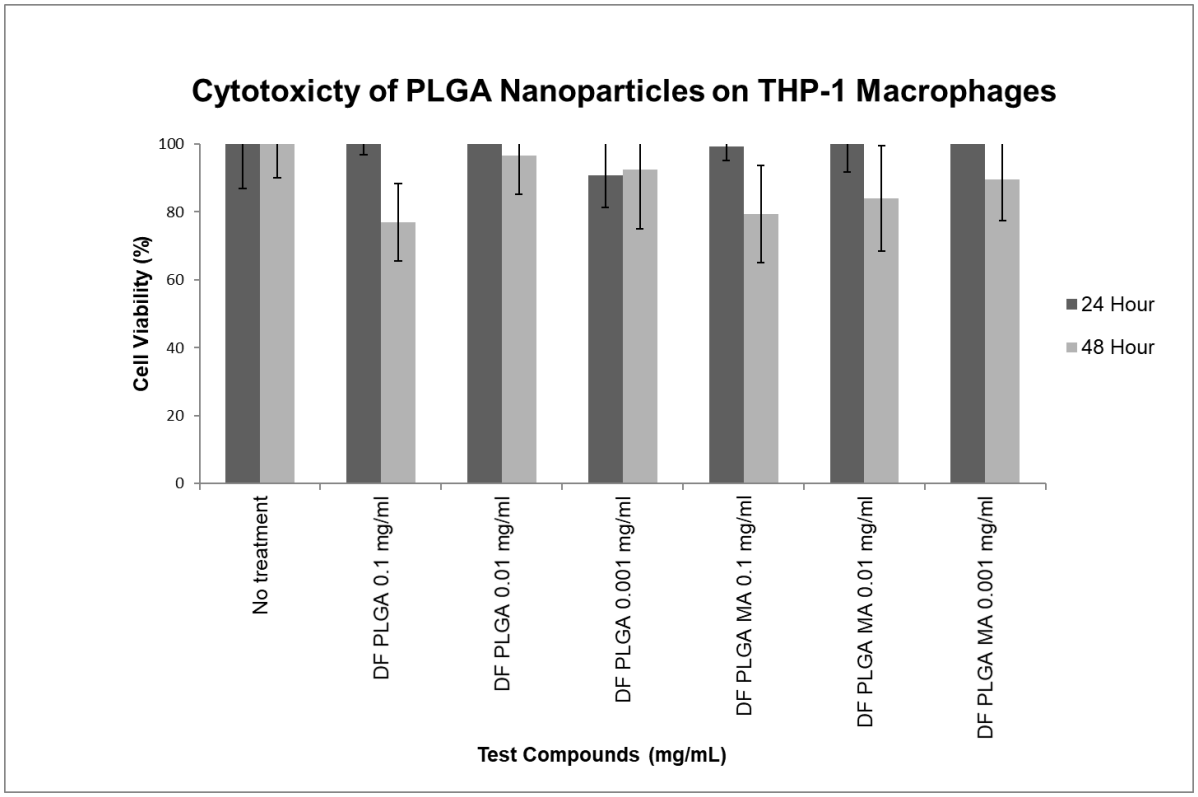


Figure 3.3: The cytotoxic evaluation of THP-1 macrophages after the treatment of DF PLGA formulations with and without MA at different concentrations; no treatment = cells only, DF PLGA = drug free PLGA NPS, DF PLGA MA = drug free PLGA NPs with mycolic acid. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation.

In THP-1 macrophages, the calculated cell viability values were similar to the control (100%) after 24-hour incubation (Figure 3.3). However, after 48 hours of incubation the NPs without MA induced moderate cytotoxicity of 25% at the highest concentration of 0.1 mg/mL. The particles with MA induced a slight change in viability after 48 hours of exposure.

The *in vitro* cytotoxicity of ETB was evaluated alone and in combination with the PLGA and PLGA MA NPs (Figure 3.4). For free drug particles the cell viability varied between 80 – 100%, indicating that the ETB itself does not induce cytotoxicity for HeLa cells at all test conditions. When evaluated in combination with the PLGA and MA did not induce cytotoxicity in HeLa cells as all calculated viability was above 90% and similar to the control.

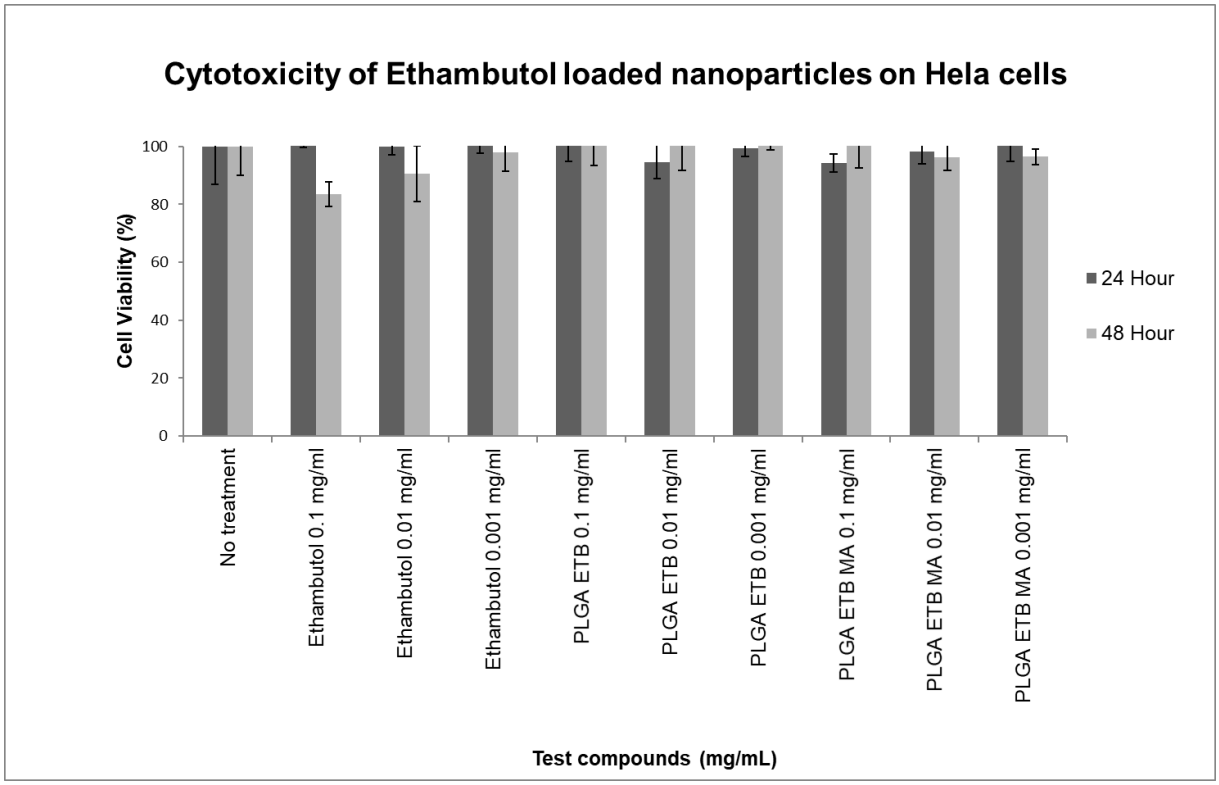


Figure 3.4: The cytotoxic evaluation of HeLa cells after the treatment of PLGA ETB formulations with and without MA at different concentrations; no treatment = cells only, PLGA ETB = PLGA NPs loaded with ethambutol, PLGA ETB MA = PLGA with mycolic acid ethambutol NPs loaded with ethambutol. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation.

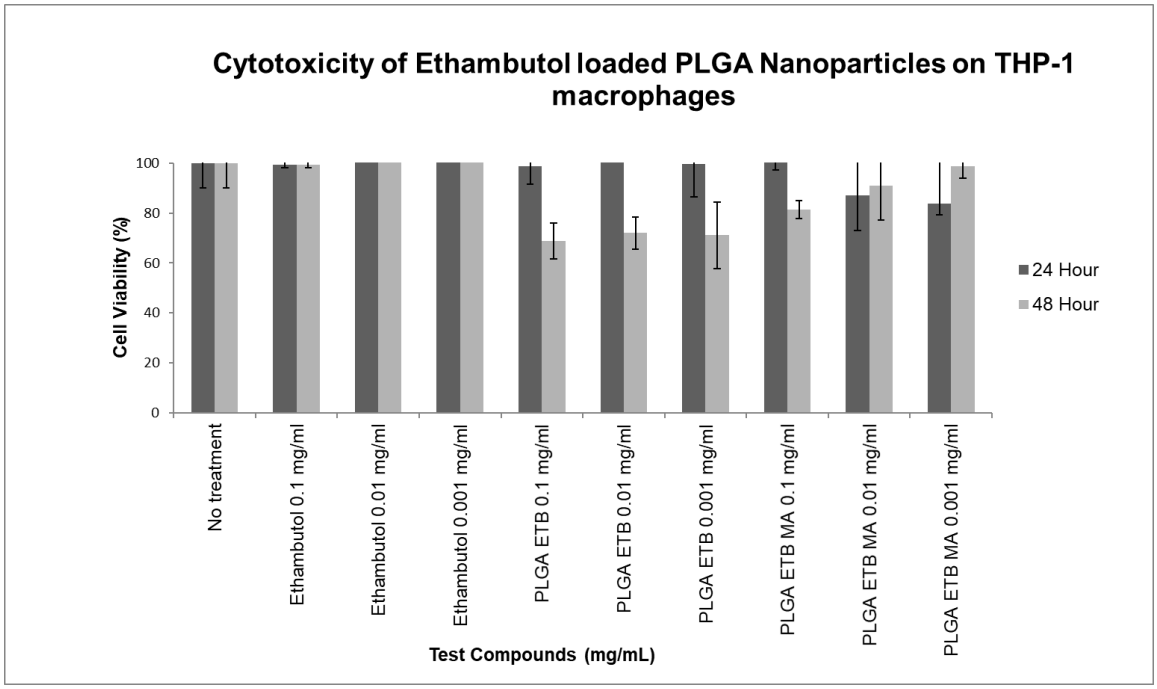


Figure 3.5: The cytotoxic evaluation of THP-1 macrophages after the treatment of PLGA ETB formulations with and without MA at different concentrations; no treatment = cells only, PLGA ETB = PLGA NPs loaded with ethambutol, PLGA ETB MA = PLGA NPs with mycolic acid loaded with ethambutol. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation

However, the cytotoxicity evaluation of ETB in combination with PLGA and MA on THP_1 cells, displayed a different relationship when compared to the HeLa cells. Free ETB displayed no cytotoxic effect at all concentrations at all test conditions. The combination of ETB and PLGA with and without MA induced minimal cytotoxicity at all concentrations at 24 hours. However, when evaluated at 48 hours, the ETB in combination induced some cytotoxic effect. The ETB with PLGA had a cell viability of approximately 65-70% at all concentrations. When compared to the formulations with the addition of MA, the lowest viability was approximately at 80% at the highest concentration. The cell viability was mitigated with the decrease in concentration. It appears that the inclusion and presence of MA reduced the slight cytotoxic effect of the PLGA in the formulation.

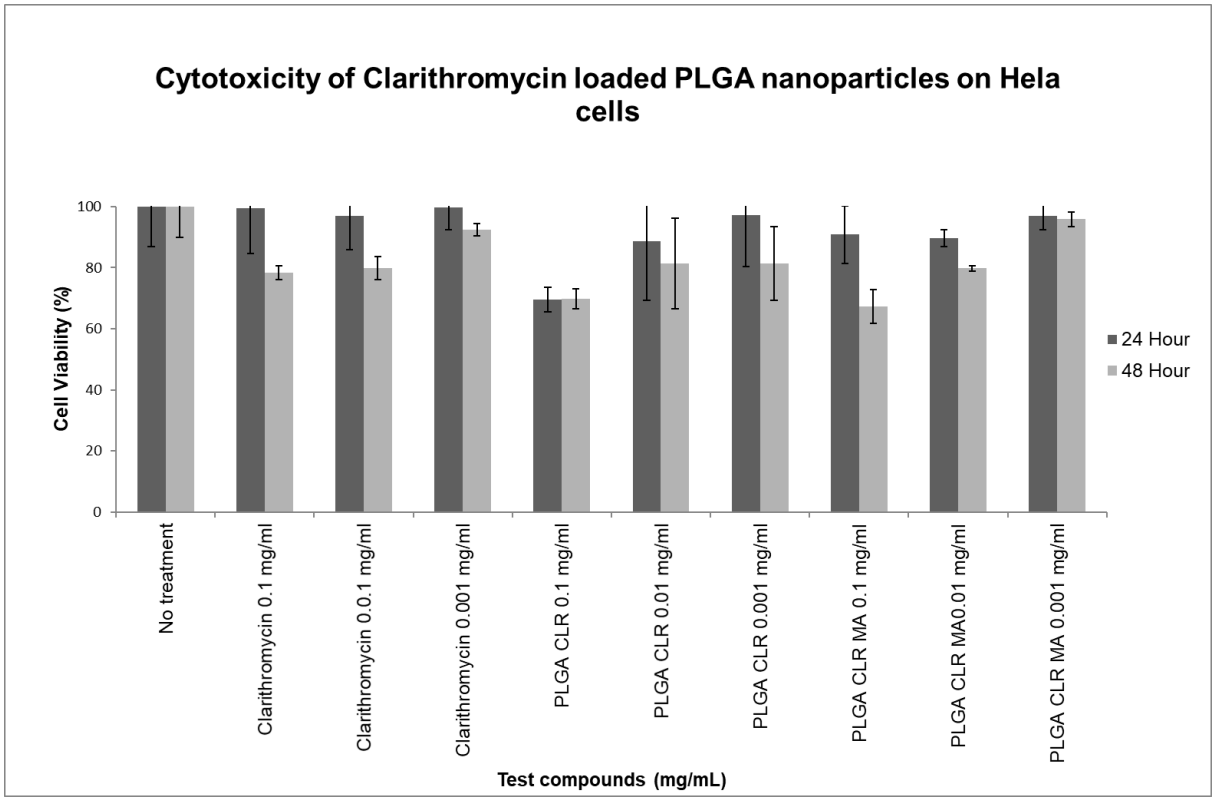


Figure 3.6: The cytotoxic evaluation of HeLa cells after the treatment of PLGA CLR formulations with and without MA at different concentrations; no treatment = cells only, PLGA CLR = PLGA NPs loaded with clarithromycin, PLGA CLR MA = PLGA NPs with mycolic acid loaded with clarithromycin. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation.

The cytotoxic effect of free CLR together in combination with PLGA and MA was evaluated on HeLa cells, Figure 3.6. Free CLR at 24 hours possessed no cytotoxic effect on HeLa cells at all test concentrations. However, after 48 hours of exposure, a noticeable reduction in viability from 100 to below 80% was observed at the highest concentration. When compared with formulations with PLGA and MA, a greater effect was observed on cytotoxicity at 24 and 48 hours of exposure. However, 48 hours of exposure was more cytotoxic than 24 hours. The calculated cytotoxic viability decreased with a decrease in concentration.

A drastic change in viability was observed with PLGA and MA NPs when compared to the free drug. This may be attributed to the fact that CLR is a hydrophobic compound and was incorporated into the polymer matrix thus providing better exposure to the cells than when the free drug is suspended in the aqueous environment. In the NP system, the uniformity of CLR incorporation as well as the extent at which CLR drug was bound to the surface was not controlled or investigated.

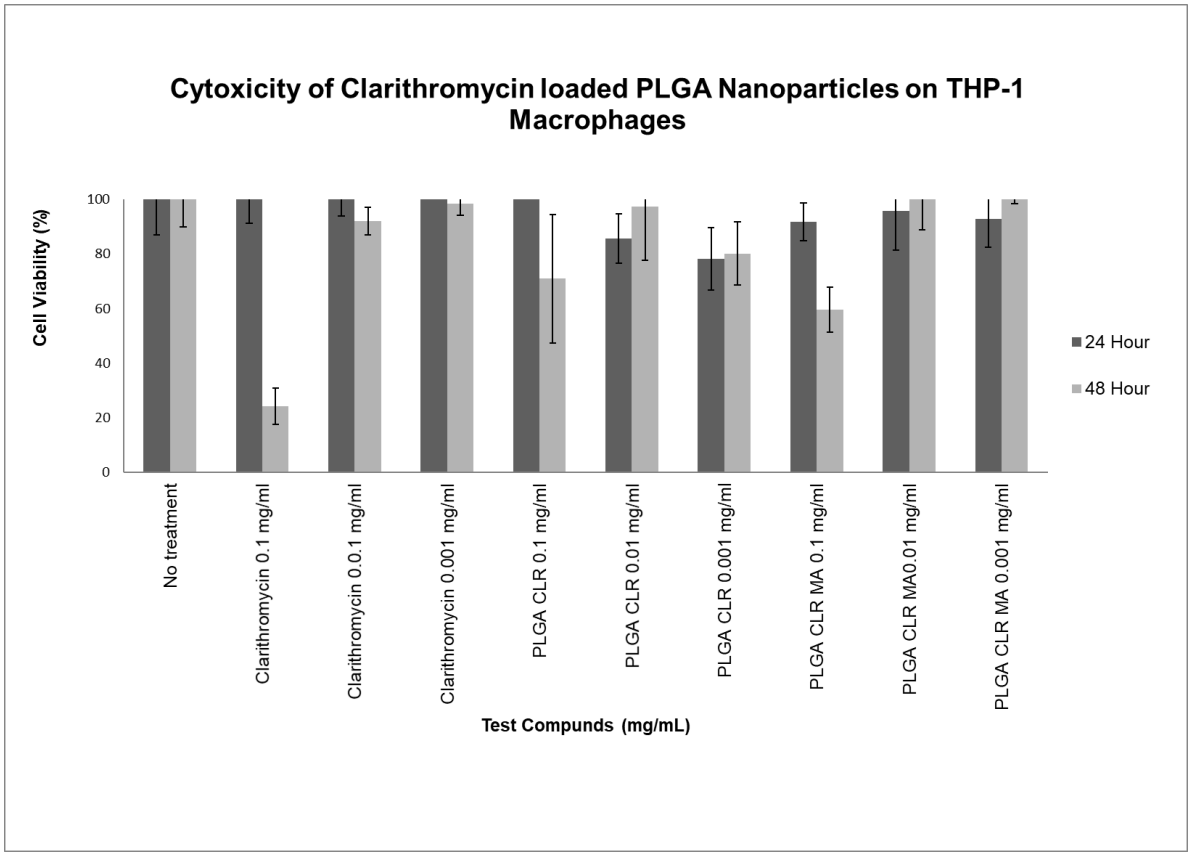


Figure 3.7: The cytotoxic evaluation of THP-1 macrophages after the treatment of PLGA CLR formulations with and without MA at different concentrations; no treatment = cells only, PLGA CLR = PLGA NPs loaded with clarithromycin, PLGA CLR MA = PLGA NPs with mycolic acid loaded with clarithromycin. The data are representative of one experiment of n = 16 and the error bars indicate standard deviation.

The pattern observed with CLR formulations were similar to that observed for HeLa cells. Free CLR at 0.1 mg/mL was highly cytotoxic with a reduction in viability from 100% to approximately 20% when after 48 hours of exposure. Formulations in combination with PLGA and MA at 24 hours did not have a great effect on viability ranging from 80 to 100%. After 48 hours of exposure the viability of PLGA and PLGA with MA in combination with CLR had a greater effect displaying viability of 70% and below. No apparent pattern was observed for the evaluations of 0.01 and 0.001 mg/mL which may be a result of non-uniformity of bound CLR on the NP surface.

3.5 Uptake of PLGA MA NPs into THP-1 macrophages

In order to study the potential of the macrophages to take up and accumulate the CLR and ETB containing PLGA nanoparticles with and without MA, visual observations were made via confocal microscopy. The utilisation of fluorescently labelled NPs viewed CLSM or fluorescent

microscopy for cellular uptake is widely found in literature and is an accepted approach (Win and Feng, 2004).

The uptake capability of DF, ETB loaded and CLR loaded PLGA NPs with and without MA was investigated on THP-1 macrophages. NP formulations at 0.1 mg/mL were incubated with THP-1 macrophages for 1 hour and were thereafter fixated to view cellular uptake with CLSM. In addition, particle uptake was investigated using coumarin-6 and 5-BMF as fluorescent markers for the particles to evaluate the uptake of PLGA NPs loaded with MAC therapeutics with and without MA

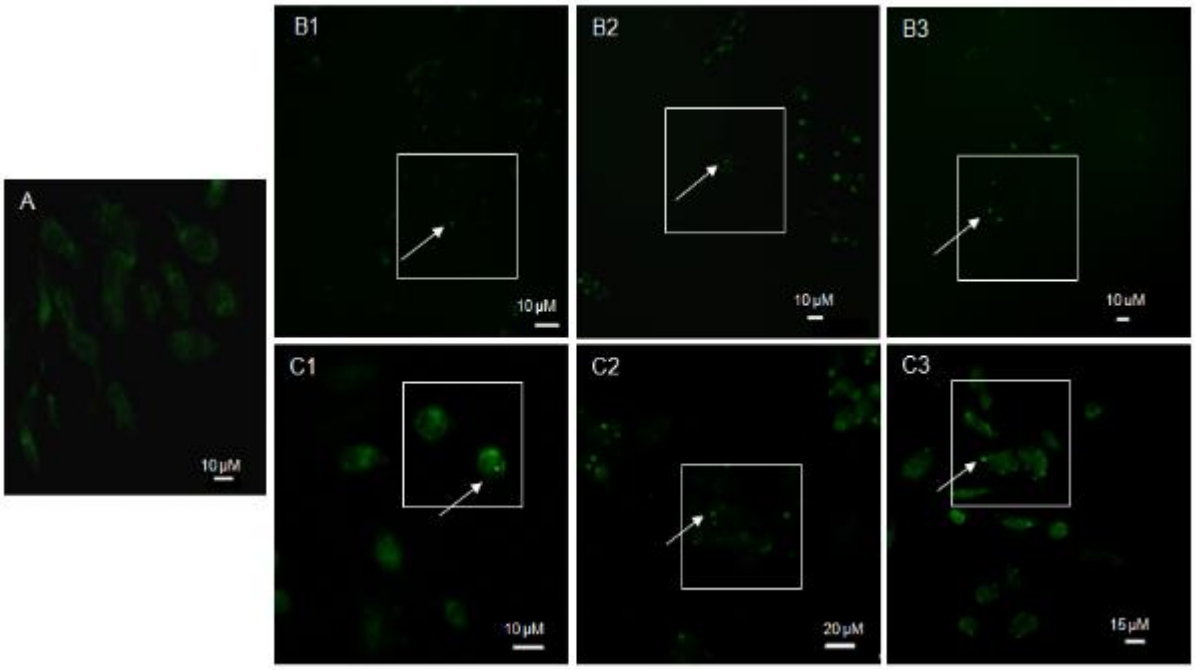


Figure 3.8: Confocal images of PLGA NPs taken up by THP-1 macrophages, i.e. A: Control, B1: DF PLGA C6, B2: PLGA ETB C6, B3: PLGA CLR C6, C1:DF PLGA MA, C2: PLGA ETB MA, C3:PLGA CLR MA

Figure 3.8, illustrate the confocal images of fluorescent marked PLGA NPs into THP-1 macrophages. The control (untreated cells) indicated by Figure 3.8A exhibited auto-fluorescence of THP-1 macrophages in the green channel (Ex: 488 nm, Em: 515 nm) with no particles. Figure 3.8(B1-3) illustrated cells treated with DF, ETB and CLR loaded PLGA NPs particles dyed with coumarin-6. Despite the cytotoxic effect that coumarin may have exhibited onto the cells, the dye was a good representation for initial uptake of particles (Lemmer, 2010). The highlighted areas are representation of particles taken up by the macrophages. Owing to the physicochemical properties of the particles (size, charge and shape), cellular uptake by

macrophages was expected. No observable difference was noticed for the dyed C6 PLGA formulations.

Figure 3.8 (C1-3) illustrated DF, ETB and CLR loaded PLGA particles together with MA for targeted delivery of the particles to macrophages. The uptake of particles with MA is illustrated by the highlighted region. The MA labelled NPs displayed no visual cytotoxic effect at the test concentration of 0.1 mg/mL as the overall appearance of the cells were similar to that of the untreated cells. From the images, it is not clear whether the uptake of the particles into the macrophage was enhanced by the presence of the mycolic acids. To determine this, a time interval study was considered as important for future investigations. However, it was speculated that this would be the case as research recently published by Lemmer et al. (2015) supported this theory.

3.6. Discussion

The aim of this work was to provide a preliminary screening for the development of a drug delivery system to aid in the shortfalls currently experienced in MAC treatment. High dosages with a high dose frequency are required to maintain therapeutic levels as both clarithromycin and ethambutol have low bioavailability hence impacting on drug efficacy (Jonnson et al., 2011; Rae et al.,2017). These high dosages are the main contributing factor to low patient compliance which could contribute towards the development of drug resistance (Tesfahuneygn et al., 2015).

We proposed to mitigate these shortfalls with the encapsulation of these drugs into polymeric NPs with a targeting ligand to improve drug efficacy, lower side effects and increase compliance, thus eliminating long treatment duration. PLGA has been investigated to treat various diseases as it has shown to be biocompatible and provide optimal degradation for sustained drug release. Although previous studies have shown the encapsulation of CLR and ETB into PLGA NPs with alternative methods where CLR showed improved activity and therapeutic effects (Lotifpour et al., 2016) and ETB showed increased bioavailability (Pandey et al., 2006) however, the end goal in mind was never MAC. Therefore, this system is new and different especially with the use of robust and stable MA which is found in mycobacterial cell wall (Lemmer et al., 2015) to enhance the cellular uptake of these particles.

Our results showed the easy encapsulation of CLR and ETB into PLGA NPs labelled with and without MA with an average size of 305–498 nm with a high negative zeta potential ranging from -26 to -30 mV. Particles displayed a smooth spherical morphology and an EE of 94.4% for CLR and 96.6%.

The physical characteristics of the particles are good candidates for drug delivery. The size and shape alone has been shown to efficiently be internalised by cells (Champion and Mitragotri, 2006).

The increased potential positively affects the drug delivery system as these values are indicative of stable NPs in aqueous suspensions (Schramm, 2005). Negatively charged NPs has also shown preferential interaction with phagocytic cells because of its ingestion of bacteria, which also displays a negative net charge (Fröhlich, 2012). Macrophages are phagocytic cells therefore the high negative zeta potential may positively assist MA with enhanced cellular uptake.

Although a high EE was determined for the NPs, for future and further *in vivo* and *in vitro* investigations a direct method of quantification would be more ideal. The indirect method provided sufficient information for this work as it merely served as a screening to observe the encapsulation ability of the drug carrier system and its effect with therapeutics on cellular uptake in macrophages.

The preliminary screening of cell viability revealed that PLGA and MA alone did not pose any cytotoxic effect of HeLa cells as well as THP-1 macrophages. Although a slight decrease in viability was observed after 48 hours. The combination of PLGA and MA with the drugs had a greater cytotoxic effect on the cells which may be attributed to targeting ability of MA as well as the increase negative zeta potential which was shown to may cause cytotoxicity owing to its enhanced interaction with phagocytic cells (Fröhlich, 2012). However, these tests should be repeated in triplicate to draw solid conclusions.

The uptake studies indicated that the particles entered the macrophages for both PLGA particles dyed with coumarin-6 or labelled with mycolic acids. This study was not conclusive as to whether the mycolic acids facilitated uptake into the cells. To determine this, flow cytometry, fluorometry and a time interval study was considered as important for further future investigations. However, it is speculated that this the case as research published by Lemmer et al., (2015) supported the theory that PLGA NPs with MA had a significant increase in phagocytic uptake by mycobacterium infected macrophages which in turn may result in improved delivery and concentration of CLR and ETB into the infected cells.

A regime that can improve the bioavailability of CLR and ETB with a lower therapeutic dose is required. Achieving this regime is not straightforward, as many factors should be considered. Therefore, further studies are needed on the applicability of this delivery system

for the treatment of MAC. However, this system does show promise in terms of its easy modification and tunability for the desired outcome.

3.7 Conclusion

A new drug delivery system was developed in the hope to improve the current treatment regimen for MAC. With the preliminary results obtained it can be said that the physical characteristics, the preliminary cellular viability results and uptake ability into macrophages provide a basis for making this system a possible candidate for further development and investigation that can substantiate the results we have shown.

3.8 Acknowledgements

We would like to take this opportunity to thank the CSIR, PCDDP and India Brazil and South Africa trilateral (IBSA) for funding this study. We would personally like to thank Mr. Brendon Naicker for his assistance in the LCMS method development for EE quantification as well as Dr. Matthew Glyn for his assistance in obtaining confocal images for the uptake study.

References

Champion, A., Mitragotri, S. 2006. Role of target geometry in phagocytosis. Proc. Natl. Acad. Sci. U.S.A. 103(13), 4930-4934.

Fröhlich, E. 2012. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. Int. J. Nanomedicine. 7, 5577-5591.

Griffith, D.E., Aksamit, T., Brwon-Elliot, B.A., Catanzaro, A., Daley, C., Gordin, F., Holland, S.M. Horsburgh, R., Huitt, G., Lademarco, M.F. 2007. Mycobacterial Diseases Subcommittee; American Thoracic Society; Infectious Diseases Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med. 175, 744-745.

Han, X.Y., Tarrand, J.J., Infante, R., Jacobson, K.L., Truongs, M., 2005.Clinical significance and epidemiologic analyses of Mycobacterium avium and Mycobacterium intracellular among patients without AIDS. J. Clin. Microbiol. 43(9), 4407-4412.

Hirenkumar, K., Siegal, M., Siegal, S.J., 2011. Poly lactic-co-glycolic acid (PLGA) nanoparticles as biodegradable controlled drug delivery carrier. Polymers (Basel), 3(3), 1377-1397.

Jönnson, S., Davidse, A., Wilkins, J., Van der Walt, J., Simonsson, U.S.H., Karlsson, M.O., Smith, P., Melleron, H. 2011. Population Pharmacokinetics of Ethambutol in South African Tuberculosis Patients. Antimicrob. Agents Chemother. 55(9), 4230-4237.

Kadota, T., Matsui, H., Hirose, T., Suzuki, J., Saito, M., Akaba, T., Kobayashi, K., Akashi, S., Kawashima, M., Tamura, A., Nagai, H., Akagawa,S., Kobayashi, N., Ohta, K. 2016. Anaylsis of drug treatment outcome in clarithromycin resistant Mycobacterium avium complex lung disease. BMC Infect. Dis. 16,31.

Kaur, M., Garg, T., Narang, R.K., 2016. A review of emerging trends in the treatment of tuberculosis. Artif. Cells Nanomed. Biotechnol. 44(2),478-484.

Kilinc, O., Ucan, E.S., Cakan, M.D.A. 2002. Risk of tuberculosis among healthcare workers: can tuberculosis be considered as an occupational disease? Respir. Med. 96, 506–510.

Kisich, K.O., Gelperina, S., Higgins, M.P., Wilson, S., Shipulo, E., Oganesyan, E., Heifets, L. 2007. Encapsulation of moxifloxacin within poly (butyl cyanoacrylate) nanoparticles enhances efficacy against intracellular Mycobacterium tuberculosis. Int. J. Pharm. 345,154-162.

Ladavière, C., Gref, R. 2015. Toward an optimized treatment of intracellular bacterial infections: input of nanoparticulate drug delivery systems. Nanomedicine. 10(19), 3033-3055.

Lamprecht, A., Ubrich, N., Perez, M.H, Lehr, C-M., Hoffman, M., Maincent, P., 1999. Biodegradable monodispersed nanoparticles prepared by pressure homogenization-emulsification. Int. J. Pharm. 184(1), 97-105.

Lemmer, Y., Kalombo, L., Pietersen, R.D., Jones, A.T., Semete-Makokotlela, B., Van Wyngaardt, S., Ramalapa, B., Stoltz, A.C., Baker, B., Verschoor, J.A., Swai, H.S., De Chastellier, C., 2015. Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis. J. Control. Release. 211, 94-104.

Lemmer, Y., 2010. Assesment of mycolic acids as ligands for nanoencapsulated anti-tuberculosis drug targeting. Pretoria: UP. (Thesis – PhD).

Lotfipour, F., Valizadeh, H., Milani, M., Bahrami, N., Ghotasiou, R. 2016. Study of Antimicrobial Effects of Clarithromycin Loaded PLGA Nanoparticles against Clinical Strains of Helicobacter pylori. Drug Res. 66(1), 41-45.

Ma, W., Chen, M., Kaushal, S., McElroy, M., Zhang, Y., Ozkan, C., Bouvet, M., Kruse, C., Grotjahn, D., Ichim, T., Minev, B., 2012. PLGA nanoparticle-mediated delivery of tumor antigenic elicits effective immune responses. Int. J. Nanomed. 7, 1475-1487.

Meng, F.T., Ma, G.H., Qiu, W., Su, Z.G. 2003. W/O/W double emulsion technique using thyl acetate as organic solvent: effects of its diffusion rate on the characteristics of microparticles. J Control Release. 91(3), 407-416.

Miwa, S., Shira, M., Toyoshima, M., Shirai, T., Yasuda, K., Yokomura, K., Yamada, T., Masuda, M., Inui, N., Chida, K., Suda, T., Hayakawa, H. 2014. Efficacy of Clarithromycin and Ethambutol for Mycobacterium avium Complex Pulmonary Disease. A preliminary Study. Ann. Am. Thorac. Soc. 11(1), 23-30.

Muller, R., Keck, C., 2004. Challenges and solutions for the delivery of biotech drugs- a review of drug nanocrystal technology and lipid nanoparticles. J. Biotech. 113(1),151-170.

Mwandumba, H.C., Russell, D.G., Nyirenda, M.H., Anderson, J., White, S.A., Molyneux, M.E, Squire, S.B. 2004. Mycobacterium tuberculosis Resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. J. Immunol. 172(7), 4592-4598.

Naik, J.B., Lokhande, A.B., Mishra, S., Kulkarni, R.D. 2012. Development of sustained release micro/nano particles using different solvent emulsification techniques: A Review. Int. J. Pharm. Bio. Sci. 3(4), 573-590.

Nirbhavane, P., Vemuri, N., Kumar, N., Khuller, G.K.,2017. Lipid Nanocarrier-Mediated Drug Delivery System to Enhance the Oral Bioavailability of Rifabutin. AAPS PharmSciTech. 18(3), 829-837.

Palmino, J.C., Anandi, M. 2014. Drug Resistance Mechanisms in *Mycobacterium tuberculosis.* Antibiotics. 3(3), 317-340.

Pandey, R., Sharma, S., Khuller, G.K., 2006. Chemotherapeutic efficacy of nanoparticle encapsulated antitubercilar drugs. Drug Delivery.13(4), 287-294.

Panyam, J., Labhasetwar, V., 2003. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv. *Drug Deliver.* Rev. 55, 329-347.

Pinheiro, M., Lucio, M., Lima, J., Reis, S., 2011. Liposomes as drug delivery systems for the treatment of TB. Nanomedicine. 6(8),1413-1428.

Qin, Z. 2012. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis. 221,2-11.

Rae, N., Singanayagam, A., Stuart, S., Chalmers, J.D. 2017. Oral versus intravenous clarithromycin in moderate to severe community-acquired pneumonia: an observational study. Pneumonia. 9(2), 1-5.

Salouti, M., Ahangari, A. 2014. Nanoparticle based Drug Delivery Systems for Treatment of Infectious Diseases. Application of Nanotechnology in Drug Delivery. Intech, pp. 155-192.

Schramm, L.L. 2005. Colloid Stability. In Emulsions, Foams and Suspensions: Fundamentals and Applications. Weinheim: Wiley-VCH, pp. 117-152.

Semete, B., Booysen, L., Kalombo, K., Ramalapa, B., Hayeshi, R., Swai, H., 2012. Effects of protein binding on the biodistribution of Peglated PLGA nanoparticles post oral administration. Int. J. Pharm. 424(1-2), 115-120.

Steenwinkel, J.E.M., Van Vianen, W., Kate, M.T., Verbrugh, H.A., Agtmael, M.A., Schiffelers, R.M., Bakker-Woudenberg, I.A.J.M. 2007. Targeted drug delivery to enhance efficacy and shorten treatment duration in disseminated *Mycobacterium avium* infection in mice. J. Antimicrob. Chemother. 60(5),1064-1073.

Tanaka, E., Kimoto, T., Tsuyguchi, K., Watanabe, I., Matsumoto, H., Niima, A. 1999. Effect of clarithromycin regimen for Mycbacterium avium complex pulmonary disease. Am. J. Crit. Care Med. 160,866-872.

Tesfahuneygn, G., Medhin, G., Legesse, M. 2015. Adherence to Anti-tuberculosis treatment and treatment outcomes among tuberculosis patients in Alamata District, northeast Ethiopoia. MBC Res Notes. 8 (503), 1-11.

Vinneeth, P., Vadaparthi, P.R.R., Kumar, K., Babu, B.D.J., Rao, A.V., Babu, K.S. 2014. Influence of organic solvents on nanoparticle formation and surfactants on release behaviour in-vitro using costunolide as a model anticancer agent. Int. J Pharm. Sci. 6(4), 638-645.

Vyas, S.P., Kannan, M.E., Jain, S., Mishra, V., Singh, P. 2004. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Int. J. Pharm. 269 (1), 37–49.

Win, K.Y., Feng, S.S. 2005. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials. 26(15), 2713-2722

Chapter 4: PREPARATION AND IN VITRO EVALUATION OF A HYBRID DELIVERY SYSTEM CONSISTING OF CLARITHROMYCIN AND ETHAMBUTOL POLY (D, L-LACTIC-CO-GLYCOLIC ACID) PARTICLES ENTRAPPED INTO PHEROID® VESICLES FOR POTENTIAL ENHANCEMENT OF MYCOBACTERIUM AVIUM COMPLEX THERAPY

Aeysha Jakoet[a],

Yolandy Lemmer[a],

Lonji Kalombo[a]

Anne F. Grobler[b]

a) CSIR Materials Science and Manufacturing, Polymers and Composites, Pretoria, 0001, South Africa

b) DST/NWU Preclinical Drug Development Platform, Faculty of Health Sciences, North-West University, Potchefstroom, 2531, South Africa

4. 1. Introduction to the chapter

This chapter was written for the International Journal of Nanomedicine (see Annexure B for author guidelines). This journal focuses on the application of nanotechnology in the areas of drug delivery, diagnostics and therapeutics. This journal highlights research and development that may lead to potential clinical application and treatment of various diseases. This manuscript intended for submission to the journal focuses on the application of a combined/hybrid delivery system of PLGA NPs loaded with CLR and ETB together with a targeting ligand of MA entrapped into Pheroid® vesicles. This investigation provided a preliminary screening to the possibility of effective hybrid drug delivery system that could potentially enhance the treatment of MAC, as to date, no optimum treatment regime exists.

Abstract

To overcome the challenges of conventional therapy and enhance the treatment of *Mycobacterium avium* complex (MAC) infections, there is a need for new, modern and effective delivery systems. In this preliminary study we investigated the ability of a hybrid delivery system to potentially enhance the cellular uptake of MAC therapeutics into THP-1 macrophages.

Pre-formulated clarithromycin (CLR) and ethambutol (ETB)-loaded PLGA nanoparticles (NP) with and without mycolic acids (MA) were entrapped into Pheroid® vesicles. The resultant hybrid delivery system was characterised in terms of its size and visualised by means of confocal laser scanning microscopy (CLSM) and followed by its uptake capabilities into THP-1 macrophages.

Drug free (DF) and ETB-loaded PLGA NPs were successfully entrapped into Pheroid® vesicles whereas CLR-loaded PLGA NPs deemed more challenging. The cytotoxicity results displayed Pheroid® vesicles cytotoxic to HeLa cells at concentrations of ≥ 2% (v/v). Further evaluation indicated that Pheroid® vesicles were cytotoxic at low concentrations when exposed to THP-1 macrophages after 48 hours of incubation. Visual observations made with the preliminary *in vitro* uptake studies in THP-1 macrophages revealed a greater density of PLGA-MA NPs in close proximity within THP-1 macrophages after 1 hour of incubation when compared to the control of PLGA NP formulations without Pheroid® vesicles.

In summary, the PLGA NP-Pheroid® vesicle hybrid system presents the potential to be considered as an attractive and promising approach to enhance the current conventional therapy for MAC.

Keywords: cellular uptake, THP-1 macrophages, poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles, Pheroid® vesicles

4.2. Introduction

Mycobacterium avium complex (MAC) is a group of slow-growing non-tuberculous mycobacteria (NTM) that are ubiquitous to the environment.[1] MAC is commonly treated with clarithromycin or azithromycin, ethambutol and rifamycin, but to date, an optimum treatment has not yet been established.[2]

Drug delivery could potentially solve and overcome the limitations of conventional therapy by its ability to adapt the physical properties surrounding the drug.[3] A drug delivery system can be modified in terms of its biocompatibility, bio-distribution and target-specificity which would in turn modify the treatment regimen and duration.[4] A few of these drug delivery systems include polymers, liposomes, nanocrystals, Pheroid® and dendrimers[5,6] each with varying advantages and limitations.

Owing to the adaptability of these systems, one could propose the modification of drug delivery systems with the implementation of 2 different types of systems, i.e. a hybrid system. A hybrid system has the potential to exploit the advantages and resolve the disadvantages presented by the single systems. In this study we investigated a lipid-polymer hybrid nanoparticle (LPN) system encapsulated within a Pheroid® a similar approach as was shown by Chelopo and co-workers.[7]

LPN hybrid systems are advantageous as they are thought to assist in the enhancement of biocompatibility, stability and favourable pharmacokinetic profiles of varying active ingredients[8]. Furthermore, it is presumed that the presence of polymeric and liposome domains may contribute to an increase in drug loading and encapsulation efficiency of hydrophilic and hydrophobic therapeutic actives.[9]

There are several applications of these hybrid systems reported in the literature. Recent literature published by Zeng and co-workers showcased this hybrid system as a promising application to overcome cancer drug resistance[10]. Further applications include the encapsulation of docetaxel together with a targeting ligand into a LPN which has demonstrated excellent tumour targeting and significantly lowered side effects when compared to free drugs, therefore suggesting its potential use in clinical cancer treatment[11]. Varying actives known to be delivered by LPNs include; docetaxel,[12] paclitaxel[13] and curcumin[14]. Research published by Wong and colleagues has also shown that doxorubin in a LPN, displayed an 8-fold increase in cytotoxicity when compared to free drug, however cellular uptake and drug retention by the targeted cells was significantly enhanced[15]. Zhang and co-workers published results that utilised poly (D,L-lactic-co-glycolic acid) (PLGA) particles to form the hydrophobic core and

lecithin as the lipid monolayer. This hybrid system featured high drug loading, good sustained drug release, good serum stability and good cellular targeting ability[16].

Delivery of actives from these hybrid systems can be further enhanced with the addition of targeting ligands. Owing to the versatility of both these delivery systems, targeting ligands can be added to the surface of the lipid or polymeric system. Research by Zhang and co-workers have produced paclitaxel loaded polymeric NPs encapsulated into a folate modified lipid shell. Folate was chosen as the targeting ligand to cancer cells. With the addition of the folate, there was efficient internalization and increase cytotoxicity when compared to folate free LPN[17]. In this present work, we focused on the targeting ligand on the surface of the polymeric system. An interesting and novel targeting ligand combined with a PLGA polymeric NP system for the treatment of tuberculosis has been investigated by Lemmer and co-workers[18]. Mycolic acids (MA) which are found in the mycobacterial cell wall have been exploited owing to the hypothesis that the MA on the surface of the NP may interact with anti-MA antibodies in the area of infection and promote an enhanced uptake of NPs of infected and uninfected macrophages[18]. With this novel concept, we propose that the inclusion of MA in PLGA NPs loaded with ETB and CLR combined with the Pheroid® delivery system as the lipid component for the LPN hybrid system, offers a unique and promising approach to overcome the current flaws presented by the current treatment regime.

Lipids in combination with polymeric particles have shown to enhance physiochemical properties of the active ingredients. The Pheroid® delivery system is an essential fatty acid system that has shown better membrane penetration, lower cytotoxicity, enhanced bioavailability and increased encapsulation efficiency[6]. With its application to malaria, this technology has resulted in increasing the anti-malarial activities of azithromycin, mefloquine, and quinine significantly *in vitro* [19,20]. The *in vivo* pharmacokinetic profile of artemisone was improved as well[21]. Recent work published by Chelopo and team has demonstrated the development of a Pheroid®-PLGA NP hybrid system with PLGA NPs of positively and negatively charged NP surfaces entrapped into Pheroid® vesicles via two encapsulation methods, i.e. the NPs are added in the hydrophobic phase of the Pheroid® preparation (pre-mix) or the NPs are added to freshly prepared vesicles and mixed (post-mix)[7].

Herein we used previously prepared PLGA NPs loaded with MAC therapeutics together with MA that were prepared via a double emulsion solvent evaporation technique which was furthermore encapsulated into Pheroid® vesicles via a post mix approach. The particles were in the range of 305-413 nm for drug free (DF), ETB loaded and CLR loaded PLGA particles with and without MA. All particles were negatively charged and in the range of -8.2 mV for DF NPs and -26.5 – 29.8 mV for ETB and CLR loaded NPs with and without MA. Particles were

spherical and smooth in shape. The physiochemical properties for this combined system was visualised and confirmed with dynamic light scattering (DLS) and confocal laser scanning microscopy (CLSM). The cytotoxicity performance of the Pheroid® vesicles was investigated against 2 different cell lines and the ability of these formulations to be taken up into THP-1 macrophages was assessed via microscopy.

4.3. Materials and methods

4.3.1 Materials

Pheroid® vesicles were prepared with Kolliphor ® RH 40 (BASF SA, (Pty) Ltd, South Africa), DL-α-tocopherol (Chempure (Pty) Ltd., South Africa), vitamin F ethyl ester CLR (Chemisches Laboratorium, Dr. Kurt Richer GMbH, Germany) and medical nitrous oxide (N_2O) was supplied from Afrox South Africa.

For cytotoxicity and uptake evaluation, foetal calf serum (FCS), penicillin/streptomycin, Roswell Park Memorial Institute (RMPI)–1640 medium with L-glutamine and Dulbecco's modified Eagle's low glucose medium (DMEM) were obtained from Life technologies, South Africa. Phorbol 12 myristate 13-acetate (PMA) used for cell differentiation and paraformaldehyde for cell fixation, was obtained from Sigma Aldrich Co., St Louis, USA and Merck, Darmstadt, Germany respectively.

4.3.2 Methods

4.3.2.1 Nanoparticle and Pheroid® vesicle preparation

Prepared particles were donated by the Council Scientific Industrial Research (CSIR), Materials Science and Manufacturing, Polymers and Composites, Pretoria, South Africa.

Briefly prepared as follows; a double emulsion solvent evaporation technique, followed by freeze-drying[18,22]. Briefly, 100 mg of PLGA 50:50 (Mw: 30 000-60 000) was dissolved in dichloromethane (DCM) (6 mL) with or without the MA pre-dissolved in dichloromethane. To this solution, 1% (w/v) PVA aqueous phase (2 mL) containing ETB (100 mg) was added and homogenised by means of a Silverson high speed homogeniser (Silverson L4R Buckinghamshire, UK) at 8000 rpm for 3 minutes. The resulting water-in-oil emulsion (w/o) was added to 40 mL of 2% w/v PVA aqueous solution. This mixture was further emulsified for 7 minutes at 8000 rpm resulting in the final double emulsion (water-in-oil-in-water, w/o/w). The final w/o/w emulsion was stirred overnight at room temperature to allow solvent evaporation and subsequent precipitation of NPs.

For CLR NPs:

CLR (100 mg) was dissolved in the 6 mL DCM with or without pre-dissolved MA. The emulsification was followed exactly to that described for ETB NPs. For particle collection, the evaporated emulsion underwent a double centrifugation process that assisted in the separation of particles of different sizes at 845 and 33 902 rcf for 10 and 15 minutes respectively. The supernatant was collected and further analysed for free drug. The resulting particles were dried by lyophilization in a Virtis Benchtop freeze dryer (SP Industries, Gardiner, New York, USA).

For Fluorescent labelled NPs:
50% 5-bromomethyl fluorescein labelled mycolic acid (5BMF-MA) were prepared for cellular uptake evaluation. 5BMF-MA NPs were prepared similarly to the method described earlier for ETB and CLR-loaded PLGA NPs, with a direct substitution of MA with the 50% labelled MA (5BMF).

Pheroid® vesicles were prepared by the following method. Sterile water saturated with N_2O gas was heated and maintained at 70 ^{0}C. The oil phase composed of Kolliphor EL (1.0% w/w) and vitamin F ethyl ester (2.8% w/w) was heated to 70 ^{0}C. The heated oil constituents were mixed and cooled to 55 ^{0}C followed by the addition of dl-α-Tocopherol (0.2% w/w). The heated oil phase was added to the heated N_2O water (96% w/w) followed by homogenisation with a Heidolph Diax 600 homogeniser (Heidolph, Germany) at 13 500 rpm for 4 minutes whilst cooling to 40 ^{0}C. The mixture was left in an orbital shaker set at 150 rpm and shaken overnight at room temperature.

4.3.2.2 Combination of fluorescent MA PLGA NPs at varying concentrations into Pheroid® vesicles

The donated NPs were incorporated into the Pheroid® vesicles via a post-mix approach. A series of encapsulation concentrations of PLGA NPs were evaluated. The previously prepared Pheroid® vesicles was aliquoted, followed by the addition of varying mass of NPs and shaken overnight (12 hours) to yield a final suspension with a concentration of 2, 1.5, 1 and 0.5 mg/mL (NP/Pheroid®) for each test PLGA formulation.

4.3.2.3 Size determination

The mean vesicle size was measured through light scattering with a particle size analyser (Malvern Mastersizer, Malvern Instruments, UK). Pheroid® vesicles and Pheroid®-NP combinations were diluted at a factor of 100 and stirred continuously during analysis to obtain a homogenous dispersion for an accurate measurement[24].

4.3.2.4 Visualisation of fluorescently labelled PLGA NPs into Pheroid® vesicles

The morphological features and confirmation of encapsulation was viewed with confocal laser scanning microscopy (CLSM) using a Nikon Eclipse TE-3000 with the addition of 50 µl of Nile red to a 50 µl of Pheroid®-NP test formulations (0.5mg/mL) followed by sample vortexing. The microscope was equipped with a green krypton laser (wavelengths: excitation (Ex), 488 nm, emission (Em), 515 nm) and a red helium neon laser (wavelengths: Ex, 505 nm, Em, 564 nm). The formulations were placed on a glass slide and sealed with a cover slip to prevent sample loss.

4.3.2.5 Cell culture maintenance and exposure to test formulations

HeLa cells and THP-1 monocytes were grown in (DMEM) and (RPMI)-1640 medium respectively, supplemented with penicillin (50 µg/ml), streptomycin (50 µg/ml) and 10% heat inactivated FCS. Cells was maintained at a confluency of 0.5 – 5 x 10^6 cells/ml and incubated at 37 ºC in a 5% CO_2 (g) humidified incubator and passaged 2-3 times a week. For adherent HeLa cells, cells were trypsinised (0.25% trypsin) and passaged after confluency was reached.

The cytotoxicity profile of Pheroid® vesicles was evaluated against the HeLa cell line. Vesicle suspensions were freshly prepared and diluted to appropriate concentrations of 10, 8, 6, 4, 2, 1, 0.1, 0.01 and 0.001% (v/v) with serum and antibiotic free culture medium. Cells not treated with Pheroid® vesicles served as controls in each experiment. Owing to the cytotoxic results obtained from the serial dilution investigation on the HeLa cell line, the lowest concentration (0.001%, v/v) was used for an additional study to investigate whether the vesicles induced cell death after an extended period of exposure (48 hours). Furthermore, the lowest concentration (0.001%, v/v) of vesicle suspensions was exposed to THP-1 macrophages at 24 and 48 hours.

4.3.2.6 Cytotoxicity of PLGA NPs and Pheroid® vesicles on THP-1 macrophages and HeLa cells

The effect of Pheroid® vesicle suspension on the viability of HeLa cells were evaluated using the WST [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assay. The WST assay was used in accordance to the method prescribed by the manufacturer. Briefly, HeLa cells were seeded into 96-well plates at a density of 1 x 10^5 cells/well per 100 µL of DMEM, (10% FCS) and incubated for 48 hours at 37 ºC to allow for adherence onto the wells. The THP-1 monocytes were differentiated into macrophages with the addition of 50 nM PMA to wells containing a concentration of 1 x 10^6 cells/ml with a total volume of 2 ml per well and was incubated for 48 hours to allow for cell differentiation[25]. The formation of macrophages was confirmed with microscopy.

After cell exposure, 20 µL of the WST-1 solution was added to each well and incubated for 45 min for HeLa cells and 2 hours for THP-1 cells. The tetrazolium salt that was reduced to an orange formazan by cellular mitochondrial dehydrogenase present in viable cells was measured with a spectrophotometer (Infinite F500, Tecan Group Ltd, Mannedorf, SC) with a measurement wavelength of 492 nm and a reference wavelength of 620 nm. The result obtained from this assay was given as relative values to the untreated control in percentage. All experiments consisted of a single sample set of n= 16, to provide a preliminary indication of the cell viability.

4.3.2.7 Uptake of fluorescent labelled particles into THP-1 macrophages

For the uptake of PLGA and Pheroid® hybrid, neat Pheroid® was diluted with excess nitrous oxide water to achieve a concentration of 0.01%. The 0.01% was further diluted with serum free RPMI-1640 to achieve a concentration of 0.001 %. 0.1 mg of fluorescent particles was added to 1 mL of the 0.001 % Pheroid® suspension to achieve the final test concentration of 0.1mg/mL and left to agitate overnight. The final test suspension was added to newly differentiated macrophages on glass slides and incubated for 1 hour to evaluate the uptake capability.

After treatment, the slides were washed in triplicate with phosphate buffer solution (PBS) with1 mM $CaCl_2$ and 1 mM $MgCl_2$ and subsequently washed with distilled water. The macrophages were then fixated on the glass slides by the addition of paraformaldehyde (3%) in PBS followed by a 20 minute incubation period. The cover slip was drip-dried and mounted face down with fluoroshield (mounting medium), which protected the NP fluorescence. The cover slip was sealed onto the glass slide with clear nail polish. The prepared slides were viewed with a confocal microscope to determine the possible uptake of NPs.

4.4 Results and Discussion

4.4.1 Characterisation of Pheroid® vesicles

The size distribution of the Pheroid® vesicles (control) and the potential hybrid system is represented in Figure 4.1. The varying of concentration was investigated to observe the effect of the NP/Pheroid® combination with and without the targeting ligand, MA, on the size.

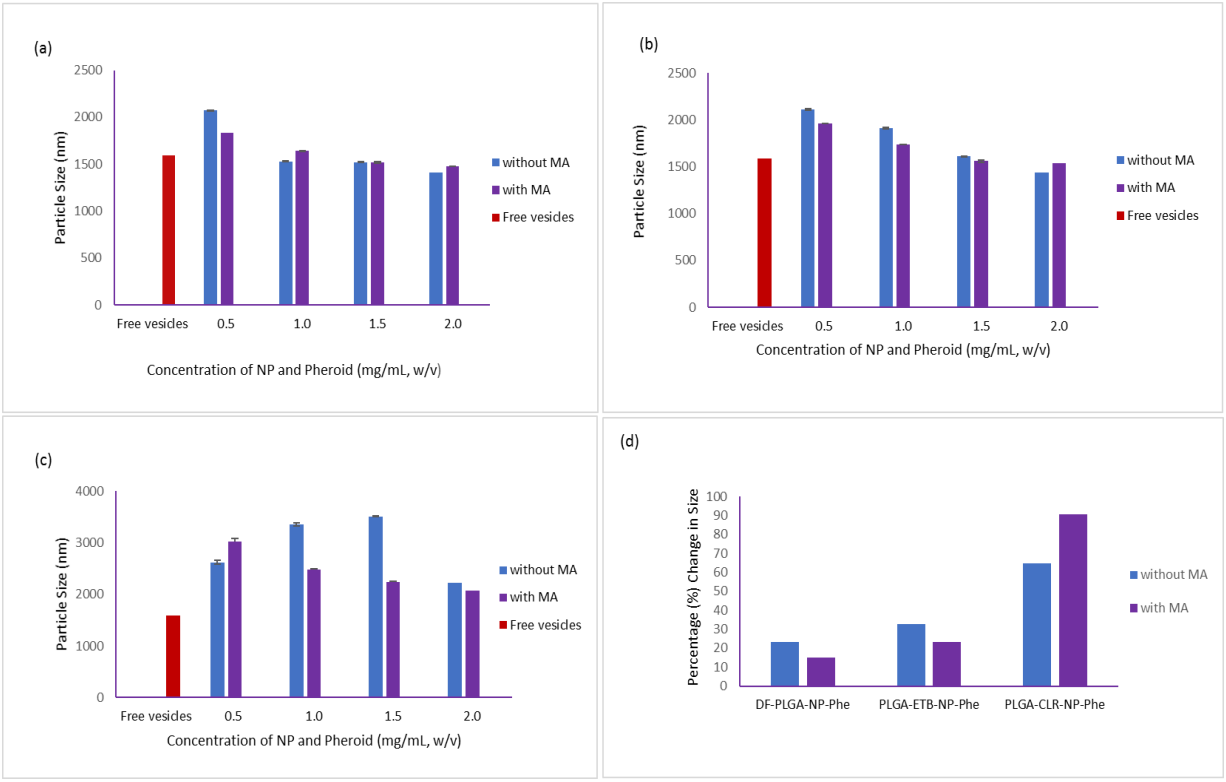


Figure 4.1: Graphs displaying the effect on Pheroid® vesicle size when varying the NP/Pheroid® (mg/mL) mixing concentration, 1a: DF PLGA NP with and without MA, 1b: PLGA-ETB NPs with and without MA and 1c: PLGA-CLR NPs with and without MA, 1d: Percentage change in size of Pheroid® vesicles post NP addition

An increase in size of the control Pheroid® vesicles could suggest the successful encapsulation of the NPs into the vesicles but will be confirmed with CLSM. The lowest concentration of 0.5mg/mL (NP/Pheroid®) had greatest influence of the size when compared to the higher concentrations for DF and ETB encapsulation NPs.

For DF-PLGA hybrid systems (0.5mg/mL), the median displayed a 30% and 15% increase (Figure 4.1, d) for formulations with and without MA, respectively, when compared to the control. The same trend was observed for PLGA-ETB hybrid systems with and with MA, with

an increase in 33% and 23% respectively. No noteworthy change in size was observed with the addition of MA to the system, however a decrease in size was observed with increasing concentration, when compared to free vesicles suggesting that higher concentration may interfere with the synergy of the combined system.

PLGA-CLR NPs hybrid systems (0.5mg/mL), however had the highest change in size with an increase of 65% and 97% for formulations with and without MA, respectively, when compared to the control. These results differed vastly from DF and ETB loaded PLGA NPs. However, for all CLR systems a phase separation was observed which was indicative of instability. The increased size may be attributed to the breaking down of vesicles and a result of vesicle coalescence. During the synthesis of the PLGA NPs, CLR was co-dissolved with the PLGA owing to its hydrophobic nature and insolubility in water[26]. Thus, suggesting that CLR may have been expressed onto the polymeric surface and resulted in an unfavourable surface modification. This may have interfered with the stability of the NP-Pheroid® hybrid system as the stability of LPNs have shown to be dependent on the chemical composition and size of the hydrophobic segments between the polymers and lipids as well as the chemical composition of the nanoparticles[27,28]. The CLR may have resulted in the incompatibility of the thermodynamics of the two systems which may have resulted in the phase separation which is true for LPN hybrid systems[28].

To confirm the size distribution of the combined systems for all three NP formulations, confocal microscopy was used to provide a visual perspective of the hybrid system. Owing to the greatest change in free vesicle size, 0.5 mg/mL of the MA fluorescent labelled PLGA NPs; 5BMF-MA PLGA NPs (DF, ETB and CLR) combined with Pheroid® vesicles was viewed with microscopy.

4.4.2 Microscopy of combined NPs into Pheroid® vesicles

The morphological features and adjustments were observed with CLSM by the means of a fluorescent probe, Nile red (a phenoxazine dye) that was capable of red lipid staining.

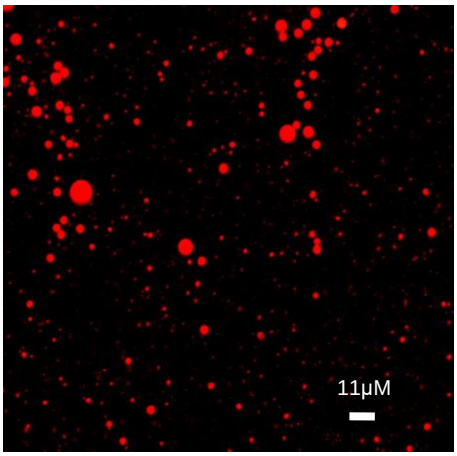


Figure 4.2: Confocal image of control Pheroid® vesicles stained with Nile red. (Scale bar 11 µm), wavelengths: Ex: 505 nm; Em: 564 nm.

Figure 4.2 is representative of free Pheroid® vesicles without the addition of NPs. The vesicles were spherical and smooth in shape with strong red fluorescence owing to the binding of nile red to the lipid layer of the vesicles.[29] A poly-dispersed system with a multimodal size distribution was observed.

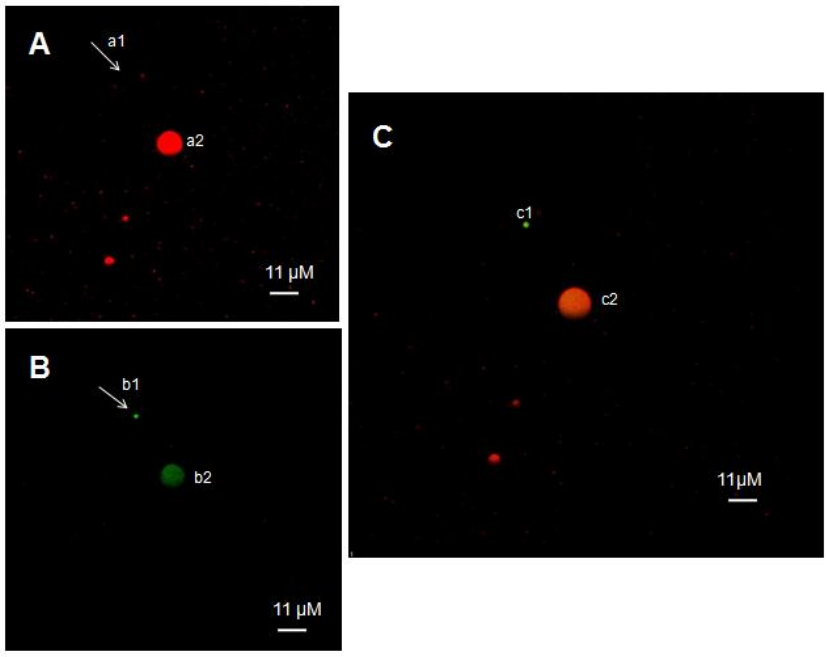


Figure 4.3 (Drug free loaded PLGA NPs): Confocal images of 0.5mg/mL (w/v) of DF-PLGA-MA combined with Pheroid® vesicles. Image **4.3(A) and 4.3(B**) viewed in the red (Ex: 505 nm; Em: 564 nm) and green (Ex: 488 nm; Em: 515 nm) channel respectively and **4.3(C)** merged red and green channels. (Scale bars 11 µm).

For the mixture of Pheroid® vesicles and fluorescently labelled NPs the hybrid sample was viewed in the red and green fluorescent channel as well as the merged channel to evaluate potential NP encapsulation.

Image 4.3(A) illustrates the sample viewed in the red channel. Pheroid® vesicles of different sizes was viewed and was in agreement with the Mastersizer results as a multimodal distribution was observed. Image 4.3(B) was viewed in the green channel.

In the merged channel (Image 4.3(C)), an orange vesicle was observed at the position c2. When compared to Image 4.3(A) and 4.3(B), a red and green fluorescence spot was observed at same position of the orange vesicle observed in Image 4.3(C) thus inferring that a Pheroid® vesicle and PLGA NPs was present at position c2 therefore accounting for the orange vesicle observed. These results suggest that 5BMF-DF-PLGA-MA NPs was encapsulated into the Pheroid® vesicles. A vast difference in size existed between the NPs and vesicles thus resulting in difficulty in visualisation of single NPs encapsulated into smaller vesicles. Very few free NPs and orange vesicles were observed which was best explained by the potential clustering of 5BMF-DF-PLGA-MA NPs into singular vesicles. Further speculation lead to the assumption that a large number of NPs were encapsulated into a Pheroid® vesicle in order to emit from within the vesicle and have an effect on the red fluorescence. The successful encapsulation of PLGA NPs (green fluorescent labelled) into Pheroid® vesicles (stained with nile red) was too confirmed with confocal microscopy by Chelopo and team[7].

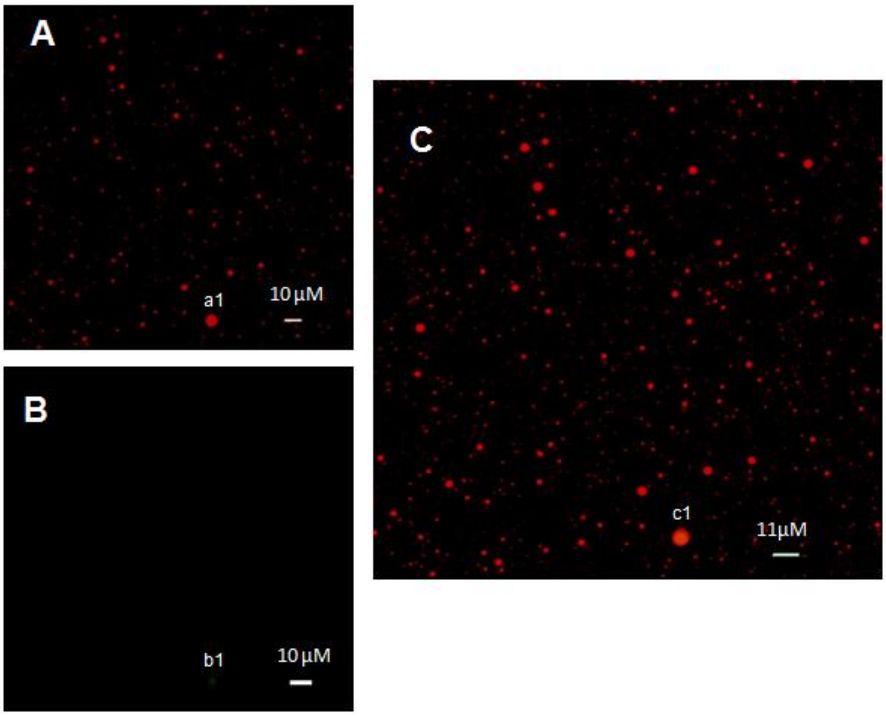


Figure 4.4(Ethambutol loaded PLGA NPs): Confocal images of 0.5mg/mL (w/v) of PLGA-ETB-MA combined with Pheroid® vesicles. Image **4.4(A) and 4.4(B)** sample viewed in the red (Ex: 505 nm; Em: 564 nm) and green (Ex: 488 nm; Em: 515 nm) channel respectively and **4.4(C)** merged red and green channels. (Scale bars: 11 µm).

Image 4.4(A), depicts the sample of 5BMF-PLGA-ETB-MA NPs mixed with Pheroid® vesicles viewed in the red fluorescent channel. Image 4.4(B) was representative of the sample viewed in the green channel. In the merged channel, an orange vesicle was observed at position c1 of Image 4.4(C) which was identical to the red spot (a1) and green spot (b1) observed in the red and green channel respectively. The orange vesicle was indicative of the potential encapsulation of 5BMF-PLGA-ETB-MA NPs into Pheroid® vesicles. From this observation, it was concluded that the hydrophilic nature of the encapsulated ETB did not affect the encapsulation process into Pheroid® vesicles

Figure 4.5, depicts the confocal images of 5BMF-PLGA-CLR-MA NPs into Pheroid® vesicles. Image 4.5 (A) depicted the sample viewed in the red fluorescent channel. Pheroid® vesicles of different sizes was observed which was agreement with the Mastersizer results. Image 4.5 (B) is representative of the sample viewed in the green channel. Multiple small fluorescent spots was viewed in this channel owing to 5BMF-PLGA-CLR-MA NPs.

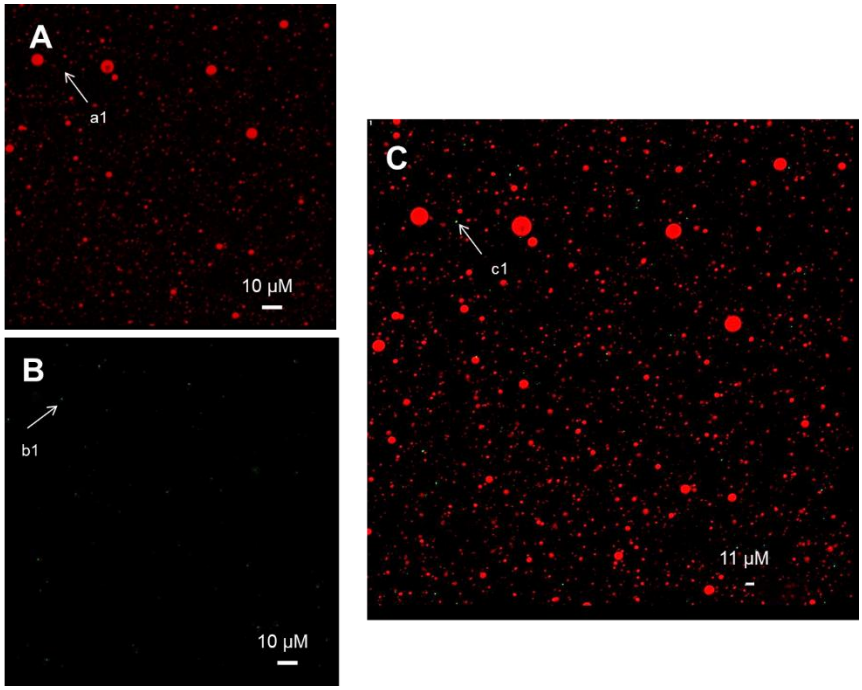


Figure 4.5: Clarithromycin loaded PLGA NPs: Confocal images of 0.5mg/mL (w/v) of PLGA-CLR-MA combined with Pheroid® vesicles. Image 4.5 (A) and 4.5(B) sample viewed in the red (Ex: 505 nm; Em: 564 nm) and green (Ex: 488 nm; Em: 515 nm) channel respectively and 4.5 (C) merged red and green channels. (Scale bars 11 µm).

In the merged channel (Image 4.5 (C)), no orange vesicles were observed. The red spots observed in red channel was observed at different positions to the green spots observed in the green channel. No overlapping occurred. These results infer that 5BMF-PLGA-CLR-MA NPs did not encapsulate into Pheroid® vesicles, despite having undergone the same experimental conditions, i.e. encapsulation time and concentration. These microscopy images substantiate the theory that the size distribution and phase separation was indicative of instability of the hybrid system[26].

Additional experiments are warranted to conclude the observations made. However, Cheow and team have speculated the formation of the lipid layer around polymeric NPs was driven by the electrostatic interactions between the vesicles and polymeric NPs.[30] Therefore, for the case of the CLR loaded PLGA NPs, the CLR together with MA may have resulted in unfavourable interactions between the NP and Pheroid® vesicle.

4.4.3 The cytotoxicity profile of Pheroid® vesicles on HeLa cells

The WST-1 assay was normalised by the viability of 'untreated' cells cultured without the exposure to the NPs and thus a value close to 100% was indicative of non-toxic cell culture conditions

The cytotoxic profile of Pheroid® vesicle suspensions at varying concentrations (v/v) were initially evaluated in HeLa cells as they are easily cultured and resilient [31].

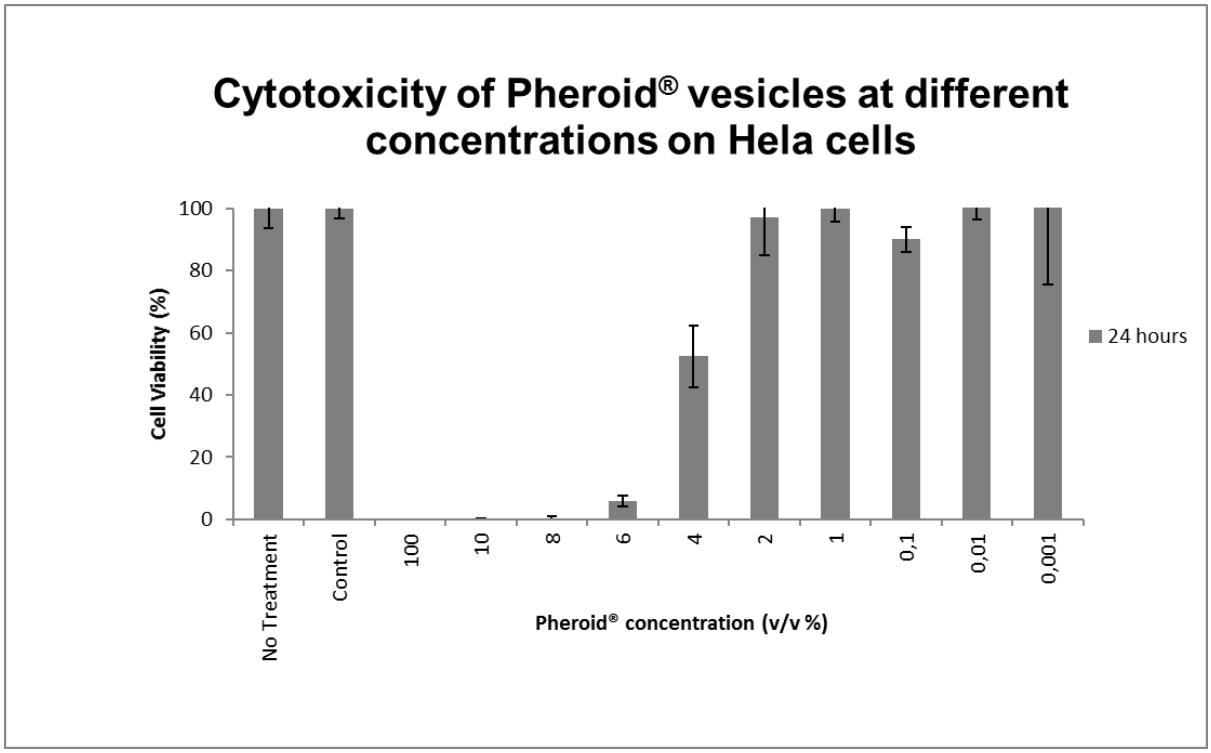


Figure 4.6: Cytotoxicity profile of Pheroid® vesicles against HeLa cells at different concentrations; No treatment = untreated cells, control = 0.1 mg/mL of ETB which was previously established as non-cytotoxic (n= 16).

Figure 4.6, is representative of the cytotoxicity profile of Pheroid® vesicles at varying concentrations on HeLa cells. At concentrations of ≥ 2% (v/v) of vesicle suspensions, a greater cytotoxic effect was observed on the cells whereas concentrations of ≤ 1% a non-cytotoxic effect was observed. The great cytotoxic effect at high concentrations was best explained by the high concentration of water used in its preparation. After exposure of Pheroid® vesicles at high concentrations for 24 hours of incubation, the cells experience a drastic change in viability. During an investigation by Selzner and team, it was shown that cells experienced cell death after exposure to 100% distilled water (hypotonic stress) for time intervals of 1, 3 and 5 minutes.[31] A plausible assumption is that HeLa cells may have experienced the same fate, owing to the Pheroid® vesicle's composition of 96% water. However, in this investigation, lower concentrations were used but with a great increase in exposure time; 24 hours instead of minutes. Owing to the low cytotoxic effect displayed at low concentrations of Pheroid® vesicles, only the lowest concentration (0.001%) was evaluated for a longer duration (48 hours). Furthermore, the cell viability of the lowest concentration was tested and observed on

THP-1 macrophages to test and identify if a cytotoxic risk existed despite its low concentration as they have been shown to be sensitive to many stimuli[32].

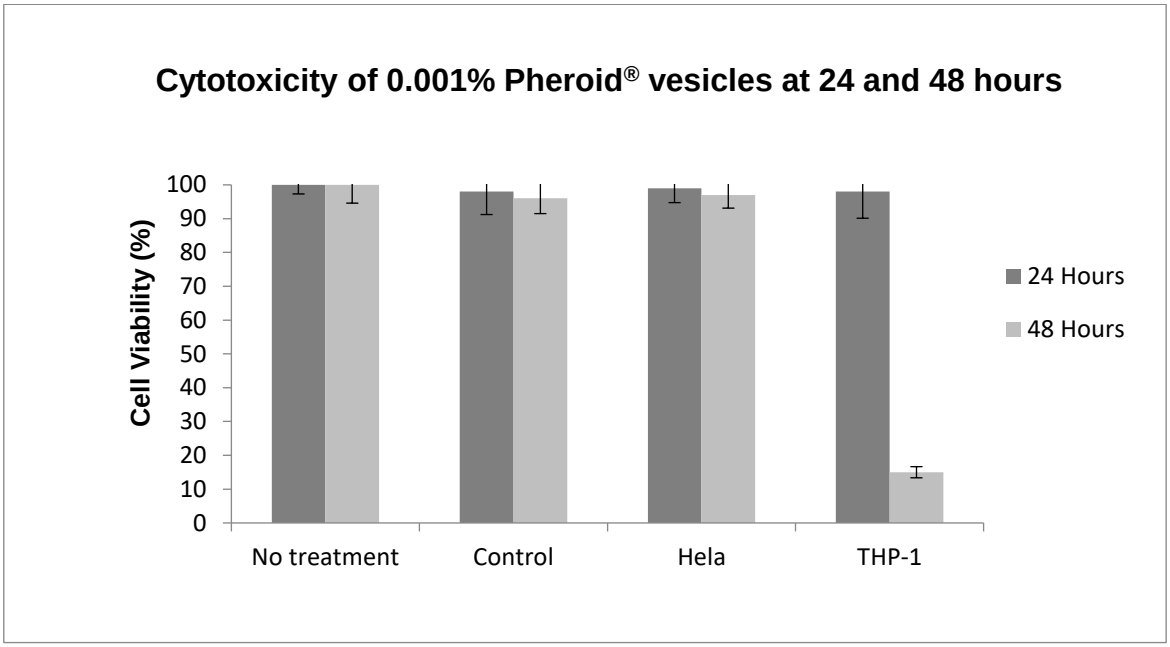


Figure 4.7: The cytotoxicity profile of Pheroid® vesicles against HeLa cells and THP-1 macrophages at 24 and 48 hours of incubation. No treatment = untreated cells, control = 0.1 mg/mL of ETB which was previously established as non-cytotoxic.

Figure 4.7 shows the cell viability of HeLa and THP-1 macrophages at 24 and 48 hours after the treatment of 0.001% (v/v) vesicle concentration. There was no cytotoxic effect on HeLa cells and THP-1 macrophages after 24 hours. In contrast to the 24-hour data a drastic decline of cell viability was observed at 48 hours for the THP-1 cells. The viability dropped to an overall low of 15%. The drastic difference in cellular viability between the two cell lines was attributed to the different internalisation/uptake mechanisms exhibited by HeLa cells and THP-1 monocytes that were differentiated into macrophages. HeLa cells are not macrophages and macrophages are phagocytes[33]. Therefore, it was inferred that the high cellular death of THP-1 macrophages was a result of its ability to randomly phagocytose any foreign material by engulfing it and therefore potentially resulting in a higher concentration of Pheroid® vesicles within cells when compared to HeLa cells which may have not experience its full cytotoxic effect at 48 hours[34]. As this preliminary screening provided a good indication of the cytotoxic behaviour of the Pheroid® vesicles, the repeat of this screening in triplicate together with fluorescent labelling of Pheroid® vesicles for future work is encouraged before further conclusions may be drawn.

Despite the cytotoxicity of Pheroid® vesicles at 0.001% (v/v) on THP-1 macrophages at 48 hours, it was hypothesised that the low vesicle concentration would be non-cytotoxic after 1 hour of exposure owing to the minimal cell viability effect after 24 hours. Therefore, this concentration was evaluated for the subsequent uptake studies.

4.4.4 Uptake of labelled MA-PLGA NPs with Pheroid®

To evaluate the uptake capability of PLGA-Pheroid® hybrid system, 0.1 mg of 5BMF-MA PLGA NPs with and without ETB was added to 0.001% Pheroid® vesicles. Only DF and ETB-loaded PLGA NPs were investigated owing to previously reported confocal microscopy evaluation where the CLR-loaded particles appeared not to be encapsulated.

The uptake ability of 5BMF-DF-PLGA-MA (0.1 mg/mL) and 5BMF-PLGA-ETB-MA (0.1 mg/mL) NPs was viewed with confocal microscopy after 1 hour of incubation and served as a control to the formulations coated with Pheroid® vesicles. Figure 4.8 represents the successful uptake of DF particles (A1) and ETB particles (A2), after 1 hour into macrophages. Figure 4.8(B1) and 4.8(B2) represents the hybrid 5BMF-DF-PLGA-MA NPs and 5BMF-PLGA-ETB-MA NPs taken up THP-1 macrophages.

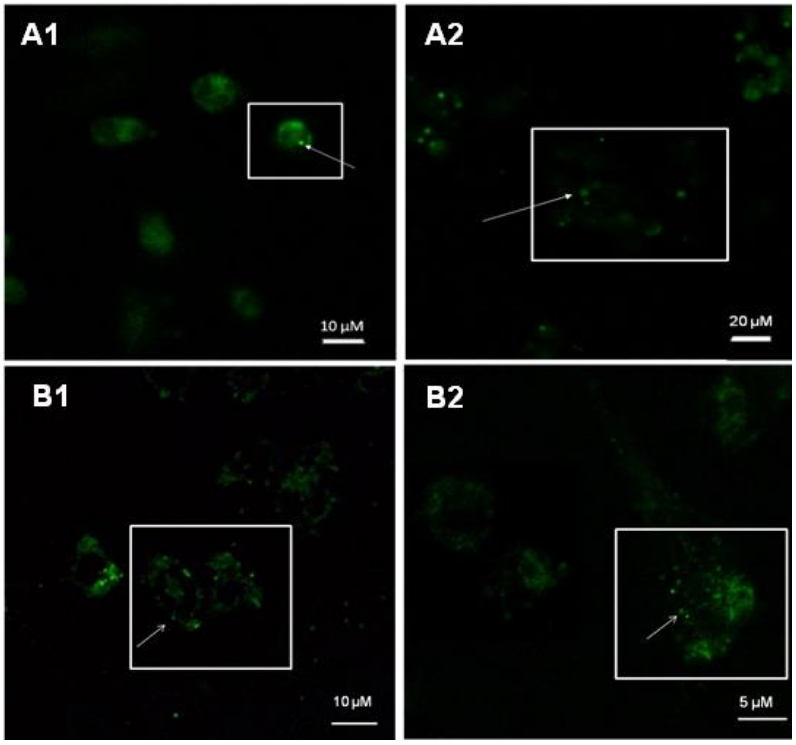


Figure 4.8: Fluorescent microscopic images of PLGA-Pheroid® vesicle hybrid system taken up by THP-1 macrophages, A1:5BMF-DF-PLGA-MA NPs, A2: 5-BMF-PLGA-ETB-MA NPs, B1: 5BMF-DF-PLGA-MA-Pheroid® hybrid system and B2:5BMF-PLGA-ETB-MA-Pheroid® hybrid system. Wavelength: Ex: 488 nm; Em: 515 nm.

Both hybrid systems (B1 and B2) were taken up by THP-1 macrophages after 1 hour of incubation. It is not clear from the images whether the Pheroid® vesicles remained with the particles. Nile red for Pheroid® visualisation was not used as it non-specifically stains any lipids. Visually a greater density of NPs was observed in close proximity to the macrophages after 1 hour of incubation for hybrid formulations when compared to control. This was hypothetically attributed to the ability of the Pheroid® to cross physiological barriers[6]. However, further investigations are needed in order to accept or reject this assumption. It was further suggested that the attachment of a fluorescent probe directly to one of the components of the Pheroid® vesicle system would shed light to whether the Pheroid® vesicle assisted in cellular uptake[11].

The images further revealed that the THP-1 macrophages experienced cell death after 1 hour of incubation. This was unexpected as the individual systems were non-cytotoxic at 0.1 mg/mL of nanoparticles (results not shown) and 0.001% (v/v) Pheroid® vesicle concentration after 24 hours of exposure. A study by Wong and team reported a similar result where individual components of a hybrid system exhibited little cytotoxicity however, when these components were combined an 8-fold increase in cytotoxicity was observed[15].

4.5 Conclusion

The encapsulation of PLGA NPs into Pheroid® vesicles was prepared via a post mix approach with pre-formed delivery systems. DF and ETB loaded particles were successfully encapsulated into Pheroid® vesicles after 12 hours of incubation. This was confirmed with confocal microscopy, however the images further revealed the unsuccessful encapsulation of CLR NPs.

The preparation of Pheroid® vesicles was successful. During cytotoxic evaluation the individual Pheroid® system exhibited little to no cytotoxicity on HeLa at concentrations lower than ≤ 2 % (v/v) after 24-hour exposure. Despite the lowest concentration of Pheroid® vesicles being non-toxic, the hybrid system showed higher cytotoxicity after 1 hour of NP-vesicle exposure. This was unexpected, as lowest concentration of Pheroid® vesicles was used to prepare the hybrid system. The confocal images further revealed that a higher density of NPs was in close proximity to the THP-1 macrophages.

The PLGA NPs in Pheroid® vesicles most likely entered the THP-1 macrophages by a combination of simple diffusion and phagocytosis[11]. This is best explained by the presence of the Pheroid® vesicles which have been reported to facilitate transfer through physiological barriers acid membrane binding proteins and dependent on the shape, size, geometry and fatty acid ratios of the Pheroid®[6].

This preliminary study is still in its infancy stage, but with further investigation this hybrid system may potentially alleviate the challenges experiences by MAC therapy.

4.6 Acknowledgments

We would like to take this opportunity to thank the CSIR for the donated PLGA NPs and the PCDDP and India Brazil and South Africa trilateral (IBSA) for funding this study. We would like to thank Dr. Matthew Glyn personally for his assistance in obtaining confocal images for the uptake study.

References

1. Tortoli E. Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. *Clin Microbiol Rev.* 2014; 27:727–752.

2. Miwa S, Shirai M, Toyoshima M, et al. Efficacy of clarithromycin and ethambutol for Mycobacterium avium complex pulmonary disease. A preliminary study. *An Am Thorac Soc*. 2014;11(1): 23-29.

3. Mahajan M, Khurana RK, Sahaipal NS, et al. Emerging Strategies and Challenges for Controlled Delivery of Taxanes: A Comprehensive Review. *Curr Drug Metab*. 2015; 16(6): 453-473.

4. Krishna RSM, Shivakumar HG, Gowda DV, Banerjee S. Nanoparticles-a novel colloidal drug delivery system*. Indian J Pharm* Educ Res. 2006; 40:15-21.

5. Faraji AH, Wipf P. Nanoparticles in cellular drug delivery. *Bioorganic Med Chem*. 2009; 17(8):2950-2962.

6. Grobler AF. Pharmaceutical applications of pheroid™ technology. Potchefstroom: NWU. 2009; (Thesis – PhD).

7. Chelopo MP, Kalombo L, Wesley-Smith J, Grobler A, Hayeshi R. The fabrication and characterization of a PLGA nanoparticle-Pheroid® combined drug delivery system. *J Mater Sci.* 2017; 52:3133-3145.

8. Hadinoto K, Sundaresan A, Cheow WS. Lipid-polymer hybrid nanoparticles as a new generation therapeutic delivery platform: A Review*. Eur J Pharm Biopharm.* 2013; 85(3): 427-443.

9. Fang D, Chen Y, Xu B, et al. Development of Lipid-Shell and Polymer Core Nanoparticles with Water Soluble Salidroside for Anti-Cancer Therapy. *Int J Mol Sci.* 2014; 15(3): 3373-3388.

10. Zheng S, Chen Y, Chen Y, Liu H. Lipid—polymer hybrid nanoparticles for synergistic drug delivery to overcome cancer drug resistance. *New J Chem.* 2017; 41(4):1518-1525.

11. Wang Q, Alshaker H, Bohler T, et al. Core shell lipid-polymer hybrid nanoparticles with combined docetaxel and molecular targeted therapy for the treatment of metastatic prostate cancer. *Sci Rep.* 2017; 7(5901):1-8.

12. Liu Y, Ki K, Pan J, Lui B, Feng S-S. Folic acid conjugated nanoparticles of mixed lipid monolayer shell and biodegradable polymer core for targeted delivery of Docetaxel. *Biomaterials.* 2010; 31(2), 330-338.

13. Wang H, Zhao Y, Wu Y, et al. Enhanced anti-tumor efficacy by co-delivery of doxorubicin and paclitaxel with amphiphilic methoxy PEG-PLGA copolymer nanoparticles. *Biomaterials*. 2011; 32(32):8281-8290.

14. Kumar SSD, Mahesh A, Mahadevan S, Mandal AB. Synthesis and characterization of curcumin loaded polymer/lipid based nanoparticles and evaluation of their antitumor effects on MCF-7 cells. *Biochimica Biophysica Acta (BBA)- General Subjects*. 2014; 1840(6):1913-1922.

15. Wong HL, Rauth AM, Bendayan RM, et al. A New Polymer–Lipid Hybrid Nanoparticle System Increases Cytotoxicity of Doxorubicin Against Multidrug-Resistant Human Breast Cancer Cells. *Pharm Res*. 2006; 23:1574-1585.

16. Zhang L, Chan, JM, Gu FX, et al. Self-assembled lipid-polymer hybrid nanoparticles: a robust drug delivery platform. *ACS Nano.* 2008; 2:1696-1702.

17. Zhang L, Zhu D, Dong X, et al. Folate-modified lipid-polymer hybrid nanoparticles for targeted paclitaxel delivery. *Int J Nanomedicine.* 2015; 10:2101–2114.

18. Lemmer Y, Kalombo L, Pietersen RD, et al. Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis. *J Control Release*. 2015; 211:94-104.

19. du Plessis L, van Niekerk AC, Maritz MM, Kotze AF. In vitro activity of Pheroid vesicles containing against Plasmodium falciparum. *J Antibiot*. 2012; 65:609 –614.

20. du Plessis LH, Helena C, van Huysteen E, Wiesner L, Kotzé AF. Formulation and evaluation of Pheroid vesicles containing mefloquine for the treatment of malaria. *J Pharm Pharmcol*. 2014; 66:14 –22.

21. Grobler L, Grobler A, Haynes R, et al. The effect of the Pheroid delivery system on the in vitro metabolism and in vivo pharmacokinetics of artemisone. *Expert Opin Drug Metab Toxicol*. 2014; 10(3): 313-325.

22. Lamprecht A, Ubrich N, Perez MH, Lehr C-M, Hoffman M, Maincent P. 1999. Biodegradable monodispersed nanoparticles prepared by pressure homogenization-emulsification. *Int J Pharm.* 1999; 184(1): 97-105.

23. du Plessis LH, Lubbe J, Strauss T, Kotze AF. Enhancement of nasal and intestinal calcitonin delivery by the novel Pheroid™ fatty acid based delivery system, and by *N*-trimethyl chitosan chloride. *Int J Pharm.* 2010*;* 385(1–2):181–186.

24. Kisich KO, Gelperina S, Higgins MP, et al. Encapsulation of moxifloxacin within poly(butyl cyanoacrylate) nanoparticles enhances efficacy against intracellular *Mycobacterium tuberculosis. Int J Pharm.* 2007; 345(1-2):154-162.

25. Lotfipour F, Valizadeh H, Milani M, Bahrami N, Ghotasiou R. Study of Antimicrobial Effects of Clarithromycin Loaded PLGA Nanoparticles against Clinical Strains of Helicobacter pylori. *Drug Res.* 2016; 66(1): 41-45.

26. Le Meins JF, Schatz C, Lecommandoux S, Sabdre O. Hybrid polymer/lipid vesicles: State of the art and future prospectives. *Mater Today.* 2013*;* 16: 397.

27. Hall JB, Dobrovolskaia MA, Patri AK, McNeil SE. Characterisation of nanoparticles for therapeutics. *Nanomedicine*. 2007; 2(6):789-803.

28. Jana B, Ghosh S, Chattopadhyay N. Competitive binding of nile red between lipids and β-cyclodextrin. *J Photochem Photobiol*. 2013; 126:1-10.

29. Hadinoto K, Sundaresan A, Cheow WS. Lipid-polymer hybrid nanoparticles as a new generation therapeutic delivery platform: A Review. *Eur J of Pharm Biopharm.* 2013; 85(3): 427-443.

30. Lucey BP, Nelson-Rees WA, Hutchins GM. Henrietta Lacks, HeLa Cells, and Cell Culture Contamination. *Arch Pathol Lab Med.* 2009; 133:1463-1467.

31. Selzner N, Selzner MR, Graf U, Fitz JG, Clavien PA. Water induces autocrine stimulation of tumor cell killing through ATP release and P2 receptor binding. Cell Death Differ. 2004; 11:172-180.

32. Aldo P B, Craveiro V, Guller S, Mor G. Effect of culture conditions on the phenotype of THP-1 monocyte cell line. *Am J Reprod Immunol*. 2013; *70*(1):80–86.

33. Gordon S. 2007. The macrophage: past, present and future. *Eur J Immunol.* 2007; 37(1): 9-17.

34. Beachey W. Respiratory Care Anatomy and Physiology: Foundations for Clinical Practice. 2012. Mosby, 3rd edition.

Chapter 5: LCMS method development

5.1 Introduction

Clarithromycin (Figure 5.1 A) and ethambutol (Figure 5.1 B) are commonly used to treat MAC (Miwa *et al.,* 2014). These two drugs are different in molecular size and in chemical nature. Although its difference in chemical nature; CLR, hydrophobic and ETB, hydrophilic, they are in fact similar, as they are both absent of chromophores.

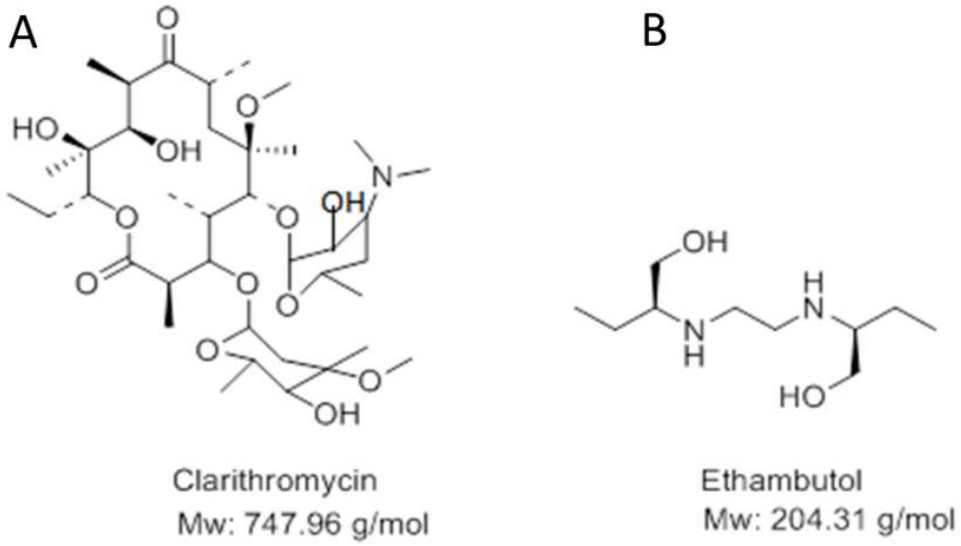


Figure 5.1 Chemical structure and molecular weight (Mw) of CLR (A) and ETB (B). Images drawn with ChemBioDraw 11.0 Software.

The quantification of CLR and ETB using analytical methods are not new. There are numerous reports on the successful quantification of CLR with high-performance liquid chromatography (HPLC) (Foroutan *et al.,* 2013); mass-spectrometry (Jiang *et al.,* 2007) and UV detection (Amini & Ahmadiani, 2005).

Various other methods have been explored for the quantification of ETB as well. Quantification methods include; the derivatisation of ETB with copper actetate to form a chromophore for UV detection (The International Pharmacopoeia, 2011); precolumn derivatisation method to quantify by HPLC (Yan *et al.,* 2007) and a LCMS method showcased by Gong *et al*., (2009) where a simultaneously quantification method was developed together with pyrazinamide in human plasma (Gong *et al.,* 2009).

All these methods possess its own unique properties and advantageous, but for the scope of this study, a LCMS method was chosen, owing to the absence of chromophores (Figure 5.1), the availability of the equipment, as well as the advantages it poses, such as selectivity, as peaks can be isolated in terms on its mass, easy identification of eluted analytes without retention time validation, sample matrix adaptability which decrease sample preparation time and its able to identify the correct fragmentation pattern of an analyte in a complex matrix (Elmashni, 2013).

LCMS could potentially assist in the accurate determination of the DL and EE which could aid in formulation optimisation (Castro Frabel do Nascimento *et al*., 2012). The EE can be determined by mean of a direct and indirect method which was briefly elaborated on in Chapter 2, section 2.12.5. However, for the intended purpose of this investigation an indirect method was chosen.

Both qualitative and quantitative information can be obtained from mass spectrometry (Poon *et al.,* 1999). The respective analytes are initially ionised into its positive or negative states followed by its detection depending on it mass/charge (m/z) ratio.

The initial part of the method development consisted of four parameters; mobile phase (1), ionising agent (2), flow rate (3) and column determination (4). These parameters are important as they affect the separation of the analytes and their respective detection (Sage *et al.,* 2013).

The aim of this work was to develop a simultaneous drug quantification method for the EE determination of CLR and ETB in the PLGA NPs. The EE was determined via an indirect method, where the difference between the total drug used in preparation and the amount of non-encapsulated drug (free drug) present in the supernatant after isolation of particles was calculated. The method development included the determination of the optimum mobile phase and ionising agent for these two drugs owing to its difference in chemical nature. Thereafter, 2 different columns (C8 and C18) with different flow rates were investigated such that good separation could be achieved. After the mobile phase, ionising agent, column and flow rate was determined; the calibration curves were generated, and the samples were analysed and quantified.

5.2 Materials and Methods

5.2.1 Materials

The reagents; acetonitrile LC-MS Chromosolv®, ammonium acetate ≥ 99.9 %, ammonium formate ≥ 99.9 %, formic acid eluent additive for LCMS and methanol Chromosolv® ≥ 99.9 % were purchased from Sigma-Aldrich, Steinheim, Germany

5.2.2. Methods

5.2.2.1 Instruments

CLR and ETB quantification was performed on a Shimadzu ultra-performance liquid chromatography (UPLC) interfaced with ABSciex 3200 Q–Trap triple quadrupole mass spectrometer detector and an electron spray ionisation source (ESI). Data was collected and processed using an Analyst software 1.6.1 package.

5.2.2.2 Chromatographic conditions

Chromatographic separations for both CLR and ETB samples as well as the calibration curve generation for CLR and ETB were performed using a Gemini 5 µm C8/C18 110 Å column 250 x 4.6 mm. The oven was set at room temperature (25 °C) and the mobile phase consisted of 10 mM ammonium acetate in methanol and was isocratically eluted at a flow rate of 1 mL/min with an injection volume of 20 µL.

5.2.2.3 Mass spectrometer conditions

The positive protonated molecule and precursor ions for the analytes (i.e. ETB and CLR) were determined using an AB Sciex 3200 Q-trap quadrupole mass spectrometer (MS) which included an electronically controlled, integrated syringe pump. The MS conditions for ETB and CLR were optimised separately with automatic Flow Injection Analysis (FIA) with the aid of Analyst 1.6.1 Software. The analytes were infused into the MS at a flow rate of 20 µL/min, whilst MS parameters were adjusted to achieve optimal intensity. For detection, electron-spray ionisation (ESI) in positive and negative mode was investigated using infused standard solutions. ESI in positive mode (ESI+) was chosen for ionisation as the base peak intensity and efficiency of ionisation was higher to that of the precursors in negative mode. Selective reaction monitoring (SRM) mode was chosen for detection because it has high sensitivity and high selectivity. The optimised precursor ion pairs were m/z 205.24 → 116.20 for ETB and 748.51 → 158.30 for CLR. The optimum source and gas parameters were; Curtain gas (CUR): 40 L/min, Ion voltage spray: 5000 volt, Temperature: 500 °C, Ion source gas (GS1): 60 psi, Ion source gas 2 (GS2): 60 psi.

5.2.2.4 Analytical conditions: Preparation of stock solutions and calibration curves

Individual standard stock solutions of ETB and CLR were prepared with a concentration of 1000 µg/mL. The solutions were prepared by weighing the appropriate amount of the analytes and dissolving them in the mobile phase. The two solutions of CLR and ETB were mixed at a 1:1 ratio to yield a 500 µg/mL stock solution.

The calibration curves were prepared by diluting the stock solution with additional mobile phase to yield the final standard concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µg/mL. Injections were carried out in triplicate.

5.2.2.5 Sample preparation

The supernatant collected from the NP preparation after centrifugation as described in Chapter 3 (see section 3.3.2.1), was subjected to further analysis for the quantification for the drug loss and furthermore the EE.

Briefly, the supernatant (500 µL) was added to the mobile phase (500 µL) followed by agitation with a vortex for approximately 1 minute to ensure thorough mixing. The sample was centrifuged at 10 000 rpm for 15 minutes at 4 °C to allow for sedimentation of residual particles. 200 µL of the sample was aliquoted for analysis. Samples were prepared in triplicate.

5.3 Results and Discussion

5.3.1 Method Development

5.3.1.1 Chromatographic conditions: Mobile phase and ionising agent determination

The mass spectrometer (MS) was coupled with an electrospray ionisation (ESI) source therefore volatile compounds were needed for detection (Garcia *et al.,* 2005).

This is usually achieved with the mobile phase consisting of an organic solvent (methanol or acetonitrile) and a volatile buffer (Garcia *et al.,* 2005). These solvents have similar dielectric constants (a measure of polarity) of 37.5 and 32.7, respectively (Maryott & Smith, 1951). Acetonitrile is a polar aprotic solvent which aids in the dissolution of ionic and polar compounds whereas methanol, being a protic solvent is capable of hydrogen bonding and aids in dissolution of polar as well as non-polar compounds (Huffman *et.al*., 2012).

ESI is a desorption ionisation technique that assisted the analyte in reaching a nonvolatile/thermally unstable state before being subjected to mass spectrometric analysis (Ho *et. al*., 2003). Hence, an ionising agent was needed to assist in the ionisation of the neutral analytes (CLR and ETB). Three ionising agents at a concentration of 10 mM was investigated and included ammonium acetate, ammonium formate and formic acid. A concentration of 10 mM was the initial starting point as the concentration of the ionising agent affects the analyte signal. A high signal may result in the suppression, while a low concentration may result in poor peak shape and efficiency (Garcia *et al.,* 2005). After the mobile phase was determined, the chromatograms were inspected and evaluated in terms of its signal strength and peak shape.

Table 5.1: Solubility results of CLR and ETB and ionisation agents in different mobile phases

Solvent	Acetonitrile (CH_3CN)	Methanol (CH_3OH)
Therapeutic (1 mg/mL)		
ETB	Soluble	Soluble
CLR	Insoluble	Soluble
Ionising Agent (10 mM)		
Ammonium Acetate	Insoluble	Soluble
Ammonium Formate	Insoluble	Soluble
Formic Acid	Soluble	Soluble

As represented in Table 5.1, CLR is insoluble in acetonitrile and soluble in methanol (MeOH). Both drugs were soluble in methanol contributing to the choice of methanol as the mobile phase. Despite the solubility of all three ionising agents in methanol, ammonium acetate was selected as it was previously shown to assist in good ionisation for ETB (Chaitanya *et.al*., 2012) and CLR (Chen *et.al*., 2006). The mobile phase was used for all subsequent development and analysis; flow rate determination, the generation of calibration curves and quantification.

5.3.1.2 Chromatographic conditions: Column and flow rate determination

For all development and testing, an isocratic elution was chosen over a gradient elution as it was deemed more advantageous in terms of separation speed and analyte quantification (Schellinger & Carr, 2006).

After mobile phase determination (methanol with 10 mM ammonium acetate), 2 hydrophobic columns of varying alkyl chain length (stationary phase) was investigated against varying flow rates. The varying stationary phase and flow rate assisted in determining the optimum chromatographic conditions for CLR and ETB detection. For each of the columns, the analytes (CLR and ETB) was eluted with an isocratic flow of 0.25, 0.5 and 1.0 mL/min.

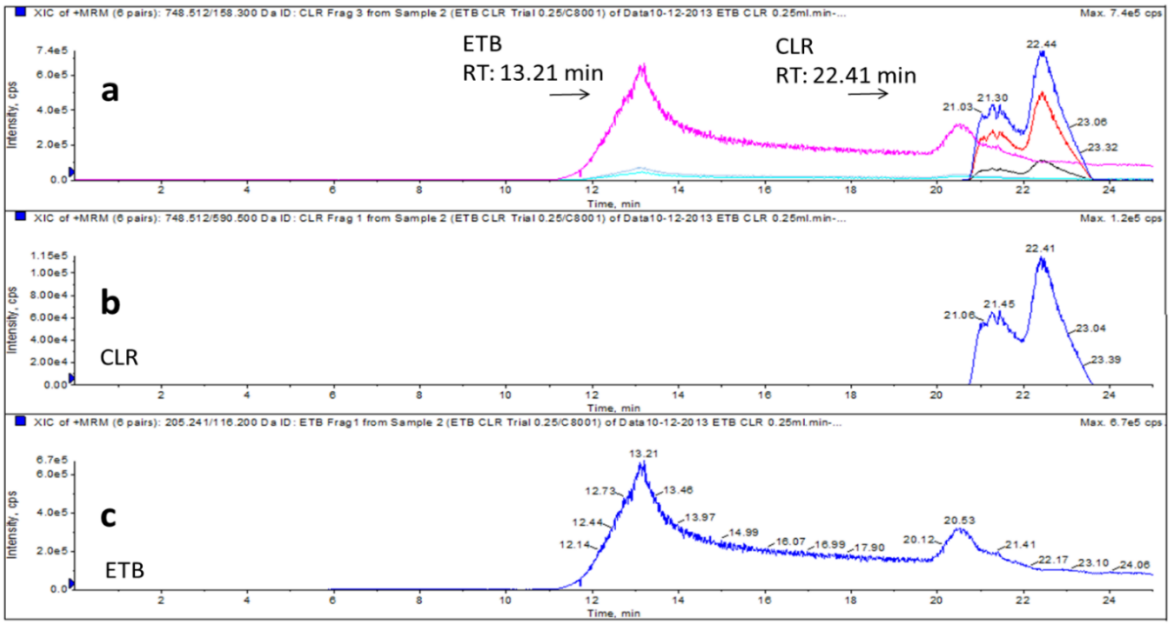


Figure 5.2: Representative LCMS Chromatogram in a C8 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, 10 mM ammonium acetate in MeOH, flow rate, 0.25 mL/min; column temperature, 25 °C; Injection volume, 20 µl.

The chromatogram above is a graphical display of the separation of CLR and ETB as they passed through the detector.

From the chromatogram above (Figure 5.2, a) it is evident that there was co-elution of the 2 analytes; CLR (blue) and ETB (pink). ETB had a shorter retention time (RT) of 13.21 min when compared to CLR, RT 22.41 min. This result was excepted as CLR is a hydrophobic molecule and therefore had more interaction with stationary phase (hydrophobic column).

Both analytes experienced poor elution, as the separation peaks for both analytes were broad and asymmetric.

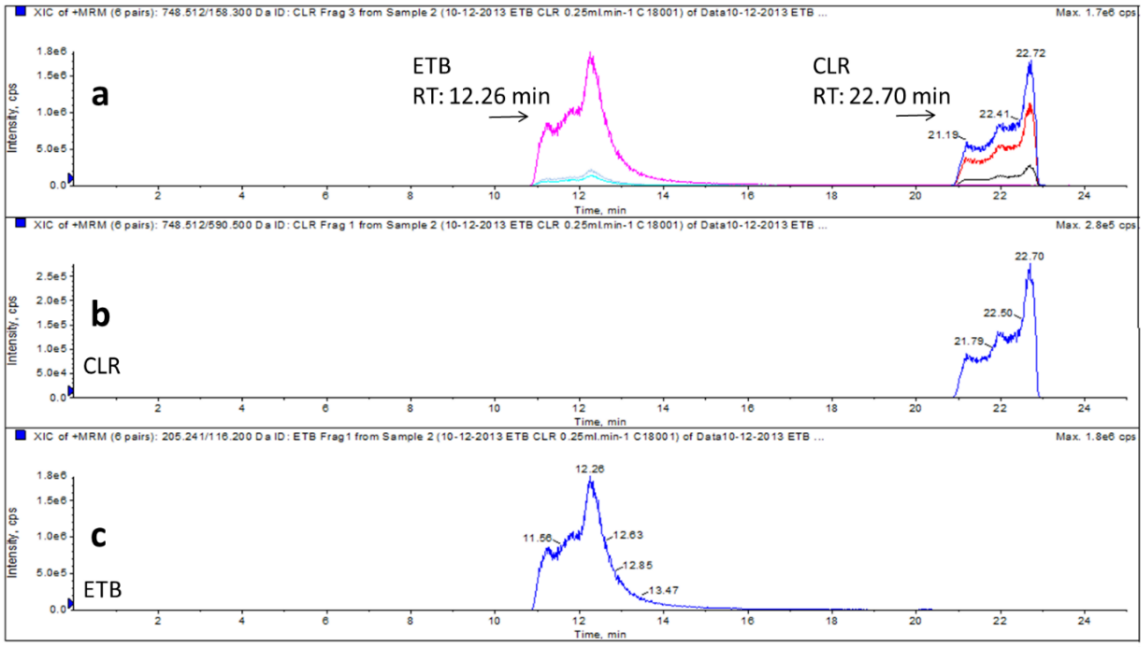


Figure 5.3: Representative LCMS Chromatogram in a C18 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, methanol: ammonium acetate (99:1, m/m); flow rate, 0.25 mL/min; column temperature, 25 °C; Injection volume, 20 µl.

Figure 5.3 is representative of the separation of CLR and ETB with the same chromatographic conditions but with a more hydrophobic stationary phase (C18 column). The RT is similar to that experience with the C8 column with a flow rate of 0.25 mL/min. Less tailing was observed from ETB (pink) which indicated a more efficient separation to that observed with the C8 column. However, the chromatogram further indicated a poor peak shape, broad width, asymmetry and tailing for both analytes, causing their quantification to be less accurate.

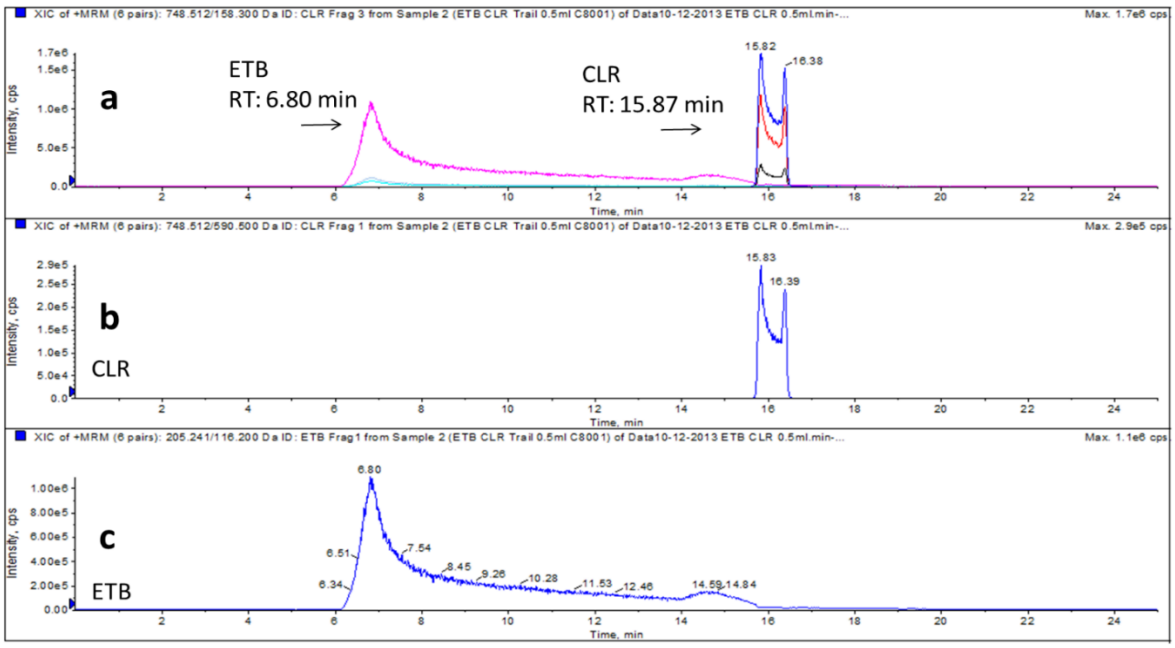


Figure 5.4: Representative LCMS Chromatogram in a C8 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, methanol: ammonium acetate (99:1, m/m); flow rate, 0.50 mL/min; column temperature, 25 ˚C; Injection volume, 20 µl.

The chromatogram of the analytes with a C8 column and 0.5 mL/min flow rate is represented by Figure 5.4. With an increase in the flow rate, a decrease in the RT for both analytes was observed as the mobile phase carried ETB and CLR faster through the column. ETB is a polar molecule and has poor retention on the C8 column when compared to CLR. With an increased flow rate, ETB (pink) had less tailing with compared to the chromatogram in Figure 5.2

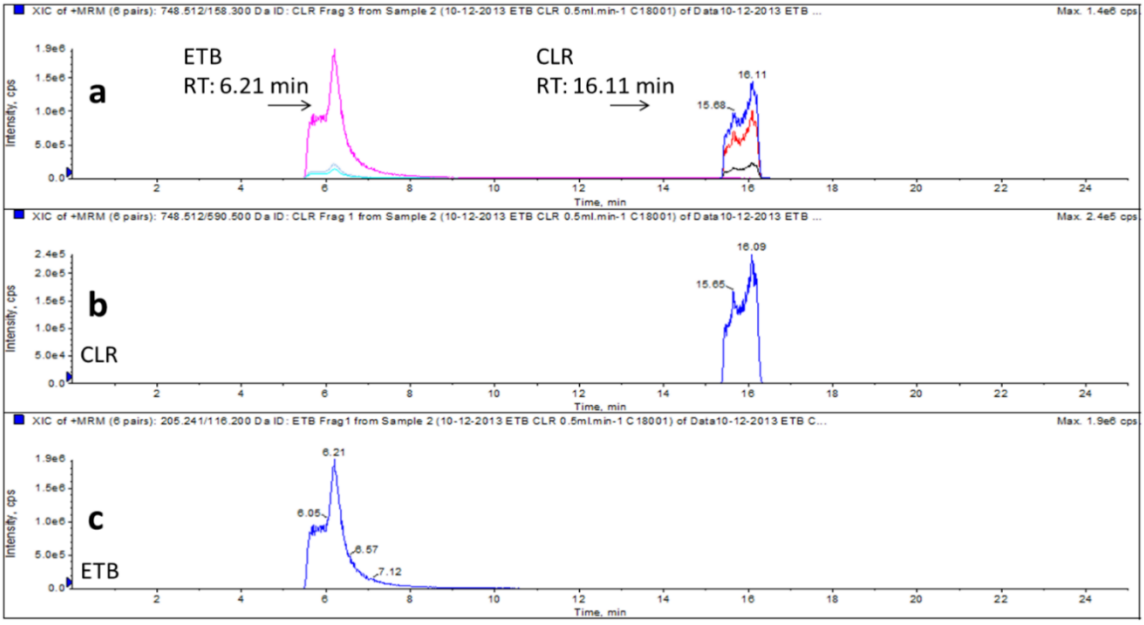


Figure 5.5: Representative LCMS Chromatogram in a C18 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, methanol: ammonium acetate (99:1, m/m); flow rate, 0.50 mL/min; column temperature, 25 °C; Injection volume, 20 µl.

The chromatogram obtained for ETB and CLR using a C18 column with a flow rate of 0.5 mL/min is given in Figure 5.5. This column and the chosen flow rate, displayed a better separation of the drugs. The chromatogram indicated a better peak shape with less tailing, however, a split peak was observed for CLR.

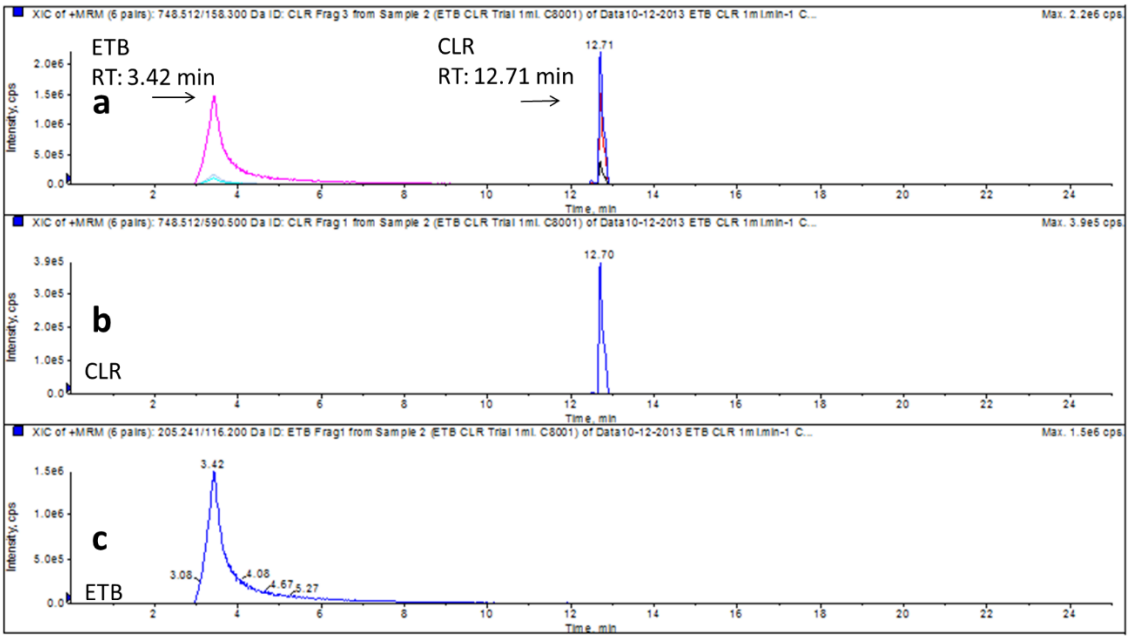


Figure 5.6: Representative LCMS Chromatogram in a C8 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, methanol: ammonium acetate (99:1, m/m); flow rate, 1.00 mL/min; column temperature, 25 °C; Injection volume, 20 µl.

The chromatograms obtained for ETB and CLR with a flow rate of 1 mL/min and a stationary phase of C8 is given by Figure 5.6. This flow rate and stationary phase displayed separate elution of the drugs. The increased flow rate reduced the contact time of both analytes on the column and thus attributed to less interaction with the column and hence sharper and narrower peaks.

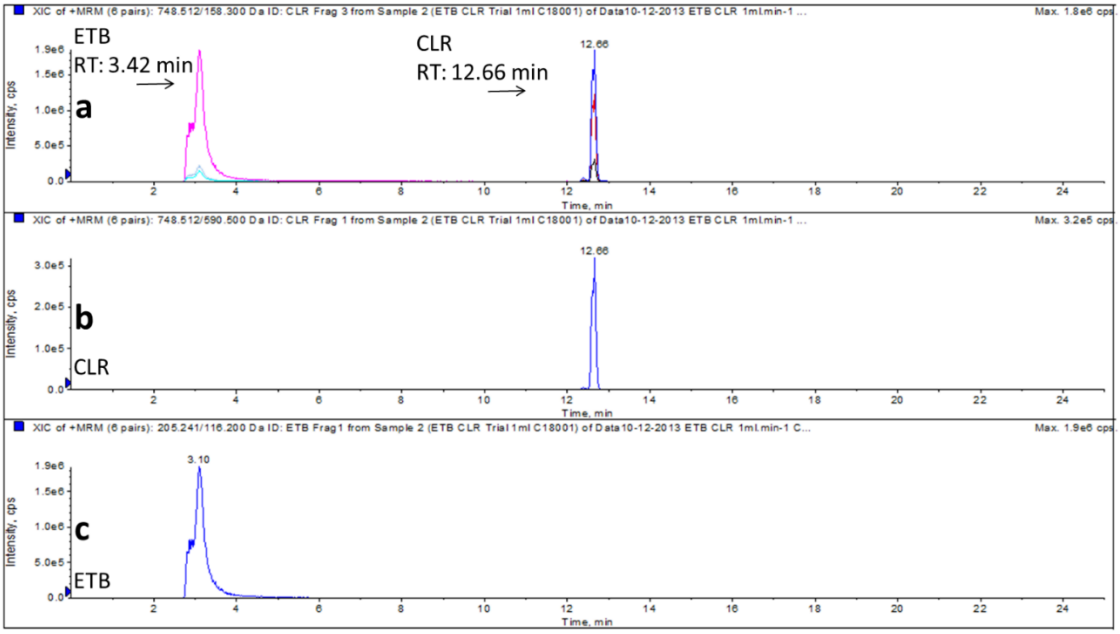


Figure 5.7: Representative LCMS Chromatogram in a C18 column of ETB (pink) and CLR (blue) samples (a) and supernatant from both samples, (b) chromatogram of CLR, (c) chromatogram of ETB. Conditions, mobile phase, methanol: ammonium acetate (99:1, m/m); flow rate, 1.00 mL/min; column temperature, 25 °C; Injection volume, 20 µl.

The chromatogram in Figure 5.7 was obtained for the separation of ETB and CLR with a C18 stationary phase and a flow rate of 1 mL/min. The elution peaks indicated a narrower, sharp split peak with less tailing and had better elution with an increase in flow rate. The retention time was reduced for both analytes.

From the chromatograms generated it can be concluded that an increase in flow rate improved the elution of both drugs. ETB experienced poor elution at lower rates on the C8 column.

After consolidation of all the chromatograms, the C18 column with a flow-rate of 1.00 mL/min showed the best separation of both drugs. The good peak shape displayed by Figure 5.7 was important for an improved resolution and accurate quantification of drug.

5.3.2 Mass spectrometry

The mass spectrum below in (Figure 5.9) is representative of the intensity vs m/z (charge to mass ratio).

CLR and ETB were analysed by ESI. Both analytes; CLR and ETB, responded to the positive ionization $[M+H]^+$ form and was detected with a QTRAP quadrupole mass spectrometer detector.

The fragment ions at m/z 590.5 and 116.2 were chosen for Multiple reaction monitoring (MRM) acquisition of CLR and ETB for identification and quantification. MRM is a technique used by a quadrupole mass spectrometer whereby the targeting of the CLR and ETB ion (parent ion) as well its subsequent fragmented of the parent ions into daughter ions is possible.

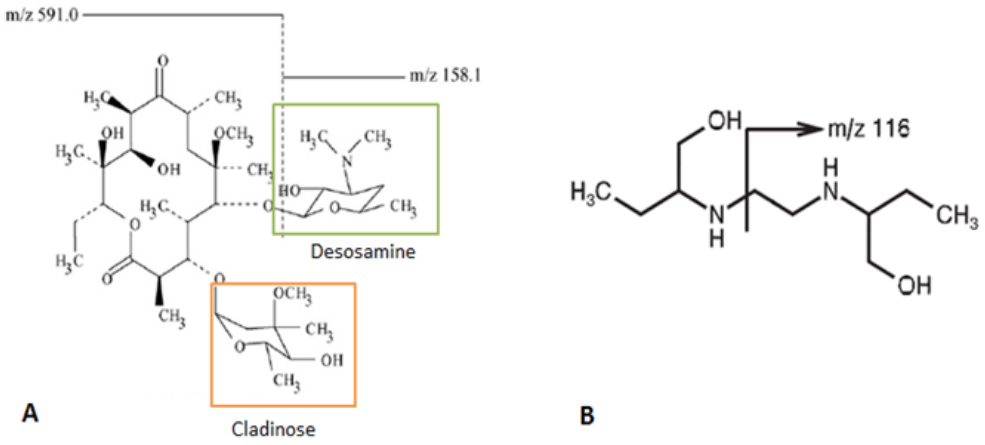


Figure 5.8: Proposed mass fragments from CLR, Mw): 748.51 g/mol (Jiang *et al.,* 2007) and ETB, Mw: 205.24 g/mol (Chen *et al.,* 2005). Images drawn with ChemBioDraw 11.0 Software.

CLR holds a 14-membered macrocyclic lactone ring with two sugar moieties (desosamine and cladinose) attached with a hydroxyl group in its 6' position (Kanfer *et al*., 1998). There are two possible points of fragmentation, however the cleavage of the desosamine sugar moiety (Figure 5.8, green) was speculated, resulting in the m/z 590.2 precursor ion. This fragmentation was proposed by Jiang and co-workers (2007) and best explains the m/z 590.2 precursor ion observed during analysis.

For ETB, the parent and precursor ion were proposed by Chen and co-workers (2005) whereby a covalent bond cleavage was observed at the position depicted in Figure 5.8 B. This proposed fragmentation substantiated the precursor ion m/z of 116.2 observed during analysis.

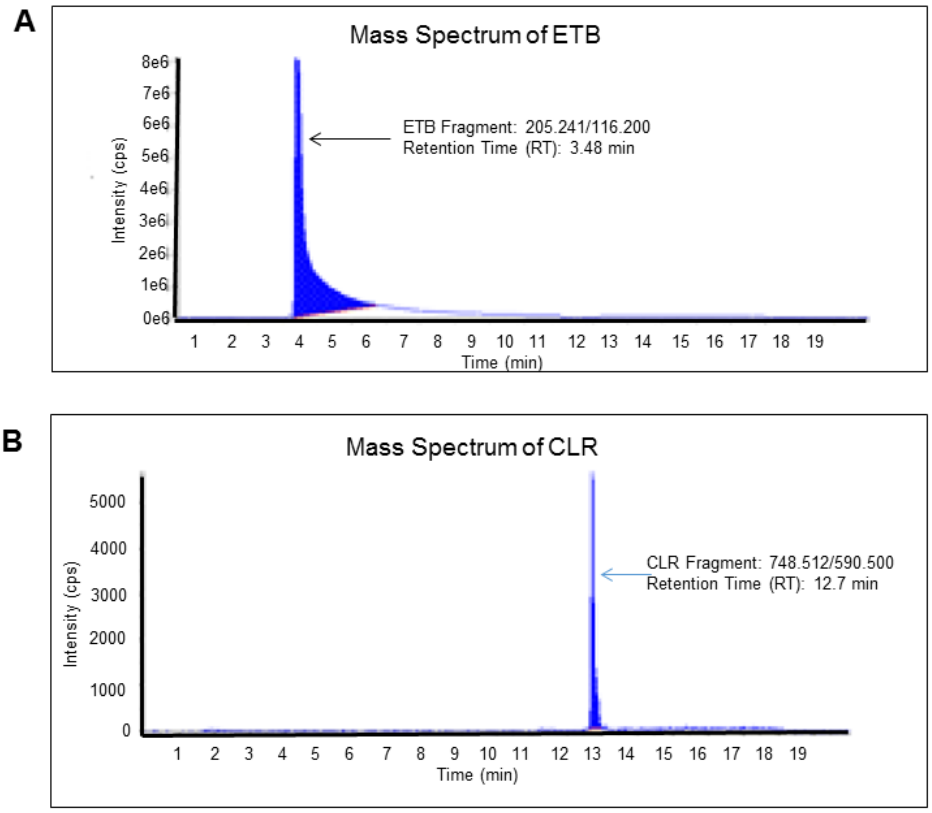


Figure 5.9: The mass spectrums of ETB (A) and CLR (B). Conditions: mobile phase 10 mM ammonium acetate in methanol; flow rate, 1 mL/min; column temperature, 25 ºC; injection volume, 20 µL

The mass spectrum for CLR and ETB is depicted in Figure 5.9. ETB and CLR were quantified in MRM mode using m/z 748.5 → 590.5 and m/z 205.2 → 116.2 respectively.

5.3.4 Analytical conditions: Calibration curves

5.3.4.1 Matrix Determination

NPs loaded with CLR and ETB were prepared to that described in Chapter 3, section 3.3.2.1. Briefly, after NP synthesis, the emulsions were left to stir overnight to allow for organic solvent (DCM) evaporation. The particles were subsequently collected after centrifugation. The supernatant was collected and stored for EE determination. The supernatant consisted of a 2% PVA solution with residual DCM.

An appropriate matrix was prepared as described below.

5.3.4.2 Method

A 2 % PVA solution (m/V) was prepared with a DCM concentration of 0.2 % (v/v) to best fit the liquid components in the NP preparation. To this solution approximately 50 mg of CLR and ETB each was added to make stock solution of 1000 ppm (µg/ml).

With the addition of DCM to the PVA, 2 distinct layers were formed. With increased stirring speed, an emulsion was formed. The emulsion was left to stir overnight to encourage DCM evaporation. After subjected to overnight evaporation, 1 or both of the drugs precipitated out of solution. It was suspected that ETB remained in solution and CLR precipitated out owing to its hydrophobic nature, however further quantification was needed to confirm this.

As the aim was to develop a simultaneous drug quantification method, problems will continue arise, as the dispersed phase of the emulsion is aqueous and CLR is insoluble in aqueous mediums. Therefore, owing to this characteristic, it was decided that the mobile phase Methanol (10 mM ammonium acetate) will be used for the preparation of stock solutions for the calibration curves.

5.3.2.2 Calibration Curves

The calibration curves was obtained to that described in 5.2.2.4. Briefly, stock solutions were prepared with the mobile phase and diluted serially with additional mobile phase to prepare final standard concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µg/mL. After injection, the actual concentrations were achieved using the Analyst software that identified the respective parent and precursor ions in a mass spectrum owing to MRM selection followed by an automatic calculation of the area under the peak for each respective peak as well as the regression analysis.

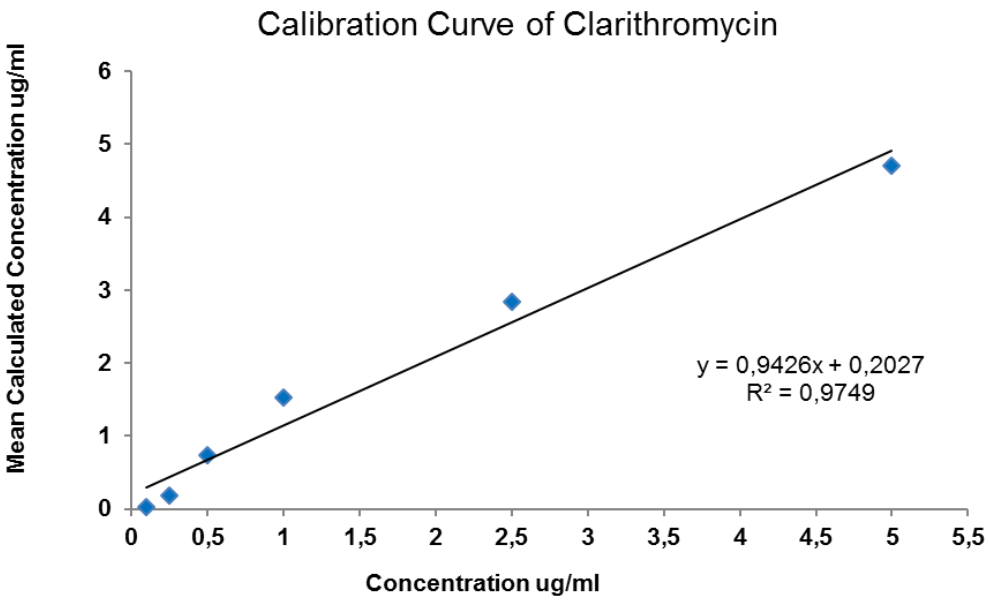


Figure 5.9: The calibration curve for CLR. Conditions: mobile phase 10 mM ammonium acetate in methanol; flow rate, 1 mL/min; column temperature, 25 ºC; injection volume, 20 µL

For CLR, the calibration curves showed an overall accuracy of 95.1 – 110.5% with a coefficient of variance (CV) of 1.1 – 4.7%. The calibration curve displayed a linear nature with a correlation coefficient (R^2) of 0.9749 (Figure 5.9).

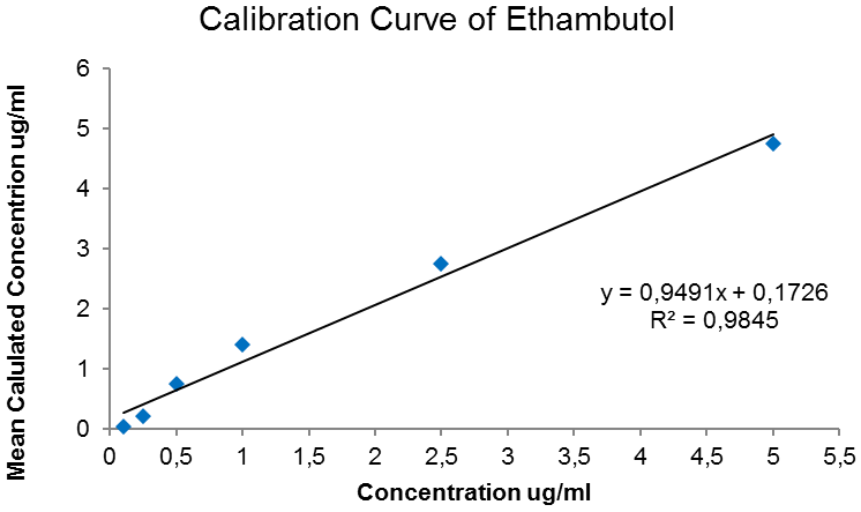


Figure 5.10: The calibration curve for ETB. Conditions: mobile phase 10 mM ammonium acetate in methanol; flow rate, 1 mL/min; column temperature, 25 ºC; injection volume, 20 µL.

The relatively low CV for both calibration plots implies that the actual concentrations were relatively close to the predicted concentrations.

With a R^2 close to 1 for both CLR and ETB, a strong positive correlation exists between the predicted and actual concentrations of the calibration curves. The positive value indicates a relationship between the x (predicted concentration) and y (actual concentration) variable such that when values for x increase, values for y increase as well.

5.4 Drug Quantification

The generated calibration curves and Analyst software was used to quantify the free drug present in the analysed samples**.**

Table 5.2: The calculated concentration of the drugs present in the analysed samples

Analyte Supernatant from NP	**Calculated concentration (ug/mL)***	**Retention time (min)**
PLGA-CLR	70.6	12.7
PLGA-CLR	72.0	12.7
PLGA-CLR	83.9	12.7
PLGA-CLR-MA	70.0	12.7
PLGA-CLR-MA	76.6	12.7
PLGA-CLR-MA	72.7	12.7
PLGA-ETB	49.3	3.45
PLGA-ETB	46.5	3.45
PLGA-ETB	45.5	3.44
PLGA-ETB-MA	48.8	3.43
PLGA-ETB-MA	47.0	3.44
PLGA-ETB-MA	47.0	3.45

*concentration calculated from analyst software

Table 5.2 is representative of the amount of CLR and ETB that was present in the diluted sample from the supernatant of all the particles prepared in Chapter 3, section 3.3.2.1. Samples were prepared in triplicate to ensure uniformity within the batch. No noteworthy difference in concentration was observed for the drug quantification across the CLR samples. The same is observed for ETB samples. However, a difference between ETB and CLR samples exists. It is evident from the data that more CLR drug was present in the supernatant after particle collection to that of observed in the ETB samples. This difference in drug loss may be attributed to the differences in the synthesis of these particles. CLR owing to its hydrophobicity was dissolved with the PLGA carrier and therefore theoretically embedded into the polymeric shell of the NPs. ETB was added in the internal water phase, and therefore

theoretically encapsulated into the polymeric core. The results further showed that the incorporation of MA into the NP did not affect the loss of drug during the preparation process.

The final EE was calculated to that described in Chapter 3, section 3.3.2.4. The drug loss in the centrifugation process was determined, followed by the calculation of the drug remaining in the collected pellet. This was subsequently divided by the initial drug added into the NP preparation to calculate the final EE (results shown in Chapter 3, Table 3.2).

5.5 Conclusion and future work

A LCMS method was developed for simultaneous detection of CLR and ETB in an aqueous matrix. This was possible with a 100% organic mobile phase with ammonium acetate as an ionising agent. This method had simple sample preparation, quick column separation and was efficient in simultaneous detection and separation of both analytes. This method allowed for the quantification of CLR and ETB indirectly with an EE of 94.4% and 96.6% respectively (results shown in Chapter 3, Table 3.2).

This LCMS method shows potential for the further utilisation for the determination of DL directly and furthermore the detection in blood plasma and tissue after potential *in vivo* work. To validate the robustness of this method, further method development is suggested. The further method validation should include the following: selectivity and sensitivity: which would assist in the interference evaluation of the analyte at the retention time, matrix factor: to evaluate the effect of the matrix at high and low concentrations to ensure precision and recovery: to evaluate the loss of analyte during sample extraction and stability: to evaluate the stability of the analyte in different matrices at different conditions.

References

Amini, H. & Ahmadiani, A. 2005. Sensitive determination of clarithromycin in human plasma by high performance liquid chromatography with spectrophotometric detection. *Journal of Chromatography B*, 817(2):193-197.

Castro Frabel do Nascimento, T., Meza Casa, D., Facco Dalmolin L., Cristina de Mattos, A., Maissar Khalil, N. & Mara Mainardes, R. 2012. Development and Validation of an HPLC Method Using Fluorescence Detection for the Quantitative Determination of Curcumin in PLGA and PLGA-PEG Nanoparticles. *Current Pharmaceutical Analysis*, 8(4): 324-333.

Chaitanya, K.A., Saravanan, R.S., Sathiyaraj, M., Jeevanatham, S. & Baskaran, R. 2012. Determination of ethambutol in presence of fixed dose combination molecules from human plasma by direct injection to liquid chromatography tandem mass spectrometry. *Clinical Pharmacology and Biopharmaceutics,* 1(2):1-6.

Chen, X., Song, B., Jiang, H., Yu, K. & Zhong, D. 2005. A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of isoniazid and ethambutol in human plasma. *Rapid Communications in Mass Spectrometry*, 19(18):2591-2596.

Chen, B.M., Liang, Y.Z., Chen, X., Liu, S.G., Deng, F.L. & Zhou, P. 2006. Quantitative determination of azithromycin in human plasma by liquid chromatography-mass spectrometry and its application in a bioequivalence study. *Journal of Pharmaceutical and Biomedical Analysis,* 42(4):480-487.

Elmashni, D. 2013. LC/MS detection: powerful tool for organic compound analysis. http://www.scientistlive.com/content/13865. [30 November 2017].

Foroutan, S.M., Zarghi, A., Shafaati, A., Madadian, B. & Abolfathi, F. 2013. Rapid High-Performance Liquid Chromatographic Method for Determination of Clarithromycin in Human Plasma Using Amperometric Dectection: Application in Pharmacokinetic and Bioequivalence Studies. Iranian *Journal of Pharmaceutical Research*, 12:64-69.

Garcia, M.C. 2005. The effect of the mobile phase additives on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B,* 825(2):111-123.

Ho, C.S., Lam, C.W.K., Chan, M.H.M., Cheung, R.C.K., Law, L.K., Lit, L.C.W., Suen, M.W.M. & Tai, H.L. 2003. Electron spray ionisation mass spectrometry: principles and clinical applications. *The Clinical Biochemist Reviews,* 24(1):3-12.

Huffman, B.A., Poltash, M.L. & Hughey, C.A. 2012. Effect of polar and polar aprotic solvents on negative-ion electrospray ionization and chromatographic separation of small acidic molecules. *Analytical Chemistry*, 84(22):9942-9950.

Jiang, Y., Wang, J., Li, H., Wang, Y. & Gu, J. 2007. Determination of clarithromycin in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 43(4):1460-1464.

Kanfer, I., Skinner, M.F. & Walker, R.B. 1998. Analysis of macrolide antibiotics. *Journal of Chromatography A*, 812(1-2):255-286.

Maryott, A. & Smith, E.R. 1951. Table of dielectric constants of pure liquids. Washington. D.C: US Government Print Office.

Miwa, S., Shira, M., Toyoshima, M., Shirai, T., Yasuda, K., Yokomura, K., Yamada, T., Masuda, M., Inui, N., Chida, K., Suda, T. & Hayakawa, H. 2014. Efficacy of Clarithromycin and Ethambutol for Mycobacterium avium Complex Pulmonary Disease. A preliminary Study. *Annals of the American Thoracic Society*, 11(1):23-30.

Poon, G.K., Kwei, G., Wang, R., Lyons, K., Chen, Q., Didolkar, V. & Hop, C.E.C.A. 1999. Integrating Qualitative and Quantitative Liquid Chromatography/Tandem Mass Spectrometric Analysis to Support Drug Discovery. *Rapid Communications in Mass Spectrometry*, 13: 1943-1950.

Sage, A., Wolff, C., Mussell, C., Neville, D., Lord, G., Saeed, M., Lad, R., Godfrey, R., Hird, S., & Barwick, V. 2013, Guide to achieving reliable quantitative LC-MS measurements. 1st ed. RSC Analytical Methods Committee.

Schellinger, A.P. & Carr, P.W. 2006. Isocratic and gradient elution chromatography: A comparison in terms of speed, retention reproducibility and quantitation. *Journal of Chromatography A,* 1109(2): 253-266.

The International Pharmacopoeia. 2011. 2nd Supplement. 4th Ed. World Health Organisation, Geneva, Switzerland.

Yan, M., Guo, T., Song, H., Zhao, Q. & Sui, Y. 2007. Determination of ethambutol hydrochloride in the combination tablets by precolumn derivatization. *Journal of Chromatographic Science,* 45(5): 269-272.

Chapter 6: Conclusion and future prospects

6.1 Conclusion

The aim of this research investigation was to potentially improve the current conventional treatment of *Mycobacterium avium* complex with a combined/hybrid drug delivery system that includes a targeting agent (MA), a polymeric core and a Pheroid® vesicle coating. Theoretically, we propose that with the utilisation of; Pheroid® technology, MA, polymeric (PLGA) NPs with CLR and ETB we could facilitate enhanced uptake, have uptake into macrophages and provide a sustained release of drugs which would in turn enhance the current treatment.

The preparation of PLGA NPs with CLR and ETB with and without MA was successful. These were prepared via a double emulsion solvent evaporation technique, followed by a double centrifugation step to obtain the desired size particles for further cytotoxic and uptake investigations. The incorporation of MA did not significantly affect the overall charge and physical characteristics of the selected drugs into the NPs.

Despite achieving the ideal size of NPs recommended for cellular uptake by means of the differential centrifugation process, further formulation development is encouraged aiming at reducing the particle size distribution in a single step. Various process and formulations factors might influence the particle size and particle size distribution. These include the solvent, surfactant type and its concentration, homogenisation speed and time, solvent evaporation rate as well as the adopted drying technique (Rao & Geckeler, 2011). Therefore, further work should include a trial of different solvent systems as demonstrated by Song *et al*, (1997) who showcased the effect of a mixture of DCM and acetone at a ratio of 8:2 (v/v), yielding particles sizes ranging from 60-200 nm with PVA as a stabiliser. Other research has shown that a relatively high concentration (3-5%) of PVA as a polymeric surfactant, resulted in good emulsification and led to small particle sizes with a narrow distribution (Zambaux *et al*., 1998; Rao & Geckeler, 2011). These types of investigations are valuable and applicable as we utilised a DCM solvent for PLGA and MA dissolution and PVA as a stabiliser at 2%. Further, investigations on manufacturing parameters e.g. homogenisation, as it has shown that an increased intensity and duration of the second homogenisation step may lead to a significant decrease in particle sizes (Bilati *et al*., 2003). These few examples illustrate the necessity of a comprehensive experimental design that encompasses the effects of the various key process and formulation parameters in order to achieve an optimal delivery system. An extension of this preliminary study would include the optimisation of the investigated delivery system by attempting to achieve a narrow size distribution with a single NP isolation step.

A LCMS method was successfully developed for the simultaneous detection of unbound CLR and ETB remaining in the supernatant after NP preparation. Although a high EE was observed, it is recommended to substantiate these finding via a direct method approach, with the dissolution of the PLGA carrier with an appropriate solvent such that the encapsulated drug is free for quantification. With the quantification of drug via a direct method, the determination of the DL would be possible. This would shed light on the exact quantity of drug that the HeLa and THP-1 cells were exposed to during cytotoxicity evaluation which would assist in drawing concrete conclusions. This new information would influence future work by making the determination of the delivery system dosage possible for any future *in vivo* work.

During the cytotoxic evaluation with the WST-1 assay, results revealed PLGA and MA alone do not to pose any cytotoxic effect of HeLa cells as well as THP-1 macrophages. However, a small decrease in viability was observed after 48 hours. The combination of PLGA and MA with CLR and ETB had a greater cytotoxic effect on the cells which may be attributed to proposed targeting ability of MA and an increase in negative zeta potential which has been previously shown to enhance interaction with phagocytic cells such as THP-1 cells (Blanco, 2015). No conclusion could be drawn to whether the PLGA carrier reduced the cytotoxicity of the free drugs on cell lines as the DL was not determined.

Drug free, ETB and CLR loaded PLGA NPs with and without MA were successfully taken up by THP-1 macrophages after 1 hour of incubation. These results were not conclusive to whether the MA facilitated in the cellular uptake, however it was speculated that this was the case as it was previously shown that there was a significant increase in PLGA NP uptake by mycobacterium infected macrophages (Lemmer *et al.,* 2015).

To validate that MA facilitated in the uptake, future studies should include fluorescence-activated cell sorting (FACS), that could quantify a mixture of unaffected cells and cells that has taken up NPs, based upon the specific light scattering and fluorescent characteristics of each cell (Ducat *et al*., 2011). With these results a conclusion could be made to whether MA indeed facilitated cellular uptake and therefore improve the delivery of the CLR and ETB into the infected cells.

All particles with and without active (CLR and ETB) as well as with and without MA, were subjected to encapsulation into previously prepared Pheroid® vesicles via a post mix approach. DF and ETB-loaded PLGA NPs with and without MA were successfully encapsulated into the Pheroid® vesicles, however the same was not true for CLR loaded NPs as phase separation during preparation was observed as well as a drastic increase in the size percentage change was observed. Interference from CLR may have arose owing to the

method of preparation for the PLGA-CLR NPs. CLR is hydrophobic in nature and was co-dissolved with PLGA which may have resulted in an unfavourable polymeric surface modification. This may have interfered with the stability of the NP-Pheroid® hybrid system, as the conditions of the hybrid system are dependent on the hydrophobic segments between the polymers and lipids as well as the chemical composition of the nanoparticles (Le Meins *et al.,* 2013; Hall *et al.,* 2007). Alternatively, the PLGA and Pheroid® hybrid system could be prepared via a pre-mix approach which was showcased by Chelopo *et al*., (2017) where previously prepare NPs are added to the oil phase of the Pheroid® system and subsequently added to the nitrous oxide water phase. No noteworthy change in the mean size was observed with the inclusion of MA, however a decrease in size was observed with increasing concentration of the NP inclusion.

DF and ETB PLGA NPs were successfully labelled with fluorescent MA (5-BMF-MA). Confocal microscopy confirmed the successful encapsulation of these particles into the Pheroid® vesicle. CLR loaded particles were not investigated as it was previously shown to influence the stability of the Pheroid® system. The focus of the current delivery system was not to determine whether the incorporation of MA influenced the encapsulation of polymeric particles into the vesicles, therefore particles not containing MA was not investigated. As a part of a larger, in-depth study, this can be tested to determine the influence of MA on this hybrid system.

Both DF and ETB hybrid systems were successfully taken up by THP-1 macrophages. Visually a greater density of NPs was observed in close proximity to the macrophages after incubation for hybrid formulations when compared to control. The encapsulation into the Pheroid® vesicles may have influenced this but further investigations are warranted to accept or reject this assumption. It was not clear from the images whether the Pheroid® vesicles remained with the particles. To confirm this, it is recommended to attach a fluorescent probe directly to one of the constituents of the vesicle.

The successful linking of a fluorescent probe, e.g. Dipyrromethene boron difluoride (BODIPY) to α-tocopherol, was shown by West *et al.,* (2010) This serves as an ideal starting point for Pheroid® labelling, as α-tocopherol is one of the constituents for Pheroid® formation. This would enable positive conclusions to be drawn to whether it assisted in the cellular uptake of the PLGA NPs.

In summary, these preliminary results for the development of a novel polymeric NPs and Pheroid® vesicle hybrid system, have shown to be a promising alternative to the conventional treatment for MAC. As was demonstrated by recent work published on a lipid and polymeric

hybrid system that has been synergistically combined, to overcome cancer drug resistance (Zeng *et al.,* 2017) and further modified with a targeting ligand, that demonstrated excellent tumour targeting and significantly lower side effects with compared to free docetaxel for cancer treatment (Wang *et al*., 2017).

6.2 Future Prospects

This preliminary investigation paved the way for further and extensive development to be conducted such that the synergy and functionality of this hybrid system can be proved.

Recommendations for further studies on PLGA-MA NPs with MAC therapeutics combined with Pheroid® vesicles may include the following:

- Optimise the PLGA NPs preparation method
- Determine the EE and DL via a direct method, such that drug dosing can be calculated for further *in vitro* and *in vivo* screening
- Complete a FACS assay to confirm MA facilitated in cellular uptake
- Investigate the electrostatic interactions between the PLGA NPs and Pheroid® delivery system
- Conjugate a lipid probe to the Pheroid® to confirm its participation in cellular uptake

If all of these suggestions are attended to, this hybrid system could be further evaluated in terms of its drug release profile as well its ability to behave *in vivo and* prove its worth in improving the treatment for MAC.

References

Bilati, U, Allemann, E. & Doelker, E. 2003. Sonication parameters for the preparation of biodegradable nanocapsules of controlled size by the double emulsion method. 2003. *Pharmaceutical Development and Technology*, 8:1–9.

Blanco, E., Shen, H. & Ferrari, M. 2015. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nature Biotechnology*, 33:941-951.

Chelopo, M.P., Kalombo, L., Wesley-Smith, J., Grobler, A. & Hayeshi, R. 2017. The fabrication and characterization of a PLGA nanoparticle-Pheroid® combined drug delivery system. *Journal of Material Science*, 52:3133-3145.

Ducat, E., Evrard, B., Peulen, O. & Piel, G. 2011. Cellular uptake of liposomes monitored by confocal microscopy and flow cytometry. *Journal of Drug Delivery and Science and Technology*, 21(6): 469-477.

Hall, J.B., Dobrovolskaia, M.A., Patrik, A.K. & McNeil, S.E. 2007. Characterisation of nanoparticles for therapeutics. *Nanomedicine*, 2(6):789-803.

Le Meins, J.F., Schatz, C., Lecommandoux, S. & Sabdre, O. 2013. Hybrid polymer/lipid vesicles: State of the art and future prospectives. *Materials Today,* 16:397.

Lemmer, Y., Kalombo, L., Pietersen, R.D., Jones, A.T., Semete-Makokotlela, B., Van Wyngaardt, S., Ramalapa, B., Stoltz, A.C., Baker, B., Verschoor, J.A., Swai, H.S. & De Chastellier, C., 2015. Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis. *Journal of Controlled Release,* 211:94-104.

Rao, J.R. & Geckeler, K.E. 2011. Polymer nanoparticles: Preparation techniques and size-control parameters. *Polymers in Polymer Science*, 36:887-913.

Song, C.X., Labhasetwar, V., Murphy, H., Qu, X., Humphrey, W.R., Shebuski, R.J.&Levy, R.J. 1997. Formulation and characterization of biodegradable nanoparticles for intravascular local drug delivery. *Journal of Controlled Release*, 43:197–212.

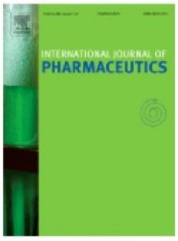
Zambaux, M.F., Bonneaux, F., Gref, R., Maincent, P., Dellacherie, E., Alonso, M.J., Labrude, P. & Vigneron, C. 1998. Influence of experimental parameters on the characteristics of poly (lactic acid) nanoparticles prepared by a double emulsion method. *Journal of Controlled Release*, 50:31–40.

Annexure a

INTERNATIONAL JOURNAL OF PHARMACEUTICS

AUTHOR INFORMATION PACK

TABLE OF CONTENTS



ISSN: 0378-5173

DESCRIPTION

The *International Journal of Pharmaceutics* is the journal for **pharmaceutical scientists** concerned with the physical, chemical and biological properties of devices and **delivery systems** for **drugs**, **vaccines** and **biologicals**, including their design, manufacture and evaluation. This includes evaluation of the properties of drugs, **excipients** such as surfactants and polymers and novel materials. The journal has special sections on **pharmaceutical nanotechnology** and personalized medicines, and publishes research papers, reviews, commentaries and letters to the editor as well as special issues.

Editorial Policy

The over-riding criteria for publication are originality, high scientific quality and interest to a multidisciplinary audience. Papers not sufficiently substantiated by experimental detail will not be published. Any technical queries will be referred back to the author, although the Editors reserve the right to make alterations in the text without altering the technical content. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the manuscript shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

AUDIENCE

Pharmaceutical Scientists, Clinical Pharmacologists, Chemical Engineers, Biotechnologists.

IMPACT FACTOR

2013: 3.785 © Thomson Reuters Journal Citation Reports 2014

ABSTRACTING AND INDEXING

BIOSIS
Chemical Abstracts
MEDLINE®
International Pharmaceutical Abstracts
EMBASE
PASCAL M
Science Citation Index
CAB Abstracts
Current Contents (Life Sciences, Clinical Medicine)
Elsevier BIOBASE/Current Awareness in Biological Sciences
Scopus

EDITORIAL BOARD

GUIDE FOR AUTHORS

INTRODUCTION

The *International Journal of Pharmaceutics* publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

Types of paper

(1) **Full Length Manuscripts**

(2) **Rapid Communications**

(a) These articles should not exceed 1500 words or equivalent space.
(b) Figures should not be included otherwise delay in publication will be incurred.
(c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

(3) **Notes**

Should be prepared as described for full length manuscripts, except for the following:

(a) The maximum length should be 1500 words, including figures and tables.
(b) Do not subdivide the text into sections. An Abstract and reference list should be included.

(4) **Reviews and Mini-Reviews**

Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Human and animal rights

If the work involves the use of animal or human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/en/30publications/10policies/b3/index.html; EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

Conflict of interest
All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Submission declaration and verification
Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/sharingolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Contributors
Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship
This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:
Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.
After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Article transfer service
This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal. More information about this can be found here: http://www.elsevier.com/authors/article-transfer-service.

Copyright
Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted third party reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. For more information see http://www.elsevier.com/copyright.

Role of the funding source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some authors may also be reimbursed for associated publication fees. To learn more about existing agreements please visit http://www.elsevier.com/fundingbodies.

Open access
This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse
• An open access publication fee is payable by authors or on their behalf e.g. by their research funder or institution
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs (http://www.elsevier.com/access).
• No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 3000**, excluding taxes. Learn more about Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

Note:
The choice of general classifications such as "drug delivery" or "formulation" are rarely helpful when not used together with a more specific classification.

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of at least four potential reviewers. Good suggestions lead to faster processing of your paper. Please note:
Reviewers who do not have an institutional e-mail address will only be considered if their affiliations are given and can be verified. Please ensure that the e-mail addresses are current. International reviewers who have recently published in the appropriate field should be nominated, and their areas of expertise must be stated clearly. Note that the editor retains the sole right to decide whether or not the suggested reviewers are contacted.
To aid the editorial process when suggested reviewers are not chosen or decline to review, ensure that the classifications chosen as the field of your paper are as detailed as possible. It is not sufficient to state "drug delivery" or "nanotechnology" etc.

PREPARATION

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract must not exceed 200 words.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Keywords
Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Chemical compounds
You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). The PubChem CIDs can be found via http://www.ncbi.nlm.nih.gov/pccompound. Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below:
Chemical compounds studied in this article
Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865)
More information is available at: http://www.elsevier.com/PubChem.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Database linking
Elsevier encourages authors to connect articles with external databases, giving their readers one-click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Image manipulation
Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications that can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the

journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

Reference links
Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One Author designated as corresponding Author:

- E-mail address
- Full postal address
- Telephone and fax numbers

All necessary files have been uploaded

- Keywords
- All figure captions
- All tables (including title, description, footnotes)

Further considerations:

- Use continuous line numbering (every 5 lines) to facilitate reviewing of the manuscript.
- Manuscript has been "spellchecked" and "grammar-checked"
- References are in the correct format for this journal
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web)
- Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print
- If only color on the Web is required, black and white versions of the figures are also supplied for printing purposes

For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal *Physics Letters B*):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor.It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article on ScienceDirect. This link can also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

AUTHOR INQUIRIES

You can track your submitted article at http://help.elsevier.com/app/answers/detail/a_id/89/p/8045/. You can track your accepted article at http://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.

Annexure B

Dovepress

Manuscript preparation

- While the editors fully understand the extra challenges posed to authors whose native language is not English, we must ask that all manuscripts be reviewed and edited by a native speaker of English with expertise in that area prior to submission
- Double-spacing
- 3-cm margins
- Page numbers
- Clear concise language
- American spelling (all components of a manuscript must be in English)
- Ensure tables and figures are cited
- The preferred electronic format for text is Microsoft Word
- Manuscripts will be accepted in LaTeX as long as the native LaTeX and a PDF is also supplied
- Use International Systems of Units (SI) symbols and recognized abbreviations for units of measurement
- Do not punctuate abbreviations eg, et al, ie
- Spell out acronyms in the first instance in the abstract and paper
- Word counts are not specified. In general, shorter items range from 1000 to 3000 words and reviews from 3000 to 7,500
- Generic drug names are used in title, text, tables, and figures
- Suppliers of drugs, equipment, and other brand-name material are credited in parentheses (company, name, city, state, country)
- If molecular sequences are used, provide a statement that the data have been deposited in a publicly accessible database, eg, GenBank, and indicate the database accession number
- Depositing laboratory protocols on protocols.io is encouraged, where a DOI can be assigned to the protocol. To include a link to a protocol in your manuscript:

  1) Describe your step-by-step protocol on protocols.io

  2) Select "Get DOI" to issue your protocol with a unique DOI (digital object identifier)

3) Include the DOI link in the Methods section of your manuscript using the format provided by protocols.io: http://dx.doi.org/10.17504/protocols.io.xxxxxxx (where xxxxxxx is the unique DOI)

- At this stage, your protocol is only visible to those with the link. This allows editors and reviewers to consult your protocol when evaluating the manuscript. You can make your protocols public at any time by selecting "Publish" on the protocols.io website. Any referenced protocols will automatically be made public when your article in published.

Manuscript templates

We have prepared two manuscript templates to help authors when submitting their manuscript to one of our journals. The first template is for all journals except Core Evidence, and the second is only for the journal Core Evidence.

Please click on the appropriate link below and 'Save As' the Word document onto your local computer.

Template for all journals (except Core Evidence)

Core Evidence template

When you are ready to submit your paper please go to our online submission form, which is designed to be as quick and painless as possible.

If you have any questions about submitting your manuscript please email our Editorial team or use the green/red 'Live Support' button on the website.

Manuscript organization

Title page

- Forename(s) and surnames of authors (see Authorship section below)
- Author affiliations: department, institution, city, state, country
- ORCID number(s) for all authors whenever available

Abstract

There are two types of abstracts - structured and unstructured. Original research papers require a structured abstract. Both types of abstracts should be no more than 300 words.

Plain Language Summary (optional)

Academic articles are generally written in a specific manner and aimed at communities of experts. It can be extremely difficult for lay audiences or people not familiar with the field of study to understand those articles.

One way to bridge the gap between providing open access to knowledge and improving its understanding is to provide an easily understandable, stand-alone plain language summary alongside the published research article. As Professor Sir Mark Walport said “Science is for everyone. It’s not just to be shared within a small, closed community. We are in the middle of an information revolution, which has been made possible by Open Access and electronic publications. Plain-English summaries are the way of the future” (Keynote speech, Access to Understanding competition awards ceremony 2014).

Not only it is useful for researchers to write plain language summaries of their articles to make them accessible to a wider audience but they can also make research accessible to professionals in nearby disciplines. Crucially, plain language summaries are beneficial to improve public engagement with science and medical research. By helping the public to understand biomedical research, researchers can contribute to raising awareness of its value and attracting further public support and involvement. Plain language summaries can help connect researchers with those, who are directly and indirectly impacted by the outcomes of the research thereby helping scientists understand the needs of patients, carers, health professionals, and other members of the public. In turn, better understanding of biomedical research can inform future research directions and funding priorities as well as increase recruitment to clinical trials.

As an author, promoting your work in an engaging way to a wider audience can help you:

- Attract more readers
- Potentially increase the number of citations to your articles
- Get noticed
- Build a strong reputation
- Connect with patients, carers, politicians, policy-makers and other decision-makers
- Attract more funding opportunities
- Expand your professional network

For the reasons described above, we now require all authors of accepted research articles, reviews, clinical trials, protocols and methodologies to write a plain language summary to be published with their article. The plain language summary should have between 150 and 250

words, be written in plain English, and be placed after the Abstract and before the Introduction. The plain language summary should be distinct from the abstract and should be written in an accessible, interesting way without spinning or exaggerating the story.
- The plain language summary should not be a “dumbed down” version of your work. You must not treat your audience as stupid or patronise the reader.
- Provide answers to the questions: Why was the study done, What did the researchers do and find, What do these results mean?
- Communicate the facts in an interesting way and put them in the appropriate context.
- Use short, clear sentences broken up into paragraphs for readability. You may use bullet points.
- Use the active voice rather than the passive voice (for example, “Dr Smith’s team report several improvements” rather than “Several improvements were reported by Dr Smith’s team”).
- Avoid jargon, complex grammatical structures or abbreviations. You should use everyday English words rather than complex words. If you need to use a technical term or abbreviation, please explain it the first time you use it.

- Phrase sentences in a positive manner rather than negatively.
- Use person-centred language rather than focussing on the condition/illness or disability.
- Ask someone, who doesn’t have any knowledge of the subject, to read your plain language summary and provide feedback. They should find it interesting and they should be able to understand what your study was, what the conclusions are and what the impact of the research may be.

For further information on how to write about biomedical and health research in plain English, please read the Access to Understanding Writing Guidance or the INVOLVE Plain English Summaries resource from the National Institute for Health Research.

Keywords

3–6 keywords

Running header (shortened title)

Corresponding author

Name, physical address, phone, fax, email

Introduction

Material and Methods

Results

Discussion

Conclusions

Abbreviations (if any)

Ethics approval and informed consent

Manuscripts reporting studies on human subjects, human data or tissue, or animals should include a statement on ethics approval and consent when humans are involved. Please include the name of the committee that approved the study and reference number if appropriate. Please see our editorial policies for more information.

Consent for publication

In cases where details, images, or videos relating to individual participants are included in the manuscript, authors should have obtained written informed consent to publish the details from the participant or parent/guardian. Authors should add a statement to this effect here. Authors should be able to provide such signed consent if required by the Editor.

Data availability (where applicable)

Please include a statement about where data supporting the results reported in the manuscript can be found and about data sharing including, where applicable, links to the publicly archived datasets. The statement of data availability should explain which additional unpublished data from the study, if any, are available, to whom, and how these can be obtained. In cases where authors do not wish to share their data or are unable to do so, they should state that data will not be shared and the reasons why. Please refer to our editorial policies for further information.

Funding

Please declare all the sources of funding including financial support. Please describe the role of the sponsor(s), if any, in any of the stages from study design to submission of the paper for publication. Please state if the sponsor(s) had no such involvement.

Competing interests

Your relationship with other people or organisations may influence the way you interpret data or present the information your study demonstrated. This is known as a competing interest. For transparency, authors are required to complete a declaration of competing interests. The author(s) of a paper submitted to any Dove Medical Press journal are required to complete a declaration of competing interest for any commercial associations or financial interests held by the author or immediate members of the author's family, which might be construed as posing a conflict of interest, including but not limited to consultancies, employment, expert testimony, honoraria, retainers, stock holdings or options, and

membership on boards of for-profit organizations with a financial interest in the paper. All competing interests will be listed in the declarations at the end of the article.
Please consider the following questions when completing your competing interest declaration:

- *Financial competing interests*

In the past three years have you received any funding from an organization that may have a financial interest in the manuscript? If so, please specify.
Do you hold any stock holdings or options in an organization that may have financial interest in the publication of this manuscript? If so, please specify.
Does the content of the manuscript relate to any patents you hold or are you currently applying for? If so, please specify.
Have you received any funding or salary from an organization that holds or has applied for patents relating to the content of the manuscript? If so, please specify.
Do you have any other financial competing interests? If so, please specify.

- *Non-financial competing interests*

Are there any non-financial competing interests to declare in relation to this manuscript? Examples of non-financial competing interests include family associations, political, religious, academic or any other.
If so, please specify.

If you are unsure as to whether you, or one your co-authors, has a competing interest please discuss it with the editor.
Dove Medical Press subscribes to the general intent of the principles adopted by the International Committee of Medical Journal Editors (ICMJE) on the control of data in publications arising from sponsored research. The author submitting a manuscript for a paper for any study funded by an organization with a proprietary or financial interest in the outcome shall have access to all the data in that study, and to have complete responsibility for the integrity and accuracy of the data, and the decision to publish.

Authors' contributions

According to the IMCJE guidelines, authorship credit should be based on:
http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html
1. Substantial contributions to conception and design, data acquisition, or data analysis and interpretation;

2. Drafting the article or critically revising it for important intellectual content;
3. Final approval of the version to be published; and
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.
Authors should meet conditions 1, 2, 3, and 4 and appropriate credit for each author's contribution should be given.
Acquisition of funding, data collection, or general team supervision alone does not constitute authorship.
All persons designated as authors should qualify for authorship, and all those who qualify should be listed.
Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content.
Increasingly, authorship of multicenter trials is attributed to a group. All members of the group who are named as authors should fully meet the above criteria for authorship/contributorship.
The group should jointly make decisions about contributors/authors before submitting the manuscript for publication. The corresponding author/guarantor should be prepared to explain the presence and order of these individuals. It is not the role of editors to make authorship/contributorship decisions or to arbitrate conflicts related to authorship.

Acknowledgements

All contributors who do not meet the criteria for authorship should be listed in the Acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chairperson who provided only general support. Authors should declare whether they had assistance with study design, data collection, data analysis, or manuscript preparation. If such assistance was available, the authors should disclose the identity of the individuals who provided this assistance and the entity that supported it in the published article. Financial and material support should also be acknowledged.

Groups of persons who have contributed materially to the paper but whose contributions do not justify authorship may be listed under such headings as "clinical investigators" or "participating investigators," and their function or contribution should be described—for example, "served as scientific advisors", "critically reviewed the study proposal", "collected data", or "provided and cared for study patients". Because readers may infer their endorsement of the data and conclusions, these persons must give written permission to be

acknowledged. For individual members of a collaboration group to be searchable through PubMed (for those journals listed on PubMed), please ensure that the title of the collaboration group is included on the title page and in the submission system and also include collaborating author names as the last paragraph of the "Acknowledgements" section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name.
As it takes PubMed additional time to code these groups these may not be present when an article is initially included on PubMed.

Please note: the Authorship and "Contributors Listed in Acknowledgments" sections are reprinted from the ICMJE Uniform Requirements for Manuscripts Submitted to Biomedical Journals. Dove Medical Press prepared this reprint. The ICMJE has not endorsed nor approved the contents of this reprint. The official version of the Uniform Requirements for Manuscripts Submitted to Biomedical Journals is located at http://www.icmje.org/. Users should cite this official version when citing the document.

Authors' information (optional)

Information about the author(s) that may be relevant to the interpretation of the article may be listed here. This may include the authors' affiliations, qualifications or other relevant background information. This section does not list any competing interests.

References

See Reference Style Guidelines

Figures and tables

- Submit as separate files and also as one combined file
- Submit figures as JPG files
- All figures and tables must be in English
- Number consecutively
- Provide a descriptive heading/legend
- Place abbreviations immediately below the table
- Use superscript a, b, c… as identifiers
- Supply Line Art 900 dpi, Combination (Line Art + Halftone) 900 dpi, Halftone 300 dpi
- Graphics downloaded from Web pages are NOT acceptable
- Submit multi-panel figures, ie with parts labeled a,b,c,d, as one file

Supplementary data

Any supplementary data should be kept to 4 typeset pages or 2,400 words. If you have any more than this you should provide a link to the supplementary data on an external website, your institute's website for example, and/or Dove Medical Press may be able to upload the raw supplementary data to the https://www.dovepress.com/ website and provide a link in your paper. We welcome video files either as supplementary data or as part of the actual manuscript to show operations, procedures, etc.

Use of Brand Names in submitted manuscripts

We require that generic drug names are used in submissions. When proprietary brands are used in research, use the generic name throughout the text. You may include the brand name(s) in parentheses after the first mention only of the generic name (once in the Abstract and once in the body of the manuscript is acceptable).

Clinical trial registration

We require the registration of all clinical trials in a public trials registry at or before the time of first patient enrolment.

Reference Style Guidelines

DMP follow the style adopted by the American Medical Association (AMA),* (pp39–79) which, in turn, is based on the style developed by the International Committee of Medical Journal Editors in 1978 in Vancouver.

Reference Management systems

Users of the EndNote® software should select the JAMA reference style when preparing references for any Dove Medical Press Journal.

Please note that authors are responsible for the accuracy and completeness of their references.

Text citations: Cite references sequentially in text, tables, and legends by superscript Arabic numerals with no parentheses, eg, [1] or [3,4] or [10–15]. Numbers should be

placed **after** punctuation marks, eg, .[3,4] **Do not use** Microsoft Word's footnote/endnotes function to build the reference list as this can introduce errors during the typesetting process.

Reference list:

List items **numerically** in the order they are cited in the text, eg, 4. Kapur NK, Musunuru K. Clinical efficiency and safety of statins in managing cardiovascular risk. *Vasc Health Risk Manag*. 2008;4(2):341–353.

Some commonly used sample references follow.

Journal articles

Standard journal article:

Kapur NK, Musunuru K. Clinical efficiency and safety of statins in managing cardiovascular risk. *Vasc Health Risk Manag*. 2008;4(2):341–353.

Abbreviate names of journals according to the journals list in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed)

List **all** authors if six or less, otherwise list **first three only**, followed by "et al.":Brandén E, Gnarpe J, Hillerdal G, et al. Detection of Chlamydia pneumoniae on cytospin preparations from bronchiolar lavage in COPD patients and in lung tissue from advanced emphysema. *Int J Chron Obstruct Pulmon Dis*. 2007;2(4):643–650.

Group as author:

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension*. 2002;40(5):679–686.

Both personal authors and an organization as author:

Munakata M, Honma H, Akasi M; for J-STOPMetS Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *Vasc Health Risk Manag*. 2007;4(2):415–420.

Volume with no issue but a supplement:

Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache*. 2002;42 (Suppl 2):S93–S99.

Issue with supplement:

Glauser TA. Integrating clinical trial data into clinical practice. *Neurology*. 2002;58(12 Suppl 7):S6–S12.

V*olume with part:*

Abend SM, Kulish N. The psychoanalytic method from an epistemological viewpoint. *Int J Psychoanal*. 2002;83(Pt 2):491–495.

Issue with part:

Ahrar K, Madoff DC, Gupta S, Wallace MJ, Price RE, Wright KC. Development of a large animal model for lung tumors. *J Vasc Interv Radiol*. 2002;13(9 Pt 1):923–928.

Issue with no volume:

Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop*. 2002;(401):230–238.

No volume or issue:

Outreach: bringing HIV-positive individuals into care. *HRSA Careaction*. 2002 Jun:1–6.

Type of article indicated as needed:

Lofwall MR, Strain EC, Brooner RK, Kindbom KA, Bigelow GE. Characteristics of older methadone maintenance (MM) patients [abstract]. *Drug Alcohol Depend*. 2002;66 Suppl 1:S105.

Article published electronically ahead of the print:

Yu WM, Hawley TS, Hawley RG, Qu CK. Immortalization of yolk sac-derived precursor cells. *Blood*. 2002 Nov 15;100(10):3828–3831. Epub 2002 Jul 5.

Foreign language:

Virchow R. Aetiologie der neoplastischen Geschwulst/Pathogenie der neoplastischen Geschwulste [Etiology and pathology of cancerous tumors]. Die Krankhaften Geschwulste. Berlin: Verlag von August Hirschwald; 1865:57–101. German.

Books and other monographs

Personal author(s):

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. *Medical Microbiology*. 4th ed. St Louis: Mosby; 2002.

Editor(s), compiler(s) as author:

Gilstrap LC 3rd, Cunningham FG, VanDorsten JP, eds. *Operative Obstetrics*. 2nd ed. New York: McGraw-Hill; 2002.

Author(s) and editor(s):

Breedlove GK, Schorfheide AM. *Adolescent Pregnancy*. 2nd ed. Wieczorek RR, ed. White Plains (NY): March of Dimes Education Services; 2001.

Chapter in a book:

Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. *The Genetic Basis of Human Cancer*. New York: McGraw-Hill; 2002:93–113.

Conference proceedings (published):

Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13–15; Leeds, UK. New York: Springer; 2002.

Conference proceedings (unpublished):

Eisenberg J. Market forces and physician workforce reform: why they may not work. Poster presented at: Annual Meeting of the Association of American Medical Colleges; October 28; 1995; Washington, DC.

Patent:

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1.

Other published material

Newspaper article:

Tynan T. Medical improvements lower homicide rate: study sees drop in assault rate. *The Washington Post*. 2002 Aug 12;Sect. A:2 (col. 4).

Prescribing information:

Ampyra® (dalfampridine) extended release tablets [prescribing information]. New York: Acorda Therapeutics, Inc; 2010.

Package insert:

Coumadin® (warfarin sodium) [package insert]. Princeton, NJ: Bristol-Myers Squibb; 2010.

Dissertation:

Etsey AN. Effects of Regular Ingestion of Natural Cocoa on Testicular Histology in Streptozotocin-Induced Diabetic Rats [dissertation]. Accra: University of Ghana; 2009.

Clinical trial:

Pfizer. A study in patients with non-small cell lung cancer testing if erlotinib plus SU011248 (sunitinib) is better than erlotinib alone (SUN1058). Available from: http://www.clinicaltrials.gov/ct2/show/NCT00265317. NLM identifier: NCT00265317. Accessed August 1, 2011.

Unpublished material

In press:

Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. In press 2002.

Avoid citing a personal communication unless it provides essential information not available from a public source, in which case the name of the person, date of communication and the form of communication (e.g. verbal or written) should be cited in parentheses in the text, not as a formal reference.

Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.

Electronic material

Journal article on the Internet:

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. *Am J Nurs* [serial on the Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 3 p.]. http://www.nursingworld.org/AJN/2002/june/Wawatch.htm. Accessed April 3, 2003.

Homepage/Website:

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/. Accessed August 29, 2003.

---

*Iverson C, Christiansen S, Flanagin A, et al. *AMA Manual of Style: A Guide for Authors and Editors.*10th ed. New York, NY: Oxford University Press; 2007.

Paper type definitions

Case report
In medicine, a case report is a detailed report of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient, but usually describe an unusual or novel occurrence. Some case reports also contain a literature review of other reported cases.

Case series
More than 1 case report. A case series (also known as a clinical series) is a medical research descriptive study that tracks patients with a known exposure given similar treatment or examines their medical records for exposure and outcome.
It can be retrospective or prospective and usually involves a smaller number of patients than more powerful case-control studies or randomized controlled trials. Case series may be consecutive or non-consecutive, depending on whether all cases presenting to the reporting authors over a period were included, or only a selection.

Commentary
Short, decisive observations and findings that generally relate to a contemporary issue, such as recent research findings, but can also include the discussion of difficulties and possible solutions in a field of research.

Corrigendum
Correction to an error in published paper; due to author's error.

Editorial
An opinion piece written by the senior editorial staff or publisher. Editorials may be supposed to reflect the opinion of the journal. Guest Editorials may only be submitted when an Editor-in-Chief has approached the author to write one directly. Regular submissions cannot be made as Editorial pieces.

Erratum
Correction to an error in published paper; due to publisher's error.

Expert opinion
Where experts in their field can promote rigorous research that makes a significant contribution to advancing knowledge.

Hypothesis
A hypothesis (plural hypotheses) is a proposed explanation for a phenomenon. For a

hypothesis to be a scientific hypothesis, the scientific method requires that one can test it. Scientists generally base scientific hypotheses on previous observations that cannot satisfactorily be explained with the available scientific theories. Even though the words "hypothesis" and "theory" are often used synonymously, a scientific hypothesis is not the same as a scientific theory. A scientific hypothesis is a proposed explanation of a phenomenon which still has to be rigorously tested. In contrast, a scientific theory has undergone extensive testing and is generally accepted to be the accurate explanation behind an observation. A working hypothesis is a provisionally accepted hypothesis proposed for further research.

Letter to the editor

Letters to the Editor will be considered for publication that are pertinent to articles recently published in Dove Medical Press (DMP) journals. Please ensure that your letter is addressed to the appropriate Editor-in-Chief of the journal concerned. All letters should be received within 30 days of the published paper appearing in a DMP journal. Letters received after this time will rarely be considered. All letters will be screened for appropriateness and significance and the Editor may assign external peer review at their discretion. DMP journals are not a vehicle for grievances or personal rebukes. DMP reserves the right to reject letters where the Editor-in-Chief has deemed it unfit on the grounds of misleading, inaccurate or inappropriate content. The Editor-in-Chief will make a final decision. Word count should not exceed 500 words of text and 5 references, 1 of which should be to the recent article, and no more than 3 cited authors. The text should include the full name, academic degrees, and a single institutional affiliation for each author and the e-mail address for the corresponding author. Letters should not duplicate other material published or submitted for publication and should not include unpublished data. Letters not meeting these specifications are generally not considered for publication.

Meeting Report

Should focus on developments presented at the meeting, particularly any new research discoveries. The abstract of the Meeting Report should be short and unstructured giving the name, location (city and state or country) and dates, as well as an indication of the meeting. The body of the article can have subsections with short headings. If speakers are mentioned please provide their full name, institution, city and country. There should be a maximum word count of 2500 words. A reference list should not be included. If abstracts are published from the meeting a URL should be included of where these can be found.

Methodology

The systematic, theoretical analysis of the methods applied to a field of study, or the theoretical analysis of the body of methods and principles associated with a branch of knowledge. A Methodology does not set out to provide solutions but offers the theoretical underpinning for understanding which method, set of methods or so called “best practices” can be applied to a specific case.

Original research

Reports data from original research, in which the conclusions drawn from data collected,

show a major advance in understanding an important issue. Original research is the results of a study written by the researchers who did the study. The hypothesis, research method and results are detailed and the results are discussed.

Perspectives

More like a review but written with the author's point of view in mind. They focus on a specific field or discipline, and discuss current advances or future directions, and may include original data as well as personal insights and opinions.

Photo Essays

A Photo Essay should focus on the visual aspects of the topic presented. It should be a series of photographs that visually tell the story the author wishes to convey. The photos should be self-explanatory of very high quality. Photographs can be of clinical subjects, laboratory results (eg, slides, scans, magnetic resonance images, ultrasonograms) and therapeutic procedures. A Photo Essay should not exceed 300 words and should have no more than 10 references. The number of photographs is limited to 10, with a limit of 60 words for each legend. Please note that not all journals published by Dove Medical Press accept Photo Essays, please ask before submitting.

Rapid communication

Same as a Short report.

Research Letter

Research Letters are concise, focused reports of original research or observations. They should not be under consideration, submitted or published elsewhere in any form, in part or as whole. They should not exceed 600 words of text and 7 references, and up to 2 tables or figures/photographs/images (photograph format should follow research article guidelines – see Figures and Tables). An abstract is not required for a Research Letter but authors should follow the manuscript preparation and submission guidelines. Research letters are subject to external peer review and will be screened for appropriateness and significance. The Editor-in-Chief of the journal will make the final decision on publication. Research Letters not meeting these specifications are generally not considered for publication.

Retraction

In science, a retraction of a published scientific article indicates that the original article should not have been published and that its data and conclusions should not be used as part of the foundation for future research. The most common reasons for the retraction of

articles are scientific misconduct including plagiarism, serious errors, and duplicate/concurrent publishing (self-plagiarism).

Review

Literature reviews of published papers. These look in depth and discuss topics that have had significant research or progress over recent years.

Short report

Same as Rapid communication. Brief (2-3 page) reports of data from original research, focused on initial findings that will be of interest to scientists in other fields.

Study protocol

A study protocol describes in detail the plan for conducting a specific clinical study and explains the purpose and function of the study as well as how to carry it out

Invited reviews

We operate a programme that commissions reviews from leading authors around the world and across a range of subjects. We invite the submission of reviews on a particular topic and, in some instances, will even suggest a structure for the review that the authors should follow when writing their review.

Publication processing fee

These invited reviews are submitted in the normal way via our website and are exempted from paying any publication processing fee.

Editorial decision-making

Our long-standing policy has been not to let editorial decision-makers know which papers are invited and which are submitted spontaneously. Our view has always been that editorial decision-makers should not have their view clouded either for or against a paper simply because it has been invited. Good papers should be accepted and bad papers rejected irrespective of their source. As a result some invited reviews will be rejected.

Manuscripts are subject to same checks as all other manuscripts

All invited reviews that come to us are subject to all the same checks that every paper goes through. These are:

- Authors and their affiliations are checked;
- Conflicts of Interest information is sought for all authors;
- CrossCheck antiplagiarism software is used to check for re-use of materials;

- External peer-review with a minimum of two comprehensive sets of narrative comments and two numerical scores are required;
- The editorial decision-maker, often the Editor-in-Chief, will review the submitted manuscript, peer-reviewer comments and scores, and Conflict of Interest declarations before making their first editorial decision.

After first editorial review

Many manuscripts will require to be modified in order to address points raised by peer-reviewers or suggested by editorial decision-makers. It is not a case of having to address all the points raised. Rather we require that the author provide us with a revised manuscript and a point-by-point response to the points raised. If authors disagree with individual points or feel that they are misguided they should detail this in their point-by-point response.

The editorial decision-maker who reviewed the submission at first editorial review will subsequently receive the revised manuscript and the point-by-point covering letter and make a decision. This may be to reject the paper, return it to peer-reviewers for further consideration, or return it to the author directly for further points to be addressed. They may also make the decision of accept the paper for publication.

Video abstracts

We are pleased to announce the winner for the Dove Medical Press Video Abstract Award 2016

In vitro study of RRS HA injectable mesotherapy/biorevitalization product on human skin fibroblasts and its clinical utilization

Pierre-Antoine Deglesne, Rodrigo Arroyo, Evgeniya Ranneva, Philippe Deprez

Castelló d'Empúries, Spain

Annual Video Abstract Awards

Submit a video abstract with your paper and be in to win the annual Dove Medical Press Video Abstract Award. The videos with the most views will be eligible for selection and voting will be performed by published Dove Medical Press authors.
Video abstracts are an addition to papers that we offer our authors.
The videos would be presented by the author, be of 1-3 minutes duration and give an overview of their paper so readers can get an idea of the content and motivation behind the paper.

The aim is to enable authors to personally explain the importance of their work to the reader. Video abstracts will enhance the reader's understanding and appreciation of an article through the accessible presentation of the main results and conclusions reported. To maximize engagement and visibility, authors are encouraged to combine footage of themselves with other relevant material of interest—such as footage of an experiment running or a lab tour.

The English video abstracts will be published with the paper and also provided to the journal indexing databases (eg, PubMed, MedLine, Embase, Scopus, CAS).

Editorials, Expression of Concern, Corrigenda, Photo Essays and Meeting Reports do not accept video abstracts.

2017 awards: Video abstracts (papers) published before midnight on November 30th will be eligible for inclusion in the 2017 video abstract competition.

Video abstract examples:

• New modalities of cancer treatment for NSCLC: focus on immunotherapy
• Worldwide increase in diabetes: implications for tuberculosis control
• Effects of magnetic cobalt ferrite nanoparticles on biological and artificial lipid membranes
• Effect of melatonin on nocturnal blood pressure
• Racial differences in endothelial microparticles
• Superparamagnetic iron oxide nanoparticles
• Everolimus-eluting stents

Editorial guidelines

Please adhere to the following guidelines when producing your video abstract.
• No longer than 3 minutes in duration.

• Introduce the topic of the article, highlight the main results and conclusions and discuss future potential developments in the field as a result of the work.
• The presentation should be understandable and accessible to users outside of the immediate field of the article.
• Inclusion of additional relevant material such as animations and lab footage is encouraged. Please check if any figures/animations are subject to copyright and, if so, ensure you have permissions to use these in your video.
• Do not include small text that will be difficult to read.
• Video abstracts will only be accepted in English.
• Authors should speak clearly and slowly when presenting their video abstract.
• Terms and conditions regarding the use and distribution of video abstracts will apply in line with the copyright statement of Dove Medical Press.
• Use only your own original work.

Technical specifications

Video abstracts must meet minimum standards of quality for both video and audio components. In creating a video abstract, please meet the following specifications.
• Format: .mov, .mpg, .mp4, .avi, .flv, .wmv, WebM
• Maximum file size: 1 GB
• Up to 3 minutes in duration

Whilst it is always best to use professional video equipment to record your video abstract, we recognize that not all authors have access to such resources. If you have a mobile phone that produces reasonable quality video please feel free to use this. Before sending it to us always view the video yourself to ensure it is of adequate quality, ie. It is easy to see and hear everything on the video.
PLEASE NOTE: All video abstracts will be assessed for editorial suitability and quality by the editorial team. Any that are not of good enough quality will not be published.

Submission guidelines

Please send your single video abstract file via:
https://www.hightail.com/dropbox?dropbox=VideoAbstracts
Follow the instructions onscreen:
• Type in your name in the ‘Full Name' field
• Type your email address in the ‘Email’ field
• For ‘Subject’ type in “Video abstract for ID xxxxx” (replace xxxxx with the submission ID of your paper)

• Type in any specific instructions into the ‘Message’ box (optional)
• Click ‘Select File’ button and upload your video abstract
• Click ‘Send it'

If you have any queries regarding video abstracts please contact us

Rejection Rate

For 2016 the total of rejections/withdrawals across all Dove journals was 53%. This was 47% in 2015 and 42% in 2014.

Pre-submissions

Authors are welcome to send an abstract of their manuscript to obtain a view from the Editor about the suitability of their paper. Please complete the pre-submission check form here. Our Editors will do a quick review (not peer review) of your paper and advise if they believe it is appropriate for submission to their journal. It will not be a full review of your manuscript.

Submission of manuscripts

- All manuscripts should be submitted via our website (in English)
- By doing so you agree to the terms and conditions of submission
- Keep a backup and hard copies of the material submitted

Proofs

- You will receive the typeset page proofs for approval
- Check amendments made by the editor have not rendered the material inaccurate
- Check you have answered all the editor’s queries
- Ensure your corrections are minimal and absolutely necessary
- Mark the adjustments clearly in the text and margins, and keep a copy of what you send to the editor
- Notify the editorial office of all corrections within 72 hours of your receipt of the material
- Ensure all authors sign and return the Approval for Publication and final page of Publication Agreement