DEVELOPMENT OF ONCE-DAILY MYCOPHENOLATE MOFETIL SUSTAINED RELEASE ORAL NANOPARTICLES

This thesis is submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

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Abstract

Objective: To develop an oral sustained release formulation of the immunosuppressive drug, mycophenolate mofetil (MMF) for once-daily dosing, for use in organ transplant recipients as an anti-rejection drug. Formulating mucoadhesive chitosan-coated polymeric nanoparticles (CS-PNPs) of MMF presents a novel strategy for achieving sustained drug release of an essential drug for transplant patients, which could improve medication adherence and therefore transplant outcomes.

Methods: MMF CS-PNPs were prepared by a single emulsion solvent evaporation method in chloroform with slight modifications. All the formulations are evaluated for particle size, encapsulation efficiency as well as *in vitro* drug release in USP simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Differential scanning calorimetry (DSC), surface morphology by scanning electron microscopy (SEM), and *in vitro* mucin binding of the nanoparticles were performed for further characterization.

Results: Two optimal formulations [MMF: PLA: MMWC= 1:7:7 and MMF: HMWPLGA: HMWC= 1:7:7] had high encapsulation efficiency (94.34% and 75.44% respectively) and sustained drug release with a minimal burst phase. DSC experiments reveal an amorphous form of MMF in the nanoparticle formulations. The surface morphology of CS-PNPs observed by SEM showed spherical nanoparticles with minimal visible porosity. Mucin binding was assessed by changes in zeta potential after incubation of the nanoparticles in mucin.

Conclusion: Two CS-PNP formulations; MMF: HMWPLGA: HMWC= 1:7:7 (w/w/w) and MMF: PLA: MMW= 1:7:7(w/w/w) had high drug loading and sustained drug release of MMF, representing lead candidates in the effort to design a once-daily dosage form for MMF.

Acknowledgements

First and the foremost, I would like to thank the Almighty for everything

I would like to extend my gratitude to my supervisor, Dr. Ellen Wasan for giving me an opportunity to be a part of Wasan lab at University of Saskatchewan. She has provided such an invaluable support all through the program. I'm grateful to the College of Graduate and Postdoctoral studies and the College of Pharmacy and Nutrition for providing me financial support in my graduate studies.

I sincerely appreciate the help of Ms. Vicki Keeler and Ms. Dorota Rogowski with the equipment and training in the drug discovery and drug development research cluster. I cannot thank enough the support and guidance offered by my committee members; Dr. Holly Mansell, Dr. Anas El-Aneed and Dr. Ildiko Badea. My heartiest gratitude to the advisory committee of this project, Dr. Ahmed Shoker, Dr. Kishor Wasan, Dr. Holly Mansell and Dr. Ellen Wasan for their brainstorming ideas all throughout the project. I greatly appreciate the help of Dr. Jacqueline Cawthray, previous lab manager of Wasan lab for all her help. My sincere thanks to all the Wasan lab group; Jaweria Syeda, Yunyun Di, Grace Cuddihy and Tatiana Orlowski.

I greatly appreciate the help of Dr. Eiko Kawamura of the Imaging Centre, Western College of Veterinary Medicine, University of Saskatchewan for advice and assistance with the scanning electron microscopy. I would like to extend my sincere appreciations to my colleagues and fellow graduate students, Ayat Zagzoog, Mona Hamada, Mays Al-Dulaymi, Waleed Mohammed-Saeid and Shanal De Silva for their wonderful company and friendly help. I can't thank enough the love and support of my family and all my friends.

Dedication

I would like to dedicate my thesis to

- *My brother (**Mansoor Ali Mohammed**) and my father (**Ali Mohammed**), whom I lost during my graduate studies.*
- *My wife, **Jaweria Syeda** without whom I cannot imagine spending the last three years at University of Saskatchewan.*
- *My supervisor, **Dr. Ellen Wasan** for giving me an opportunity to be her first graduate student and **Dean Kishor Wasan** for the continuous support.*

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List of Abbreviations

MMF	Mycophenolate mofetil
NPs	nanoparticles
PNPs	Polymeric nanoparticles
CS-PNPs	Chitosan coated polymeric nanoparticles
MPA	Mycophenolic acid
g	gram
µg	microgram
mg	milligram
IR	Immediate release
DR	Delayed release
SR	Sustained release
CR	Controlled release
ER	Extended release
USP	United States Pharmacopoeia
GI	Gastrointestinal
nm	nanometers
PLGA	poly-D, L-lactide-co-glycolide
PLA	polylactic acid
PCL	Poly-ε-caprolactone
FDA	Food and Drug Administration
TMC	N-trimethyl chitosan chloride
DNA	Deoxyribonucleic acid

MW	Molecular weight
PSA	Poly (sebacic acid)
DOX	Doxorubicin hydrochloride
PBS	Phosphate buffer saline
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
PVA	Polyvinyl alcohol
HPLC	High performance liquid chromatography
RSD	Relative standard deviation
LOD	Limit of detection
LLOQ	Lower limit of quantification
min	minute
PDI	Polydispersity index
EE	Encapsulation efficiency
rpm	Rotations per minute
h	hour
SEM	Scanning electron microscopy
kV	Kilo volts
DSC	Differential scanning calorimetry
ANOVA	Analysis of variance

Chapter-I. Background

Transplant patients are prescribed immunosuppressive therapy after organ transplantation. Immunosuppressive therapy is needed to prevent organ rejection, which occurs when the immune system of the transplant patient recognizes the antigens on the transplant organ as foreign and signals an attack. Adherence generally describes the degree to which the patient correctly follows medical advice. Adherence to medications is essential in the field of transplant, for preventing organ rejection. Typically, a healthcare provider can expect 23 non-adherent patients out of 100 transplant patients (Dew et al. 2007a). Every day, on an average, a transplant patient takes 11 pills that include immunosuppressive and other supportive drugs (Ponticelli et al. 2010). This a huge burden on a patient, and missing doses can lead to many negative outcomes such as graft impairment, increased mortality and increased healthcare costs (Dew et al. 2007a). Decreasing pill burden may be an important strategy for improving transplant adherence.

Oral drug delivery continues to be the preferred route of administration because of the ease of administration. The time required for a drug given as an oral immediate release formulation to reach 95% of steady-state plasma concentration is 4.3 biological half-lives when equal doses are administered at equal amount of time. Mycophenolate mofetil (MMF, trade name Cellcept®) is an immunosuppressive agent used in most solid organ transplant regimens. With a half-life of 8-16h, it would take around two and half days to attain 95% of steady-state plasma concentration of mycophenolate mofetil (Banker and Rhodes 1996). As the attainment of steady-state plasma concentration is related to the clinical effectiveness of the drug, a patient taking Cellcept® must wait for two and half days to get a maximum therapeutic effect of the drug. This lag time could play a significant role in an immunocompromised patient. If a transplant patient

misses a dose or two, the steady-state would fall off and the patient has to wait for two and half days to attain the steady-state concentration. A potential approach to reduce the fluctuations in plasma drug concentrations with immediate release dosage forms is to formulate sustained release dosage forms. With sustained release dosage forms, the steady-state concentrations can be reached more promptly than immediate release dosage forms and the next doses will help maintain the steady-state concentrations effortlessly. Sustained release dosage forms release drug for a long period thereby limiting the number of doses needed per day.

Of all the sustained release formulations, nanoparticles (NPs) are more preferred due to several advantages such as small particle size, large surface area and modifiable surface. Besides these advantages, NPs are more stable in the gastrointestinal tract compared to other drug delivery systems such as liposomes and lipid-based systems, except solid lipid nanoparticles (Palacio et al. 2016). There are different kinds of nanoparticles depending on the material used to prepare them such as polymeric NP, inorganic NP and biomolecular NP. Formulation scientists face many challenges with respect to drug solubility, permeability and bioavailability. Polymeric NPs (PNPs) have been successfully employed to achieve several formulation strategies such as protection of the drug in gastric environment, enhanced bioavailability, enhanced absorption into a specific tissue and increased GI retention time (Alexis et al. 2008). Therefore, formulating PNPs of MMF with sustained release and mucoadhesive properties can help reduce the dosing frequency and release the drug for a long time. This can be achieved by preparing PNPs with PLGA/PLA as polymer and coating them with chitosan to achieve mucoadhesive properties. The MMF is entrapped in the polymeric matrix and the PNPs prepared are stable in gastric environment thereby reducing the gastric irritation caused by MMF. The NPs could help to achieve a once a day dosage formulation. Moving forward, the bigger goal of the project is to

incorporate another immunosuppressive drug such as tacrolimus, in addition to MMF to further decrease pill burden.

Chapter-II. Literature review

2.1. Organ transplantation and organ rejection

Solid organ transplantation is a lifesaving procedure for patients with end-stage organ failure. Over 126, 670 solid organ transplants were performed in 102 countries across the world in 2015, an increase by 5.7% from the previous year of 2014 (Carmona et al. 2017), indicating that transplantation is on the rise. One of the major barriers to the field of transplantation has been the development of organ rejection which can also be termed as transplant or graft rejection. This is when the immune system identifies the transplant as foreign, triggering a complex response that will ultimately destroy the new organ (Tse and Marson 2014). There are different types of organ rejection, which can occur either acutely or chronically. While organ rejection is complex, it often occurs due to the innate and adaptive immunity interactions with T cells (Ingulli 2010).

2.2. Immunosuppressive therapy

To prevent graft rejection and maintain a successful transplant, a complex regimen of immunosuppressive medications is necessary for blocking the body's immune response. The importance of adhering to these immunosuppressive regimens cannot be understated. Missing doses leads to negative outcomes such as graft impairment, increased mortality and increased healthcare expenditures (Dew et al. 2007b). Nevertheless, approximately one in four patients fails to take their medications as prescribed for a multitude of reasons, which may include high pill burden, uncomfortable side effects and/or high cost of medication (Butler et al. 2004). Immunosuppressive agents are generally categorized into two types which include induction agents and maintenance agents. Induction agents act when the host's immune system is first exposed to antigens, while

maintenance drugs help provide continuous protection against organ rejection. Induction agents include antithymocyte globulin (rATG) (Brennan et al. 1999), interleukin-2 receptor antagonists (Webster et al. 2004) and alemtuzumab (Hanaway et al. 2011). Maintenance drug include azathioprine (Tiede et al. 2003), glucocorticoids (Rhen and Cidlowski 2005; Pascual et al. 2009), calcineurin inhibitors (tacrolimus and cyclosporine) (Kehrl et al. 1986), mycophenolate mofetil and mycophenolic acid (Sollinger 1995; Mele and Halloran 2000), mTOR inhibitors (sirolimus (Webster et al. 2006), everolimus (Nashan et al. 2004)) and belatacept (Durrbach et al. 2010).

Table 1. Immunosuppressive drugs commonly used in solid organ transplantation, products, dosing regimen and mechanism of action.

Drug	**Products**	**Mechanism of Action**	**Dosage forms**
rATG	PrTHYMOGLOBULIN® Powder for solution	Polyclonal antibodies against CD2, CD4, CD8, CD11a, CD18, CD25 and CD 44.	Injection, 25mg and 5mg/mL
Belatacept	Nulojix	Blocks B7/CD28 costimulatory proteins	Injection powder for solution (250 mg).

Basiliximab	Simulect®	Chimeric human/murine monoclonal IgG against CD25	10mg and 20mg
Alemtuzumab	Lemtrada® and Mabcampath®	Humanized monoclonal IgG against CD52	Lemtrada® solution- 12 mg Mabcampath® solution- (10mg and 30 mg).
Glucocorticoids (Ex-Prednisone)	Multiple generic	Inhibits formation of free NF-kB and down-regulates expression of proinflammatory cytokines	Multiple dosage forms
Cyclosporine	Neoral® (capsule and solution)	inhibits calcineurin, prevents activation of IL-	Neoral® capsule (10, 25, 50 and 100mg) Neoral®

		2, stimulates TGF-β expression	solution 100 mg
Tacrolimus	Prograf®	inhibits calcineurin, prevents activation of IL-2, stimulates TGF-β expression	Prograf® injection solution (5 mg/mL) capsule (0.5, 1mg and 5mg) Advagraf® extended release capsule (0.5, 1, 3 and 5mg).
Mycophenolate mofetil and Mycophenolic acid	Cellcept® and Myfortic®	Inhibits inosine monophosphate dehydrogenase and de novo purine biosynthetic pathway	Cellcept® powder for suspension (200mg), capsule (250mg), tablet (500mg) and IV (500mg)

			Myfortic delayed-release tablet (180mg and 360mg).
Sirolimus	Rapamune®	Binding to mTOR complex 1	Rapamune® tablet (1mg), and solution (1mg).
Everolimus	Certican®	Binding to mTOR complex 1	Certican® tablet (0.25, 0.5 and 0.75 mg)

Mycophenolic acid (MPA) is a reversible inhibitor of inosine monophosphate dehydrogenase that inhibits the de novo pathway of guanosine nucleotide synthesis without incorporation into DNA. The pathway critical for proliferation of T and B lymphocytes. One of the most common immunosuppressant regimens in solid organ transplant recipients includes a combination of tacrolimus, mycophenolic acid derivative and prednisone. The marketed formulations of mycophenolate acid (MPA) derivative are Cellcept® (mycophenolate mofetil) and Myfortic® (mycophenolate sodium). Mycophenolate mofetil (MMF) has been developed to enhance the bioavailability of mycophenolic acid while mycophenolate sodium has been developed to reduce the GI effects caused by MMF and delivered as delayed-release tablet. The

chemical formula of MMF is $C_{23}H_{31}NO_7$ and Figure 1 depicts the chemical structure.

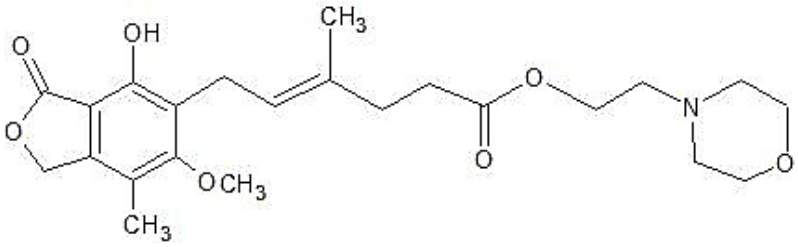


Figure 1. Chemical structure of mycophenolate mofetil.

Cellcept® is an immediate release formulation of mycophenolate mofetil. Cellcept® for oral administration is available as 250mg capsules and 500mg tablets. The oral adult dose of Cellcept® for a renal transplant patient is 1g twice a day, for cardiac and hepatic transplant patient is 1.5g twice a day. The T_{max} and C_{max} of Cellcept® in healthy volunteers are 0.8h and 24.5µg/ml respectively. Myfortic® is a delayed-release tablet formulation of mycophenolate sodium. Myfortic® is available as 180mg and 360mg tablets. The oral adult dose of Myfortic® is 720mg twice daily. A comparison of Cellcept® and Myfortic® is provided below within the discussion on sustained release formulations.

Shirali and colleagues investigated intraperitoneal delivery MPA nanoparticles and confirmed that MPA nanoparticles significantly enhances the graft survival by effectively delivering MPA at a 1000-fold lower dose compared to the conventional tablet dosage form. They also confirmed that drug toxicity was less when MPA was delivered as nanoparticles whereas conventional drug delivery led to cytopenias (Shirali et al. 2011). Similarly, nanoparticles of tacrolimus and surface modified nanoparticles of tacrolimus were prepared to evaluate the pharmacokinetics and lymphatic targeting ability of tacrolimus. These formulations along with Prograf® injectable (commercial tacrolimus product) were administered intravenously to rats and results showed that surface modified tacrolimus nanoparticles provide better therapy

than the commercial Prograf® (Shin et al. 2010). The above two examples have driven formulation scientists in developing an oral sustained release polymeric nanoparticle formulation.

2.3. Sustained release formulations

In conventional oral dosage forms, generally termed as immediate release (IR) formulations, drug release from the dosage form is prompt and achieving an effective steady-state concentration of the drug in necessitates regular dosing intervals of the IR formulations (Amidon et al. 1995), i.e. not missing doses. With IR formulations, one of the disadvantages is fluctuations in the plasma drug concentration, and therefore more difficulty in maintaining steady-state concentration for drugs with short biological half-lives drives the development of modified release formulations. Modified release oral formulations consist of various types of formulations such as delayed-release (DR), prolonged release, sustained release (SR), extended release (ER) and controlled release (CR). In DR formulations, the drug is not released immediately in the GI tract and this can be achieved by enteric-coating (Aulton 2002). SR dosage forms generally release drug slowly enough to provide therapeutic action followed by gradual release over time. While ER formulations release drug immediately for therapeutic action and sustain the drug levels for prolonged time, generally 8-12 h. CR dosage forms release drug at a constant rate so that the drug levels in plasma does not change over time. According to United States Pharmacopoeia (USP), ER, PR and SR dosage forms are generally interchangeable. A general comparison of IR and SR formulations can be seen in Figure 2. With the modified release formulations, there are several advantages over conventional formulations which include; control over drug release and maintenance of plasma drug levels, improved

patient compliance as dosing frequency is reduce, cost-effective, ability to deliver potent drugs and avoiding dose dumping (Chien 1983).

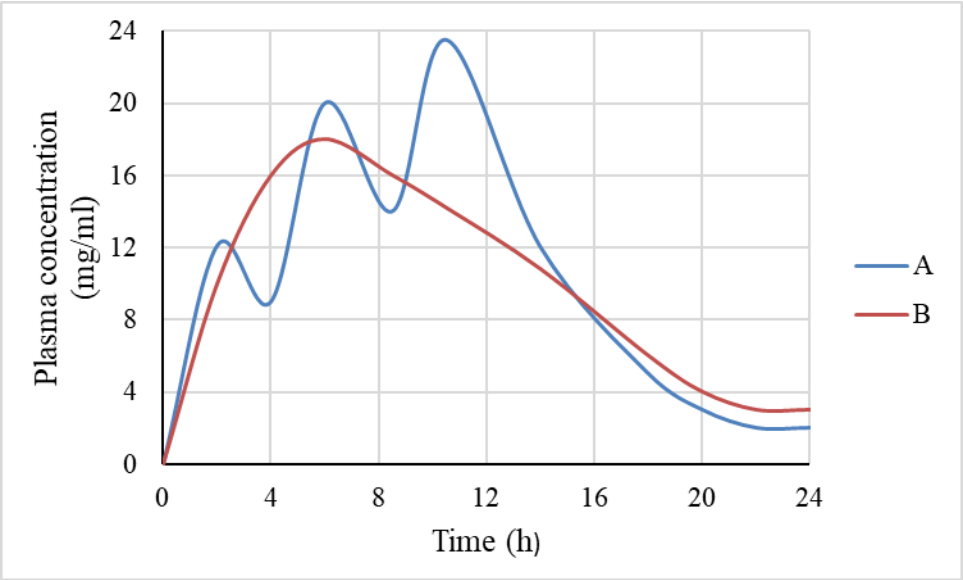


Figure 2. A graphical comparison in the plasma drug concentration levels of IR formulations (A) and SR formulation (B).

One of the important components in formulating SR dosage forms other than the drug itself is to include a drug release modifier or release controlling agent. Oral SR dosage forms can be categorized into monolithic or matrix systems, reservoir or membrane controlled systems and osmotic pump systems depending on their make. As the name indicates, monolithic systems are unit dosage forms in which the drug is dispersed in either a soluble or an insoluble matrix. In case of soluble matrices, matrix solubilizes releasing the drug which then undergoes dissolution. In the latter, the drug must dissolve *in situ* and diffuse out. Reservoir systems contain drug in the core surrounded by a rate controlling membrane. This membrane becomes permeable as it reaches GI lumen, slowly diffusing the drug out through the membrane. Osmotic pump systems are also a type of reservoir system, but the drug is either solubilized or dispersed in an aqueous

core surrounded by a hole containing a semipermeable membrane. Upon administration, the rate at which fluids cross the semipermeable membrane and expel the drug from the hole governs the drug release rate (Banker and Rhodes 1996).

Monolithic systems generally contain drug dispersed in a matrix. Several polymers are generally used in these systems such as alginates, carbomer, cellulose and its derivatives (hydroxypropyl, hydroxypropyl methyl and methyl cellulose). In monolithic systems, drug release is governed by diffusion or dissolution. When the drug is dispersed in a polymeric matrix, some extent of immediate release can be expected as a result of erosion from the outer surface followed by slow release. With the advent of nanoparticle drug delivery systems, development of additional sustained release systems was facilitated, which further improved several areas of applications (Aulton 2002). Nanosizing helps control the particle size and surface properties which further control the drug release. There are several factors that limit the applications of monolithic systems which include: permeability of the drug through GI mucosa, drug solubility, dose dumping and an absorption window in a specific area of the gastrointestinal tract. Nanoparticles have helped resolve such issues with their versatility, providing sustained release or increased residence time by mucoadhesion or improving the membrane permeability and oral bioavailability (Zhang et al. 2013).

As mentioned earlier, Myfortic® (enteric-coated mycophenolate sodium) is a delayed-release formulation of mycophenolic acid. It has been developed with an intention of reducing the GI effects and dosing frequency of Cellcept® (mycophenolate mofetil) yet maintaining the similar plasma drug concentration. The delayed-release is achieved by coating with hydroxypropyl methylcellulose phthalate. Although, literature suggests that Myfortic® can be safely administered to renal transplant patients to provide similar safe and efficacy profile as that

of MMF (Budde et al. 2004), GI side effects such as diarrhea and abdominal distention were similar with both the formulations (Aptaramanov et al. 2011), by mechanisms that are unclear. Both Cellcept® and Myfortic® are dosed twice a day. Sometimes, prescribers suggest splitting the dose for patients with severe/adverse gastric effects to increase tolerability which makes adherence towards immunosuppressive therapy difficult. An ideal formulation of a mycophenolic acid derivative would be dosed every 24 hours to improve medication adherence and have reduced gastrointestinal side effects (Daughton and Ruhoy 2013). It is possible that the reformulation of MMF into polymeric nanoparticles can be applied to this situation in order to; 1) achieve sustained drug release by mucoadhesion and slow drug diffusion in the intestine; and 2) reducing drug release in stomach to minimize direct irritant effects which may be part of the cause of nausea associated with this drug.

2.4. Nanoparticle drug delivery systems

Solid particles and particulate dispersions with a size range of 10 to 1000 nm are considered as nanoparticles (NPs). NPs offer several advantages over conventional drug delivery systems, such as control over particle size which further has an effect on drug release, control over the features of the particle surface which can be altered to achieve site-specific delivery or targeted drug delivery, as well as scalability. NPs have the ability to encapsulate hydrophilic or hydrophobic drugs because of the wide variety of materials available. Common nanoparticle drug delivery systems include: polymeric nanoparticles (PNPs), dendrimers, micelles, liposomes, carbon nanotubes, metallic nanoparticles, silica nanoparticles and quantum dots (Bhatia 2016). Of all the nanoparticle drug delivery systems, PNPs have gained significant importance because there are now commercially available polymers which are biodegradable, biocompatible and easy to formulate (Nagpal et al. 2010), and polymer chemistry has advanced to provide many

examples of synthetic modifications to impart specific properties or targeting moieties. However, targeting is not within the scope of this discussion, as it is typically applied to parenteral systems. Non-biodegradable PNPs have applications in drug delivery, implants, wound healing and antimicrobial activity but chronic toxicity is a major concern with these particles (Banik et al. 2016). This led to a focus on PNPs made with biodegradable polymers. The most commonly used polymers in formulating drug delivery PNPs include poly-D, L-lactide-co-glycolide (PLGA), polylactic acid (PLA), poly-ε-caprolactone, poly-alkyl-cyanoacrylates, chitosan and gelatin.

2.4.1. Poly (D, L-lactide-co-glycolide) (PLGA):

One of the most widely used polymers for preparing PNPs is PLGA because it is biodegradable, biocompatible and can be removed by normal metabolic pathways (Alexis et al. 2008). Both hydrophilic and lipophilic drugs can be incorporated into PLGA NPs (Barichello et al. 1999) and they are used for both oral and parenteral drug delivery (Avgoustakis 2004), and the US-FDA has approved this polymer for human use (Bala et al. 2004). Drug release from a PLGA matrix can be at a sustained rate through the dual processes of drug diffusion through the polymer matrix and polymer matrix degradation (Shive and Anderson 1997). Degradation of the polymer can be altered by changing the composition or ratio of copolymers and polymer molecular weight (S.-Y. Lin, K.-S. Chen, H.-H. Teng, 2000). Formulation of PLGA NPs can be readily scaled in industry because there are several extrusion methods which maintain small particle size (Berkland et al. 2002). PLGA is available in different forms depending on the ratio of lactide to glycolide used in polymerization. For example, PLGA 75:25 contains 75% lactic acid and 25% glycolic acid (Gentile et al. 2014). PLGA polymers are generally ester-capped and this category of polymers has an extended degradation time, over weeks. PLGA after

degradation forms glycolic and lactic acid constituents which enter into the citric acid cycle in the body thereby by eliminating the problem of accumulation (Doiron et al. 2009). For achieving faster degradation and drug delivery, PLGA polymers are acid-capped because ester-capped PLGA undergoes a long degradation time through the processes of hydrolysis and de-esterification (Bret et al. 2011; Gentile et al. 2014).

Specific examples can illustrate the flexibility of formulation approaches using PNPs with various drugs. Mittal and co-workers have successfully prepared estradiol-loaded PLGA PNPs by an emulsion–diffusion-evaporation method. The size of the PNPs prepared was ~100-150nm. The PNPs prepared were found to have sustained drug release properties (Mittal et al. 2007). Similarly, nelfinavir-PLGA NPs were prepared by a nanoprecipitation method. Drug: polymer weight ratio was varied from 1:1 to 1:5 and particle size, encapsulation efficiency and drug loading were measured. Among the drug: polymer weight ratios prepared, 1:4 ratio was chosen as optimal based on drug loading. The nelfinavir NPs were found to exhibit sustained drug release up to 24 h and there was a 4.94-fold increase in relative bioavailability compared to pure drug (Venkatesh et al. 2015). Detailed information about PLGA properties and applications are summarized in several reviews (Kumari et al. 2010; Danhier et al. 2012; Sharma et al. 2016b; Roointan et al. 2018). Delivery of a pH-sensitive drug might not be feasible as degradation of PLGA forms acids. Usually with PLGA, an initial or burst release of the drug is expected, which is generally attributed to surface-adsorbed drug (Sadat Tabatabaei Mirakabad et al. 2014). The surface charge of PLGA PNPs is negative which may reduce the appropriateness of this system for oral delivery as charge repulsion may reduce binding to the GI mucosal membrane (Zhang et al. 2012a).

2.4.2. Polylactic acid (PLA):

PLA is biocompatible and biodegradable with the main degradation product being monomers of lactic acid. PLA NPs are generally prepared by the solvent evaporation, solvent displacement and salting-out methods (Lee et al. 2016). However, PLA has many limitations which are significant in oral delivery. NPs made with PLA undergo precipitation in gastric fluids and have stability issues in gastrointestinal fluids (El-Say and El-Sawy 2017). PLA NPs of progesterone were prepared with good entrapment efficiency of 70% but the particle size was 320nm which was comparatively high compared to those of PLGA NPs (Palacio et al. 2016).

2.4.3. Poly-ε-caprolactone (PCL):

PCL is highly processible polymer that is soluble in a variety of organic solvents. It is biodegradable via hydrolysis of ester linkages. Due to its slow degradation in a biological environment, its high permeability to many drugs and non-toxic nature, it is generally employed in implantable devices. Capronor®, a contraceptive device containing levonorgestrel is made of this polymer that provides prolonged zero order release (Nair and Laurencin 2007). Taxol-loaded PCL NPs have been prepared, exhibiting high drug loading but the encapsulation efficiency was found to be low, 20.79%. Surface modification of the polymer with MePEG, Pluronics or PEO has been to show increase *in vivo* activity (Kumari et al. 2010). However, PCL by itself has not been widely employed in NP for oral drug delivery due to the very slow drug release rate.

2.4.4. Chitosan:

Chitosan is the most important derivative of chitin produced by removing the acetate moiety. It is derived from crustacean cells, prawns, crabs and cell walls of fungi. It is a naturally occurring polysaccharide, cationic, highly basic, mucoadhesive and biocompatible polymer,

Chitosan has been approved by the US-FDA for tissue engineering and biocompatible. Chitosan acts a penetration enhancer by opening the tight junctions of the epithelium, thus facilitating the paracellular transport of drugs. Chitosan interacts with mucus (negatively charged) to form a complex by any of the processes such as ionic, hydrogen and hydrophobic bonds. The pKa value of chitosan is 6.5 owing to which, chitosan is soluble in acidic pH and aggregates at neutral pH (Chen et al. 2013). The primary amine ($-NH_2$) groups in the chitosan can be modified to achieve various pharmaceutical applications (Shukla et al. 2013). The amino groups interact with sulphates, citrates and phosphates (Dambies et al. 2001) thereby increasing the stability and encapsulation efficiency (Al-Qadi et al. 2012). To improve the solubility and absorption of chitosan in intestinal media, N-trimethyl chitosan chloride (TMC), quaternized chitosan has been produced (Thanou et al. 2000). The mucoadhesive property of chitosan has been further enhanced by formulating NP with thiolated chitosan (Bernkop-Schnürch et al. 2003). Because of these properties and successful applications, chitosan is one of the most widely used polymer in the pharmaceutical industry.

As discussed earlier, the mucoadhesive property of the chitosan is attributed to its strong positive charge which helps in forming bonds with negatively charged mucus. Mucus is present in the organs of the gastrointestinal tract (GIT) and the respiratory tract. The GIT is characterized by varying pH as well as a changing enzyme environment along its length which makes it difficult for oral delivery of protein/peptide drugs and DNA. Chitosan is an excellent carrier for such drugs as it is mucoadhesive, functions as a permeation enhancer and forms a protective sheath across the drugs (George and Abraham 2006).

2.5. Properties of polymeric nanoparticles

The characteristics of polymeric nanoparticles which play a key role in drug delivery include particle size, surface charge, encapsulation efficiency, degradation rate and drug release. Particle size and size distribution are important properties of PNPs which influence drug release from the PNPs. NPs of 100 nm size range when compared to microparticles (1μm diameter) show greater uptake in a Caco-2 cell line (Desai et al. 1997). In oral delivery, studies show that NPs below 200nm can effectively cross the intestinal mucus layer (Yun et al. 2013). Particle size has a strong influence on drug release. NPs have large surface area as virtue of their small particle size, therefore provide fast drug release (Mohanraj and Chen 2006). Small particles have a greater risk of particle aggregation that may have further effect on drug release and stability. As nanoparticles have high surface area to volume ratio, strong cohesive forces and greater surface energy exist in between the particles which generally leads to aggregation (Honary and Zahir 2013). An excellent way to avoid particle aggregation is by altering the surface properties of NPs. NPs with a zeta potential of at least +/-30mV are known to prevent aggregation of particles due to electrostatic repulsion.

There are several mechanisms which govern drug release from polymeric nanoparticles such as: swelling of the polymer (Liu et al. 2017), diffusion of the adsorbed drug, drug diffusion through the polymeric matrix, polymer erosion or degradation, and a combination of both erosion and degradation (Singh and Jr 2009) as represented in Figure 3. The initial burst release from the chitosan nanoparticles is either because of swelling of the polymer, creating pores, or diffusion of the drug from the surface of the polymer (Yuan et al. 2013). Chitosan nanoparticles also exhibit a pH-dependent drug release because of the solubility of chitosan. Chitosan has solubility in acidic solutions and exhibits poor solubility in neutral conditions (Miladi et al.

2015) but molecular weight of chitosan also plays important role in the solubility of chitosan (Sun et al. 2009).

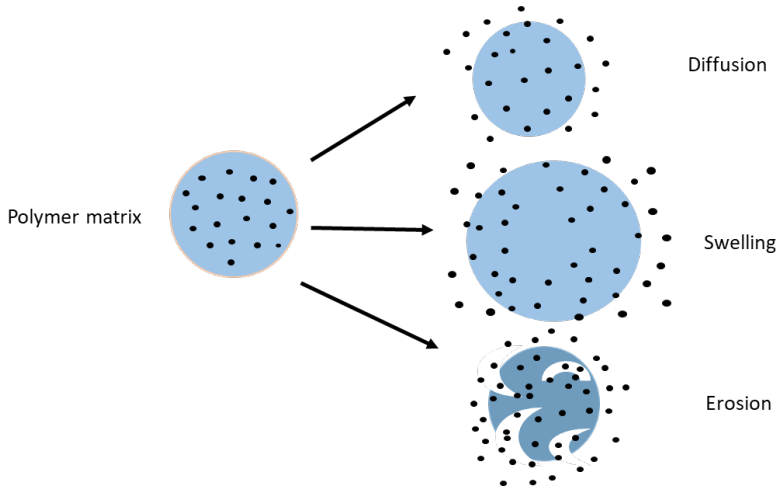


Figure 3. Diagram representing the possible mechanisms of drug release by diffusion, swelling and erosion of polymer (chitosan) matrix.

In diffusion-controlled release, drug permeates through the interior of the polymeric matrix to the surrounding medium. Polymer chains form the diffusion barrier making it difficult for the drug to pass through and this barrier serves as the rate-limiting membrane for drug release. Sometimes, diffusion is associated with polymer swelling or erosion. The mathematical representation of diffusion is given by Fick's law of diffusion.

$F = -D\frac{\partial c}{\partial x}$..... Equation 1

where F is the rate of transfer per unit area of section (flux), c is the concentration of the drug and D is the diffusion coefficient (diffusivity). To derive the parameters of Fick's law, there are a few assumptions to be made such as: pseudo-steady state is maintained during drug release, the diameter of the drug particles is less than average distance of drug diffusion through the

polymeric matrix, and sink conditions are always provided by the medium surrounding the nanoparticles (Siepmann and Siepmann 2012).

The swelling of the polymer is characterized by imbibition of water into the polymer until the polymer dissolves. This drug release mechanism is characterized by the solubility of the polymer in water, or the surrounding biological medium when administered through a specific route. When the polymer encounters the surrounding medium and swelling commences, the polymer chains detangle. This is followed by drug release from that region of the polymer matrix. Generally, the hydrophilicity of the polymer, the polymer swelling rate and the density between the polymeric chains play a key role in the drug release profile (Fonseca-Santos and Chorilli 2017). Subsequently, this will affect the rate of drug absorption from the site of delivery in vivo, as it will affect the rate at which drug is available for membrane transport or cellular uptake.

Erosion and degradation of polymers are interrelated features. Sometimes, degradation of the polymer may cause subsequent physical erosion as bonds break. Erosion of polymers is a complex phenomenon as it involves swelling, diffusion and dissolution. Erosion occurs by two ways: homogenous and heterogeneous. Homogenous erosion is erosion of the polymer at the same rates throughout the matrix while heterogenous erosion is erosion of the polymer from the surface towards the inner core. Polymer degradation may be due to the surrounding media or the presence of enzymes. The degradation of the polymer also depends on the pH of the surrounding media, the copolymer composition and water uptake by the polymer. Drug release depends on the type of polymer and internal bonding, any additives (chitosan derivatives), as well as the shape and size of the nanoparticles as this reflects surface area and free energy (Göpferich 1996).

Generally, drug release from the chitosan nanoparticles is similar to that of PLGA nanoparticles (Pawar et al. 2013) but the drug release from chitosan nanoparticles is more pH-dependent on the surrounding media (Manca et al. 2008). As an example, exendin-4 loaded PLGA and chitosan-PLGA nanoparticles have been studied. Exendin-4 is generally used for the treatment of type-2 diabetes. The NPs were evaluated for transmembrane permeability studies in MDCK cells and in an *in vivo* study in male Wistar rats. The *in vitro* transmembrane permeability studies revealed that exendin-4 was transported across the cell layer by active transport when formulated as PLGA or chitosan-PLGA NP compared to the free drug solution. The permeability coefficient (P_{app}) with PLGA and chitosan-PLGA NP was 1.52×10^{-6} and $2.5\text{-}3.0 \times 10^{-6}$, respectively, significantly greater than exendin-4 solution alone. Some of the PLGA NPs that enter the cell are trafficked into endosomes and suffer degradation while some reach the basolateral membrane via the trans-Golgi pathway. Similar such mechanisms occur with chitosan-PLGA NPs, however, due to their positive charge electrostatic interactions occur with the negatively charged cell membrane resulting in a higher P_{app}. The *in vivo* study revealed higher plasma drug levels and longer retention times of exendin-4 when administered as chitosan-PLGA NP compared to PLGA NP (Wang et al. 2013).

2.6. Mucoadhesive formulations

Mucus is basically a hydrogel composed of at least 20 proteins, carbohydrates, lipids, salts, bacteria and cellular debris. Mucus lines the gastrointestinal and respiratory tract mostly forming a protective barrier. Mucins are the primary protein component of mucus also responsible for the gel-like properties of mucus (MacAdam 1993). Besides mucin, the viscoelasticity of mucus is regulated by water, lipid and ion content. The thickness of mucus layer varies in GIT where it is thickest in stomach (to protect from gastric acid) and colon (to

protect from bacteria). Thickness of the mucus is dependent on the diet (Hollingsworth and Swanson 2004). Mucus pH is around 1-2 in stomach, neutral in the duodenum and 7-8 in colon and rectum (Kararli 1995).

Mucus acts as a barrier in the GI tract. It helps protect the stomach from acid secretions and enzymes (Cone 2009). Gastric acid cannot penetrate the mucus barrier and affect the stomach lining because mucus acts as a selective barrier against gastric acid. The presence of gastric acid and enzymes continuously degrade mucus which is why mucus is secreted continuously by goblet cells in the epithelium. Besides, mucus also acts as a protective barrier against pathogenic bacteria and foreign particles via non-specific interactions while enabling colonization of beneficial bacteria. Mucins, on the other hand, help prevent adhesion of bacteria to epithelial cells (Albanese et al. 1994). Mucus secretions contain antimicrobial agents and immunoglobulins which also act as protective mechanism (McGuckin et al. 2011). Therefore, there are two strategies of delivering the drug through mucus barrier; mucus penetrating systems and mucoadhesive systems (Olmsted et al. 2001). Mucus penetrating systems are prepared by several ways such as; using permeation enhancers (Suk et al. 2011), formulating minute particles that can avoid blocking by mucin, densely coating the nanoparticles with both negative and positive charges, formulating nanoparticles with highly hydrophilic surface (coating with polyethylene glycol) (Lai et al. 2007; Wang et al. 2008). Whereas mucoadhesive formulations adhere to the mucus thereby increasing the residence time and releasing the drug over time. Mucoadhesive formulations adhere to mucus by mechanisms such as; electrostatic attraction, hydrophobic interactions, van der Waals interactions and polymer chain intercalation (Woodley 2001). This mucus adhering strategy is employed in this study to enhance the residence time of nanoparticles in the small intestine thereby releasing drug over time.

One of the major disadvantages with oral nanoparticulate systems is rapid transit through the GIT (Kreuter et al. 1989; Galindo-Rodriguez et al. 2005) and mucoadhesive nanoparticulate systems have been successfully developed to address this issue. The applications include either enhancing the retention time in the gut or improving the oral bioavailability. Poly (sebacic acid) (PSA), Eudragit® RL-100, PLGA and PLA are some of the polymers that help mucoadhesion by hydrophobic interactions, while coating the nanoparticles with chitosan enhances mucoadhesion by electrostatic attractions. It has been reported that electrostatic attraction induces stronger mucoadhesion compared to hydrophobic/ hydrophilic interactions (Takeuchi et al. 2005; Bravo-osuna et al. 2007; Park et al. 2010).

Several cases are presented here to exemplify the use of chitosan NPs in drug delivery. Catechin and epigallocatechin are flavonoids present in green tea and are strong antioxidants. These undergo degradation in simulated intestinal fluid and are poorly absorbed across intestinal membranes. The intestinal absorption of catechin and epigallocatechin gallate was improved by encapsulating them in chitosan nanoparticles (Dube et al. 2010). Tamoxifen, an anti-cancer drug, is slightly water soluble and a good candidate for oral cancer drug delivery. Permeation of tamoxifen across the intestinal epithelium was increased by formulating tamoxifen as lecithin-chitosan nanoparticles (Barbieri et al. 2015). These NPs are mucoadhesive and increase the permeation of tamoxifen by a paracellular pathway. Feng et al. has reported oral delivery of doxorubicin hydrochloride (DOX) NPs comprised of chitosan and carboxymethyl chitosan. These nanostructures were found to enhance the intestinal absorption of DOX throughout the small intestine (Feng et al. 2013). Alendronate sodium is used in the treatment of osteoporosis and suffers from low oral bioavailability and GI side effects. Encapsulation efficiency of alendronate sodium is increased by formulating chitosan nanoparticles via an ion gelation

technique. The drug release profile of NP was different in 0.1N HCl compared to phosphate buffer saline (PBS) (pH 6.8) owing to the solubility of chitosan in different media (Miladi et al. 2015). For effective sustained delivery of sunitinib, a tyrosine kinase inhibitor, chitosan NPs were prepared by an ion cross linking method. The encapsulation efficiency of NP was 98% and sustained drug release was achieved up to 72h (John et al. 2016). The harsh conditions of the GIT denature proteins (consider the well-known example of insulin) when administered orally. Insulin-loaded chitosan NP were prepared, crosslinked with tripolyphosphate and freeze-dried. The particle size was reduced by crosslinking and the stability of the NPs was increased by freeze drying. Uptake of the insulin NPs was significant in the intestinal epithelium however, the NP were unstable in the gastric pH (Diop et al. 2015). Bhosale et al. has prepared mucoadhesive oral nanoparticles of acyclovir. The nanoparticles were prepared by solvent deposition method with PLGA as matrix polymer, polycarbophil as mucoadhesive polymer and Pluronic F68 as stabilizer. The drug release from the nanoparticles was sustained until 32h and the *in vitro* mucoadhesion studies reveal that the mucoadhesion of nanoparticles increased with increasing content of polycarbophil (Bhosale et al. 2011). In order to evaluate the ability to load proteins and to transport them across nasal and intestinal mucosa, PEG-coated PLA and chitosan coated PLGA nanoparticles of insulin and tetanus toxoid were prepared respectively (Vila et al. 2002). Coating of PLGA nanoparticles with chitosan, poly (acrylic acid) and sodium alginate was done to evaluate their mucoadhesive abilities. PLGA nanoparticles of elcatonin were prepared and coated with chitosan, poly (acrylic acid) and sodium alginate to evaluate their mucoadhesive abilities. In this attempt, it was concluded that chitosan coating of PLGA nanoparticles imparts superior mucoadhesion as demonstrated with a rat everted intestinal sac model (*in vitro*) (Kawashima et al. 2000).

Gelatin nanoparticles are another possibility to achieve enhanced oral bioavailability by mucoadhesion and increased GI residence time. Kharia and Singhai have prepared gelatin nanoparticles of acyclovir with mucoadhesive properties. The nanoparticles were prepared with gelatin (type-A) as the polymer and Pluronic F-68 as stabilizer. The optimized NPs were found to be spherical with size of 217nm, PDI of 0.268 and entrapment efficiency of 70.68%. The optimized NPs were found to have 12h residence time in gastrointestinal tract while the conventional tablet dispersion had a residence time of 8h (Kharia and Singhai 2015). It can be established that mucoadhesive formulations help in achieving sustained drug release by enhancing the residence time in the intestine and usage of polymers such as chitosan and gelatin help in formulating mucoadhesive formulations.

2.7. Preparation of polymeric nanoparticles

Ionotropic gelation, microemulsion, emulsification solvent diffusion and emulsion-based solvent evaporation are the most common methods to prepare chitosan-based polymeric nanoparticles. However, solvent evaporation and solvent diffusion methods are commonly used in formulations PLGA or PLA based nanoparticle. Usages of less organic solvent and avoidance of high force are two of the main advantages these methods offer. The key characteristics that found to affect the particle size and surface charge of PNPs prepared by these methods were found to be molecular weight and degree of acetylation of chitosan (Sreekumar et al. 2018). Entrapment mechanisms to incorporate drug within the polymeric matrix include electrostatic interaction, hydrogen bonding and hydrophobic interaction.

2.7.1. Ionotropic gelation

This is a simple technique where the chitosan solution (positively charged) is dissolved in acetic acid or any polyanionic solution (negatively charged) with or without stabilizing agent such as poloxamer. Nanoparticles were readily formed due to complexation between positive and negative charged species during mechanical stirring at room temperature resulting in precipitation of chitosan in spherical particles of different size and surface charge. Generally, the reported particle size ranges from 20-200 and 550-900nm. A few of the advantages of ionotropic gelation include; the processing uses mild conditions and an aqueous environment, low-toxicity and no change in drug chemistry. The main disadvantages of this method are its poor stability of the nanoparticles in acidic conditions and difficulty in entrapping high molecular weight drugs (Nagpal et al. 2010; Gonçalves et al. 2014).

2.7.2. Complex coacervation method

In this method, chitosan forms coacervates (precipitates) when blown into an alkali solution (sodium hydroxide, ethane diamine) with the help of a blower. The formed particles are separated and purified by filtration/centrifugation. DNA-chitosan nanoparticles are formed by coacervation of positive charge on amine groups of chitosan and negatively charge DNA phosphate groups (Zhao et al. 2011; Zhuo et al. 2014). Ionotropic gelation involves cross-linking while the coacervation method involves polyvalent interactions between the cationic chitosan and anionic polyanions. Entrapment efficiency and drug release are governed by the molecular weights of the two polymers (chitosan and nucleic acid), with a general trend of better encapsulation with higher molecular weight (Leong et al. 1998; Chen et al. 2007). A few advantages of complex coacervation include: this process can be entirely performed in an aqueous solution at low temperature and it has a better chance of encapsulating substances

because of the encapsulation method (Solanki et al. 2013). The main disadvantages of this method are poor stability of the nanoparticles, low drug loading and the fact that crosslinking of the complex by a toxic chemical reagent such as glutaraldehyde is necessary (Tiyaboonchai 2003; Huang et al. 2005; Hembram et al. 2014).

2.7.3. Coprecipitation method

The addition of chitosan solution, prepared in a low pH acetic acid solution, to a high pH 8.5–9.0 solution such as ammonium hydroxide results in coprecipitation and the formation of highly monodisperse uniform size nanoparticle. This method is similar to coacervation method but rather produces microspheres. Nanoparticles of size as low as 10 nm can be prepared with high encapsulation efficiency (Tiyaboonchai 2003; Hembram et al. 2014). The coprecipitation method was used to prepare bovine serum albumin loaded lactic acid-grafted chitosan (LA-g-chitosan) nanoparticles of ammonium hydroxide to form coacervate drops. This method yielded spherical and uniformly distributed nanoparticles (Bhattarai et al. 2006).

2.7.4. Polyelectrolyte complex (PEC)

Anionic (dextran sulfate DNA solution) solution is added to the cationic polymer (chitosan solution dissolved in acetic acid, gelatin, polyethylenimine), under mechanical stirring under room temperature followed by charge neutralization. Advantages include: a simple preparation method and the absence of harsh conditions. The nanoparticles form spontaneously upon mixing anionic solution with chitosan under mechanical stirring (Nagpal et al. 2010; Hembram et al. 2014). Low molecular weight water soluble chitosan (LMWSC) nanocarriers were developed by similar methods for insulin delivery. Insulin nanoparticles developed by the PEC method were reported to be approximately 200 nm in diameter and released drug for 120 h.

(Nam et al. 2010).

2.7.5. Microemulsion method

In this method, chitosan in acetic acid solution and glutaraldehyde are added to a surfactant in n-hexane. This mixture is subject to continuous stirring at room temperature, allowing the formation of nanoparticles, followed by overnight stirring to complete the cross-linking process. Organic solvent is removed by evaporating under low pressure. The product at this point has excess surfactant which can be removed by precipitating with calcium chloride followed by centrifugation. The final nanoparticle suspension is then dialyzed and then may be lyophilized (Maitra et al. 1999). A very narrow size distribution is seen with this method and the size can be controlled by a large amount of glutaraldehyde before stirring in the preparation on NP. Some disadvantages with this method include usage of organic solvent, a lengthy process and a complex washing step (Nagpal et al. 2010).

2.7.6. Emulsification solvent diffusion method

An o/w emulsion is prepared by mixing organic solvent with a solution of chitosan and stabilizer or surfactant (e.g. poloxamer or lecithin) under mechanical stirring followed by high-pressure homogenization (Niwa et al. 1993; El-Shabouri 2002). Polymer precipitation occurs when a large amount of water is added to the emulsion, thereby forming nanoparticles. This method is best suited for entrapment of hydrophobic drugs and the entrapment efficiency is found to be high. The major disadvantage of the method includes usage of high shear forces.

2.7.7. Emulsion based solvent evaporation method

This method is a slight modification of the above method to avoid high shear forces because high shear forces can’t help control particle size effectively. An emulsion is prepared by

adding organic solvent to a solution of chitosan with surfactant followed by ultrasonication. The emulsion formed is then added to a surfactant solution and allowed to stir until the organic solvent is evaporated, forming nanoparticles. The NP were washed and centrifuged multiple times to remove excess surfactant followed by lyophilization (Vila et al. 2002; Wang et al. 2013).

2.7.8. Reverse micellar method

The surfactant is dissolved in an organic solvent followed by the addition of chitosan, drug and crosslinking agent, under constant overnight vortexing. The organic solvent is evaporated and this results in the formation of a dry mass. The dry mass is dispersed in water and then a suitable salt is added for precipitating the surfactant and removed by centrifugation (Malmsten 2002; Zeinab Sadat et al. 2017). A very narrow size range of nanoparticles is seen. Unfortunately, however, organic solvents are used, which is less preferable because organic solvents are flammable and create hazardous waste that is costly to dispose-off (Cristina 2014). Doxorubicin-dextran conjugate loaded chitosan nanoparticles were prepared by a reverse micellar method. The surfactant used in this method was sodium bis (ethyl hexyl) sulfosuccinate (AOT), which was dissolved in n-hexane. The NP were formed by adding liquid ammonia and 0.01% glutaraldehyde to the AOT solution, containing 0.1% chitosan in acetic acid and doxorubicin-dextran conjugate, from which NP formed upon continuous stirring at room temperature (Mitra et al. 2001; Liu et al. 2007).

As per the above discussion of MMF, sustained drug release, nanoparticle properties and preparation methods, preparing nanoparticles of MMF with either PLGA or PLA (acid-capped) coating with chitosan by emulsion based solvent evaporation method was deemed a suitable approach to designing an oral controlled release drug delivery system. MMF was chosen over

mycophenolic acid because of its somewhat more hydrophobic nature which might increase its interaction with the polymers. MMF has slight water solubility, 43 mg/mL while mycophenolic acid is water insoluble. The pKa of MMF is 9.76 while the pKa of mycophenolic acid is 3.57. Acid-capped PLGA and PLA were chosen as polymers because of their properties discussed above and faster drug release compared to ester type (Doiron et al. 2009). Chitosan coating was considered to achieve a mucoadhesive NP surface and also to potentially restrict the amount of drug release in the first few hours in the acidic stomach environment. An emulsion-based solvent evaporation method was chosen because of its feasibility and advantages it offers such as simplicity, feasibility and ability to use non-toxic solvents in preparing the nanoparticles of this type.

2.8. Hypothesis

(1) MMF encapsulation in PLGA or PLA polymeric nanoparticles (PNPs) is dependant on polymer molecular weight and drug to polymer ratio.

(2) Chitosan coated nanoparticles of MMF in PLGA/PLA matrix (CS-PNPs) will achieve an entrapment efficiency of >75%.

(3) CS-PNPs will provide sustained release of MMF up to 24h in simulated gastric fluid (SGF) for 2h followed by simulated intestinal fluid (SIF), USP and minimal burst release compared to PNPs.

(4) CS-PNPs have mucoadhesive potential as demonstrated by zeta potential alteration following mucin interaction.

2.9. Specific aims

(1) To prepare PNPs with low MW PLGA/ high MW PLGA/ medium MW PLA and analyze for particle size, zeta potential, encapsulation efficiency and *in vitro* drug release.

(2) To prepare CS-PNPs with low MW PLGA/ high MW PLGA/medium MW PLA in combination with low/ medium/ high molecular weight chitosan separately and analyze for particle size, zeta potential, encapsulation efficiency and *in vitro* drug release.

(3) To assess the morphological features of optimal formulations.

(4) To evaluate mucoadhesive property of the optimal formulations by assessing changes in zeta potential following interaction with mucin.

Chapter-III. Materials and methods

3.1. Materials

Mycophenolate mofetil (MMF) (≥98% pure), D-α-tocopherol polyethylene glycol 1000 succinate (Vitamin E TPGS), polyvinyl alcohol (9000-10000 MW, 80% hydrolyzed), Resomer RG 752H (acid end polylactic-co-glycolic acid (PLGA) 75:25, MW: 4000-15000), Resomer RG 653H (acid end polylactic-co-glycolic acid (PLGA) 65:35, MW: 24000-38000) and Resomer R 203H (acid end polylactic acid (PLA), MW: 18000-24000); low (50-190kDa and 75-85% deacetylated), medium (190-310kDa and 75-85% deacetylated), high (310-375kDa and >75% deacetylated) molecular weight chitosan, acetone (HPLC grade) and methanol (HPLC grade) were all purchased from Sigma-Aldrich Canada. Cellcept® i.v. purchased from Roche Canada.

3.2. Preparation of nanoparticles

3.2.1. Preparation of PNPs.

PNPs were prepared by an emulsion-based solvent evaporation method (Sharma et al. 2016a) with slight modifications. Briefly, MMF and low MW PLGA/ high MW PLGA/ med MW PLA [at drug: polymer ratios of 1:3, 1:5 or 1:7 (w/w)] were dissolved in 1mL chloroform and added to 0.5% w/v polyvinyl alcohol (PVA) in 2mL water on a vortex mixer at a constant rate of 20mL/min with a syringe pump (NE-1000, New Era syringe pump, USA). The primary emulsion was then size-reduced by ultra-sonication for 5 min at 40% amplitude using a 1/8" probe tip (Branson Sonifier 250). Next, the emulsion was added at a constant rate of 20mL/min using a syringe pump to 5mL of 0.5% w/v PVA in a beaker and allowed to harden for 3h by constant stirring at 300rpm. After hardening, the PNPs were ultracentrifuged at 50,310×g for 25min and washed for three times to remove excess surfactant. The PNP pellet after the third wash was suspended in 4mL of deionized water. For cryoprotection 1mL of sucrose (5% w/w)

was added to each formulation. The final PNP suspension (5mL) was frozen to -80^{o}C and lyophilized for 72h. PNPs after lyophilization were stored at 4^{o}C until further characterization.

3.2.2. Preparation of chitosan coated PNPs.

Chitosan coated PNPs (CS-PNPs) were prepared in a similar manner as the above method with slight modifications (Vila et al. 2002) as follows. MMF-loaded nanoparticles (PLGA or PLA) were prepared as described above, but during the hardening step in 0.5% PVA, chitosan (low, medium or high MW as described in Materials) in dilute acetic acid (2% v/v) was added. The coating/hardening process continued with stirring 6h at 23°C. Thus, the CS-PNP compositions could be easily varied as 1:3:3, 1:5:5 or 1:7:7 drug: polymer: chitosan (w/w/w) ratios for each of the PLGA and PLA compositions. The added drug content remained constant for all formulations.

3.3. HPLC method development of mycophenolate mofetil (MMF).

An HPLC method for quantification of MMF was developed on Waters 2695 Separations module equipped with Waters 2996 photodiode array detector. MMF was separated on XTerra® C18 column (Waters, USA) with 5 µm particle size and 4.6 × 100mm dimensions. Mobile phase used was 0.3% triethylamine in water (pH 5.3, adjusted with orthophosphoric acid): acetonitrile (90:10) as solvent A and acetonitrile as solvent B at gradient level. The gradient system was set as 100% solvent A at the start of run and 50% each solvent by the end of run. A calibration curve was developed by preparing standard solutions in methanol and the range was 10-60 µg/mL and was found to be linear with r^2 value of 0.997. The retention time of MMF was found to be at 12.8 min. This method was having issues such as varied column pressure, repeatability and equipment blockade because of orthophosphoric acid. Then, another method was developed on a C8 column to reduce the retention time which is discussed in detail below.

An HPLC method for the quantification of MMF was developed on Waters 2695 Separations module equipped with Waters 2996 photodiode array detector. MMF was separated on Luna® C8 column (Phenomenex®, USA) with 5µm particle size and 30 × 2mm dimensions. The mobile phase used was 0.3% triethylamine (TEA), pH 5.3 [pH adjusted with trifluoro acetic acid (TFA)] and acetonitrile at a ratio of 70:30 (v/v) respectively, and the flow rate was 0.4mL/min. A calibration curve was developed by preparing standard solutions in methanol, and the range was 2-60µg/mL and was found to be linear with r^2 value of 0.996. The percentage relative standard deviation (RSD) of inter-day precision (0.85%) was within limits (<2%). The limit of detection (LLOD) and lower limit of quantification (LLOQ) were found to be 0.1 µg/mL and 2 µg/mL respectively. The retention times of mycophenolic acid (MPA) and MMF were found to be non-overlapping and at 2.1 min, and 2.8min respectively.

3.4. Particle size, polydispersity and zeta potential.

The mean particle size (nm), polydispersity index (PDI) and zeta potential (mV) of PNPs and CS-PNPs were determined by dynamic light scattering (Nano ZS, Malvern). Briefly, 300µg of the sample was dispersed in 1mL deionized water and ultrasonicated (1/8" probe) for 10s at 10% amplitude before analysis. All the measurements were made in triplicates at 25°C and reported as mean±SD.

3.5. Encapsulation efficiency.

The encapsulation efficiency (EE) was measured by weighing 500 µg of PNP and adding 1mL of acetone followed by bath sonication for 1h. The acetone was evaporated under a vacuum, and a triple solvent extraction was performed: in the first extraction step, methanol was added to extract the drug, followed by centrifugation at 14000 rpm for 10 min. The supernatant (first extract) was gently separated with the help of pipette and saved for quantification of drug

content. This procedure was repeated with the PNP sample twice more to maximize drug extraction, yielding second and third extract (Mu and Feng 2003). Drug content was quantified in all the extractions and the encapsulation efficiency was calculated according to equation-2.

The encapsulation efficiency of CS-PNPs was estimated in a manner similar to the above method, except for the addition of 2% (v/v) acetic acid (AA), bath sonication for 1h and evaporation of AA, prior to addition of acetone.

$$\text{EE} = \frac{(\text{MMF in 1st extract} + \text{MMF in 2nd extract} + \text{MMF in 3rd extract})}{\text{MMF added in PNP or CS-PNP}} \times 100 \quad \text{Equation 2}$$

3.6. *In vitro* drug release study.

In vitro drug release studies were performed by the dialysis bag method with slight modification (Venkatesh et al. 2015). Instead of drawing the sample from dissolution medium, PNP samples were collected from the dialysis bag to be within the quantifiable range of the HPLC analytical method (LLOQ = 2µg/mL). Pre-weighed (6mg) PNPs or CS-PNPs were suspended in deionized water (2mL) and introduced into a dialysis membrane (MW cut off 12000 -14000 Da). The dialysis bag was suspended in 900mL simulated gastric fluid, USP (SGF) for the first two hours, followed by 900mL simulated intestinal fluid, USP (SIF) for the remaining time to 24h. The media were stirred in a 1L beaker at 100rpm and maintained at 37± 2^{o}C. At predetermined time intervals; 0, 1, 2, 4, 6, 12 and 24h, 100µL of sample was collected from the dialysis bag and the drug content was determined by procedure described above.

3.7. Morphological study.

The surface morphology of PNPs and CS-PNPs was studied using a scanning electron microscope (SEM), Hitachi SU8000. In SEM, a strong electron beam focused onto a solid

sample scans point by point resulting in an image (Klang et al. 2012). SEM provides greater depth of focus while projecting areas casts shadows. Another advantage of SEM includes, individual particle analysis unlike to that of dynamic light scattering. There is no or minimal sample preparation while analyzing samples through SEM (Klang et al. 2013). Prior to the analysis, the samples were fixed on a brass stub using double-sided tape and the samples were coated with chromium (to render them electrically conductive). The images were then captured using SEM set at an excitation voltage of (3.0 kV). The magnification selected (400-10K) was sufficient to appreciate in detail the general morphology of the samples under study (Hecq et al. 2015).

3.8. Differential scanning calorimetric (DSC) study.

Thermal properties of drugs and drug formulations can be estimated by calorimetric analysis. Differential scanning calorimetric (DSC) analysis measures energy differences between a sample and a reference. It analyzes the change in physical properties upon temperature fluctuations over time (Gill et al. 2010). To study the physical state of MMF in CS-PNPs and compatibility of MMF with other excipients, thermal analysis was performed on DSC-Q2000, TA instruments, USA. A small amount of sample (<10mg) was sealed into Tzero hermetic pans and the temperature was raised at 1^{o}C/min over a scanning range of 4-350^{o}C under nitrogen atmosphere (Patel et al. 2016). The data collected was analyzed for melting points and enthalpies using TA Advantage software, version 5.5.24.

3.9. Potential mucoadhesive properties.

The mucoadhesive potential of CS-PNPs was assessed by measuring zeta potential changes upon addition of mucin protein solution (Takeuchi et al. 2005). Zeta potential is a measurement of the electrical potential difference between the surface of the particle and the

bulk phase in which it is suspended. Therefore, it represents indirectly the available charge for binding or repelling oppositely or similarly charged particles, respectively. Likewise, if a particle with a given zeta potential binds another material, such as protein, the surface charge of the particles in suspension would be expected to change. Briefly, 300µg of CS-PNP were added to mucin (porcine gastric mucus type-II, 5mg/mL), incubated for 1h at 37°C and diluted 100-fold in deionized water before analyzing zeta potential at ambient temperature (23°C). The zeta potentials of mucin alone and of the CS-PNPs in water without mucin were also measured, serving as controls.

3.10. Statistical Analysis.

The data obtained was subjected to one-way analysis of variance (ANOVA), and the significance of differences between any two formulations was calculated by Tukey's post-hoc test with SPSS software (IBM, New York, USA). The level of significance chosen was $p<0.05$.

Chapter IV. Results and Discussion

4.1. Preparation of nanoparticles.

There are several methods used for preparing PNPs or CS-PNPS such as variations on emulsion-based solvent evaporation methods, ionotropic gelation and complex coacervation techniques (Mohammed et al. 2017). PLGA and PLA are biodegradable, biocompatible polymers approved by the US FDA for human use (El-Say and El-Sawy 2017) and represent polymers particularly amenable to nanoparticle formulations because they are readily available at various MW ranges and can be obtained with acid or ester end-capping. PLGA is also available at several polylactic: polyglycolic ratios. These variables aid the formulator to make rational choices suiting the drug properties as well as permitting empiric optimization with minor compositional changes using the same particle formation method. The method utilized here for the preparation of PNPs and CS-PNPs was an emulsion-based solvent evaporation method with slight modifications. Although our aim was to develop CS-PNPs, initially uncoated PNPs were prepared with three molecular weight ranges of PLGA or PLA (low, medium high) in order to determine an optimal formulation maximizing encapsulation efficiency.

In the preliminary experiments, nanoparticles (NPs) were prepared with Cellcept® (intravenous formulation of MMF), ester end polylactic-co-glycolic acid (PLGA 75:25, lactic acid: glycolic acid), D-α-tocopherol polyethylene glycol 1000 succinate (vitamin E TPGS) as the surfactant and chloroform as solvent by single emulsion based solvent evaporation method. Each NP formulation had 7mg Cellcept® (6.6 mg of MMF), 21mg PLGA and concentration of Vitamin-E TPGS was varied at 0.1%, 0.5% and 1.0% (w/v) respectively. The NPs were then analyzed for particle size and encapsulation efficiency. the formed nanoparticles exhibited a bimodal size distribution, which can be considered as centred around two different mean

diameters (population 1, population 2) indicating heterogeneity [Figure 4]. All nanoparticle preparations had a negative zeta potential of -30 to -40 mV. More than 95% of the drug was found to be in the washes. Upon repeating these NP formulations, clumps were seen after the NP hardening step. Generally, an oil-in-water emulsion requires an HLB of approximately 8-18. Vitamin-E TPGS has an HLB value of 13.2 and it is a hydrophilic surfactant (Zhang et al. 2012b). The concentration of Vitamin-E TPGS chosen was 0.1, 0.3 and 0.5% (w/v). The PNPs prepared with Vitamin-E TPGS had very low encapsulation efficiency, suggesting it may be an unsuitable surfactant for PLGA-MMF nanoparticles by this method. The reason for low MMF encapsulation efficiency into PLGA with this surfactant can be speculated to be due to the formation of micelles of MMF and Vitamin-E TPGS, although this was not tested. So, vitamin-E TPGS was replaced with polyvinyl alcohol (PVA) in preparing further NPs. Polyvinyl alcohol (PVA) is a synthetic water-soluble polymer (log P = 0.5) often used as an emulsifier in the fabrication of polymeric nanoparticles by emulsion techniques.

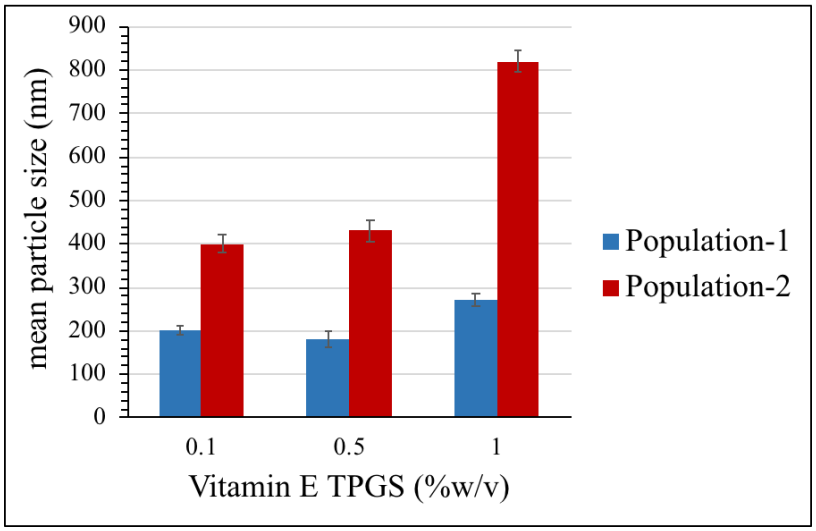


Figure 4. Particle size of nanoparticles made with Cellcept®, PLGA and vitamin-E TPGS as surfactant. NP formulations show heterogeneity.

NPs made with PVA (0.5% w/v) showed particle size below 300nm and polydispersity index below 0.4 indicating a single homogenous NP population. But, the loss of drug in the washes was still an issue. Moreover, as the goal of this project is to develop NPs with mucoadhesive nature, a second polymer, medium molecular weight chitosan (MMWC) was used and NPs were prepared by double emulsion solvent evaporation method (Giovino et al. 2012). Theoretically, NPs prepared by double emulsion method using chitosan should possess positive zeta potential but that was not the case here. Moreover, the drug loss (>95%) in washes was not reduced.

The next step to achieve high (>75%) encapsulation efficiency was a slight change in the nanoparticle preparation (modified single emulsion method) explained further (Vila et al. 2002) with slight modifications. In this modified method, MMWC was added during the hardening step. Another important factor behind achieving high encapsulation efficiency was the type of surfactant and the concentration of the surfactant. NPs were prepared by a modified single emulsion method containing Cellcept®=7mg, PLGA=21mg, MMWC= 21mg and two different concentrations of PVA; 0.5 and 2.5% w/v. Figure-5 indicates the particle size of the NPs with 0.5% w/v as 580nm and positive zeta potential (0.72 mV) in comparison with NPs prepared without MMWC but >95% of drug was lost in washes.

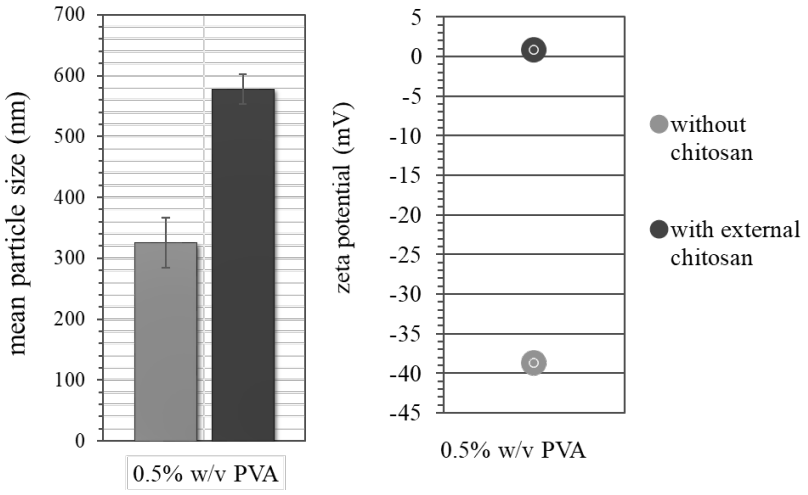


Figure 5. Particle size and zeta potential of NPs made with and without medium molecular weight chitosan.

In another trial of NP preparation with Cellcept®=7mg, PLGA=70mg and 0.5% w/v PVA particle size of 264 nm, negative (-36.4mV) zeta potential and 21±5% encapsulation efficiency was achieved. Although, 21% encapsulation is less compared to the desired, it was promising to continue further trials in enhancing the encapsulation efficiency. The main reason behind failure of encapsulating MMF was usage of Cellcept® in all the formulations as Cellcept® is an intravenous formulation of MMF which also has 25mg of polysorbate-80 per vial of Cellcept®. So, for further trials, MMF (98% pure) was used. Moreover, as MMF is slightly soluble in water, acid capped PLGA/PLA polymers were chosen to improve encapsulation efficiency.

Subsequently, CS-PNPs were prepared by adding either low, medium or high MW chitosan to the polymer nanoparticles (PLGA or PLA) loaded with MMF. The concentration of PVA to be used as a surfactant in the preparation of PNPs was then optimized to 0.5% (w/v) based on encapsulation efficiency. The hardening time for CS-PNPs was increased to 6h to facilitate the coating of chitosan onto PNPs by electrostatic attraction between PLGA/PLA and chitosan. While quantification of chitosan coating on the nanoparticles is ideal, low drug release

from CS-PNPs in the first two hours and charge reversal upon chitosan coating provide evidence to report chitosan coating on PNPs. Ninhydrin reagent is generally used in the determination of chitosan and degree of acetylation (Khan et al. 2002) and Fourier transform infrared spectroscopy (FTIR) may used to confirm chitosan conjugation with PLGA/PLA (Curotto and Aros 1993). All the PNP and CS-PNP formulations contained 7mg of MMF for ease of comparison. *In vivo* pharmacokinetic studies will be required to estimate the dose required to achieve an efficacious drug exposure level, but it is anticipated that similar to other sustained release drug formulations, a lower total drug amount per day will be required.

4.2. Particle size, polydispersity and zeta potential.

One of the key parameters for nanoparticle performance is size and size distribution. For example, controlling mean diameter affects the surface area available for dissolution from the exterior. Furthermore, monodisperse particles permit more predictable control over drug release. The polydispersity index (PDI) of most of the PNP formulations discussed here was < 0.2 [Table 2], which indicates a reasonably narrow unimodal size distribution. As the ratio of polymer to drug content was increased, the mean particle diameter also increased correspondingly, as expected. The addition of chitosan coating to the nanoparticles comprised of MMF/PLGA or MMF PLA did increase the mean diameter slightly (compare Table 2 vs. Table 3), but the particle size is still submicron at all drug: polymer ratios, acceptable for oral drug delivery. The observed negative zeta potential of the PLGA and PLA nanoparticle formulations was likewise consistent [Table 3]. An indication of successful chitosan coating was the significant increase in zeta potential, corresponding to the amount of chitosan in the formulation [Tables 3, 4 and 5]. A positive zeta potential is desirable for the CS-PNPs in order to promote mucoadhesion following *in vivo* administration (Lubben et al. 2001). The zeta potential of CS-PNPs made of either low

MW PLGA or medium MW PLA in combination with low MW chitosan was still negative, possibly due to incomplete coating with chitosan, or an insufficient amount to cause the particle surface charge to become positive [Table 3 and 5 respectively]. Similarly, CS-PNPs made with MMF, high MW PLGA and low MW chitosan in 1:3:3 and 1:5:5 ratios show a negative zeta potential [Table 4]. Other compositions incorporating medium or high MW chitosan did successfully generate cationic nanoparticles. Stress during lyophilization can significantly affect the particle size (Fonte et al. 2016). The particle size data in Table 6 indicate that there was a significant difference in particle size before and after lyophilization. Generally, cryoprotectants are added during lyophilization to reduce freezing or drying stress thereby enhancing the stability of PNPs. Trehalose, sucrose and mannitol are most commonly used cryoprotectants which yield different sized PNPs and effect their stability (Abdelwahed et al. 2006; Almalik et al. 2017). In this first foray, we chose to use sucrose, generating nanoparticles that were non-aggregated and readily redispersible. It is possible that alternative cryoprotectants may also be effective.

Table 2. Particle size, PDI and zeta potential of PNPs made with MMF and low MW PLGA/ high MW PLGA/ medium MW PLA.

Formulation	Particle size (nm)		PDI		Zeta potential (mV)	
	Mean	SD	Mean	SD	Mean	SD
MMF: Low MW PLGA = 1:3	316	33.3	0.27	0.02	-42.5	1.33
MMF: Low MW PLGA = 1:5	405	25.6	0.29	0.05	-45.2	1.75
MMF: Low MW PLGA = 1:7	752	28.2	0.32	0.03	-49.5	0.71
MMF: High MW PLGA = 1:3	230	15.4	0.38	0.04	-41	1.3
MMF: High MW PLGA = 1:5	319	27.3	0.25	0.16	-41.3	1.86
MMF: High MW PLGA = 1:7	841	26.5	0.26	0.04	-43.5	0.46
MMF: Med MW PLA = 1:3	369	19.2	0.16	0.13	-30.2	0.88
MMF: Med MW PLA = 1:5	580	24.7	0.34	0.04	-30.5	1.97
MMF: Med MW PLA = 1:7	931	12.2	0.31	0.04	-37	1.43

MMF= mycophenolate mofetil, low MW PLGA= low molecular weight poly lactic-co-glycolic acid, high MW PLGA= high molecular weight poly lactic-co-glycolic acid and med MW PLA= medium molecular weight poly(lactic) acid. PDI=polydispersity index, nm=nanometers, mV=millivolts. Data represented as n=3, mean±SD.

Table 3. Particle size, PDI and zeta potential of CS-PNPs made with MMF, low MW PLGA and three types of chitosan.

Formulation	Particle size (nm)		PDI		Zeta potential (mV)	
	Mean	SD	Mean	SD	Mean	SD
MMF: LMWPLGA: LMWC= 1:3:3	405	38.5	0.47	0.04	-29.5	0.31
MMF: LMWPLGA: LMWC= 1:5:5	600	45.8	0.62	0.02	-26.5	0.98
MMF: LMWPLGA: LMWC= 1:7:7	823	43.8	0.67	0.03	-27.1	1.13
MMF: LMWPLGA: MMWC= 1:3:3	414	43.1	0.51	0.06	31.5	0.20
MMF: LMWPLGA: MMWC= 1:5:5	568	53.4	0.43	0.04	35.6	0.79
MMF: LMWPLGA: MMWC= 1:7:7	957	57.4	0.24	0.01	39.2	1.03
MMF: LMWPLGA: HMWC= 1:3:3	471	13.3	0.33	0.05	41.8	0.85
MMF: LMWPLGA: HMWC= 1:5:5	750	22.2	0.24	0.04	45.3	1.49
MMF: LMWPLGA: HMWC= 1:7:7	955	20.6	0.60	0.02	44.2	0.80

MMF – mycophenolate mofetil, LMWPLGA: low molecular weight polylactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. PDI=polydispersity index, nm=nanometers, mV=millivolts. Data represented as n=3, mean±SD.

Table 4. Particle size, PDI and zeta potential of CS-PNPs made with MMF, high MW PLGA and three types of chitosan.

Formulation	Particle size (nm)		PDI		Zeta potential (mV)	
	Mean	SD	Mean	SD	Mean	SD
MMF: HMWPLGA: LMWC= 1:3:3	366	26.8	0.40	0.04	-27.2	0.6
MMF: HMWPLGA: LMWC= 1:5:5	420	37.4	0.45	0.02	-7.53	0.3
MMF: HMWPLGA: LMWC= 1:7:7	571	20.9	0.73	0.03	28.5	1.14
MMF: HMWPLGA: MMWC= 1:3:3	417	46	0.51	0.06	7.41	0.83
MMF: HMWPLGA: MMWC= 1:5:5	568	36.2	0.65	0.04	25.6	0.9
MMF: HMWPLGA: MMWC= 1:7:7	796	28.8	0.75	0.01	26.5	2.51
MMF: HMWPLGA: HMWC= 1:3:3	674	14.9	0.77	0.05	10.4	0.93
MMF: HMWPLGA: HMWC= 1:5:5	763	21.4	0.69	0.04	25.5	1.34
MMF: HMWPLGA: HMWC= 1:7:7	816	16.8	0.60	0.02	31	1.22

MMF – mycophenolate mofetil, HMWPLGA: high molecular weight polylactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. PDI=polydispersity index, nm=nanometers, mV=millivolts. Data represented as n=3, mean±SD.

Table 5. Particle size, PDI and zeta potential of PNPs made with MMF, PLA and LMWC/ MMWC/ HMWC.

Formulation	Particle size (nm)		PDI		Zeta potential (mV)	
	Mean	SD	Mean	SD	Mean	SD
MMF: PLA: LMWC=1:3:3	221	35.9	0.27	0.04	-22.1	1.35
MMF: PLA: LMWC=1:5:5	340	49.2	0.32	0.02	-24.8	1.53
MMF: PLA: LMWC=1:7:7	532	52.7	0.37	0.03	-14.9	2.48
MMF: PLA: MMWC=1:3:3	281	53.3	0.31	0.06	24.4	1
MMF: PLA: MMWC=1:5:5	995	34.7	0.23	0.04	53.8	1.31
MMF: PLA: MMWC=1:7:7	1194	51.4	0.14	0.01	67.6	1.29
MMF: PLA: HMWC=1:3:3	390	69	0.33	0.05	41.8	0.98
MMF: PLA: HMWC=1:5:5	1079	63.9	0.24	0.04	50.4	2.53
MMF: PLA: HMWC=1:7:7	1336	63.8	0.20	0.02	61.2	1.42

MMF – mycophenolate mofetil, PLA: medium molecular weight poly(lactic) acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. PDI=polydispersity index, nm=nanometers, mV=millivolts. Data represented as n=3, mean±SD.

Table 6. Particle size comparison of CS-PNPs before and after lyophilization.

Formulation	Particle size (nm) before lyophilization		Particle size (nm) after lyophilization	
	Mean	**SD**	**Mean**	**SD**
MMF: PLA: LMWC=1:3:3	182.7	36.5	221.8	35.99
MMF: PLA: LMWC=1:5:5	284.6	54.2	340.4	49.21
MMF: PLA: LMWC=1:7:7	447.2	46.8	532.6	52.7
MMF: PLA: MMWC=1:3:3	196.8	43	281.2	53.36
MMF: PLA: MMWC=1:5:5	257.8*	37.2	995**	34.7
MMF: PLA: MMWC=1:7:7	430.4*	24.6	1194**	51.4
MMF: PLA: HMWC=1:3:3	160.8*	66.1	390.5**	69
MMF: PLA: HMWC=1:5:5	319.4*	79.1	1079**	63.9
MMF: PLA: HMWC=1:7:7	440.2*	35.1	1336**	63.88

MMF – mycophenolate mofetil, PLA: medium molecular weight poly(lactic) acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. nm=nanometers. Data represented as n=3, mean±SD. *, ** mean particle size was significantly different before and after lyophilization ($p<0.05$).

4.3. Encapsulation efficiency (EE)

The encapsulation efficiency (EE) of PNPs made with low MW PLGA ranged from 66.7-97.3% [Figure 6a]. Similarly, the EE of PNPs made with high MW PLGA and medium MW PLA raged from 61.4-92.1% and 86.8-100% [Figures 6b and 6c respectively]. The EE of MMF increases with increasing drug: polymer ratios [Figures 6a, 6b and 6c]. Of note, both PLGA and PLA are available with carboxylic acid terminus or as the corresponding ester terminus. In the present study, the acid-terminated form of PLGA was far more effective in encapsulating MMF than the ester terminated. In theory, this may have contributed to the encapsulation of MMF by its more hydrophilic nature compared to the ester form (Félix Lanao et al. 2013); MMF with its slight water solubility (43mg/mL) showed high (>75%) EE in almost all the PNP formulations

using acid-terminated polymers. Another consideration is the ratio of glycolic to lactic acid monomers within the block copolymer. Specifically, the encapsulation of MMF in formulations comprised of MMF: low MW PLGA (1:5 w/w, 92.6% EE) was significantly different ($p<0.05$) when compared to MMF: high MW PLGA (1:5 w/w, 74.1% EE), where there is a higher (75%) lactic acid content in low MW PLGA compared to that of high MW PLGA (65%). Lactic acid (log P= -0.72) is more hydrophobic than glycolic acid (log P= -1.11). Furthermore, nanoparticles with drug: polymer ratios of 1:7 (w/w) show very high encapsulation (EE >92%) which could be due to high lactic acid content (Félix Lanao et al. 2013) or alternatively, it may simply be that at the higher MW polymer content and higher polymer: drug ratio there is a critical quantity of available polymer to incorporate the drug. However, greater polymer content did not significantly improve encapsulation for low MW PLGA (1:5 vs 1:7) or for PLA (1:5 vs. 1:7) [Figures 6b and 6c]. Hence, even with rational design, there is a need for systematic empiric testing within a range of compositions.

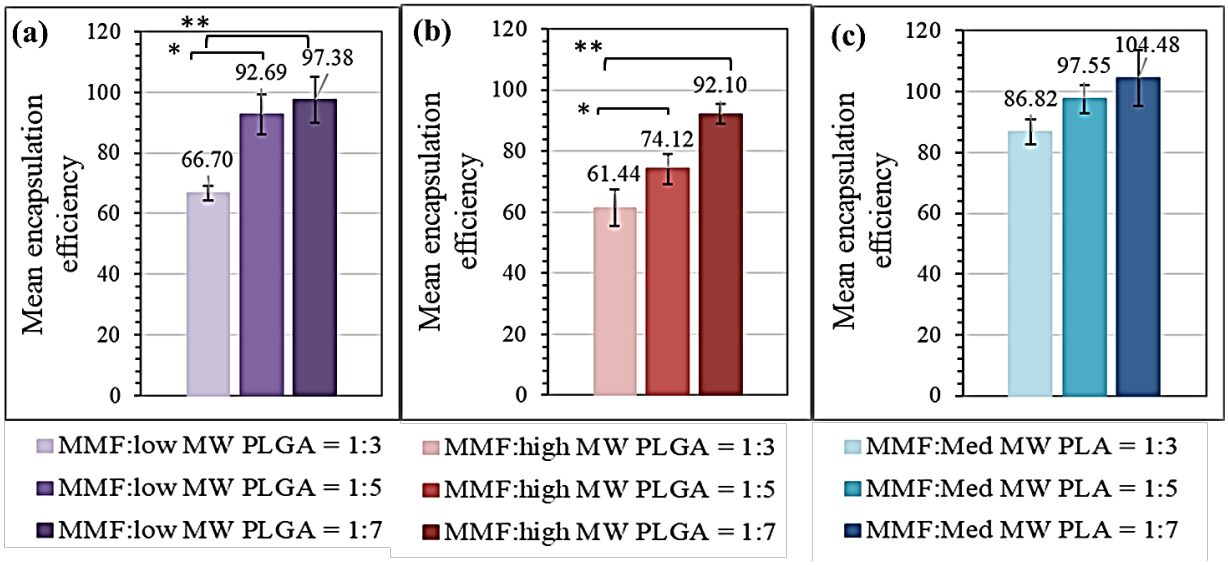


Figure 6. Graphical representation of encapsulation efficiency of PNPs made with MMF and low MW PLGA (a), high MW PLGA(b) and medium MW PLA (c). Data represented as mean±SD. MMF= mycophenolate mofetil, low MW PLGA= low molecular weight poly lactic-co-glycolic acid, high MW PLGA= high molecular weight poly lactic-co-glycolic acid and medium MW PLA= medium molecular weight poly(lactic) acid. EE was significantly different when the ratio of drug to polymer has changed within same types of polymer (*p=0.004, **p<0.001).

The chitosan coating of the nanoparticles is a critical component for mucoadhesion and sustained release. The effect of chitosan coating on the PNPs was investigated as a function of drug, type of core polymer (low MW PLGA/ high MW PLGA/ medium MW PLA) and chitosan at various molecular weights (low, medium, high) in the ratios of 1:3:3, 1:5:5 or 1:7:7 (w/w/w) respectively. The EEs of CS-PNPs made with low MW PLGA and all types of chitosan are represented in Figure 7. Here, in Figure 4a, the EE ranges from 13.5-59.4% which is comparatively low compared to PNPs in Figure 6a. With CS-PNPs made with low MW PLGA and MMWC, the EE range from 19-79% and with low MW PLGA and HMWC, the EE range

from 26.7-95.1%. The EE increased with CS-PNPs from low MW chitosan to high MW chitosan in Figure 7a-7c. The EE of CS-PNPs made with high MW PLGA in combination with LMWC, MMWC and HMWC separately ranged from 17.1-67.3%, 21.3-74% and 25.1-75.4% respectively [Figures 8a-8c]. Similarly, the EE of CS-PNPs made with medium MW PLA and LMWC, MMWC, HMWC separately ranged from 26.1-83.5%, 21.2-94.3% and 33-86.2% respectively [Figures 9a-9c]. The EE of CS-PNPs increases upon increasing the drug to polymer ratio. The EE of CS-PNPs in Figure 7a-7c are relatively low when compared to PNPs made with LMW PLGA alone [Figure 6a] except in case of MMF: LMWPLGA: HMWC= 1:7:7 (95.1 % EE). The reason might be drug loss in the washing step, however, drug loss in the washing step of nanoparticle preparation should be measured. Similarly, chitosan-coated- HMW PLGA PNPs [Figure 8] exhibited significantly less drug loading compared to PNPs comprised of HMW PLGA without chitosan [Figure 6b]. Likewise, a significant difference in EE was observed comparing nanoparticles comprised of MMF: PLA: LMWC=1:3:3 and MMF: PLA= 1:3 without chitosan, where the latter had a lower EE. However, when the core polymer was PLA, the addition of different types of chitosan (low, medium or high MW ranges) did not reduce the EE significantly at the highest polymer: drug ratios (1:7:7), achieving up to 94% encapsulation efficiency [Figure 9].

The drug loading (DL) of chitosan coated PNPs was calculated according to the following formula as indicated in table 7.

$$\mathrm{DL} = \frac{(\text{MMF in 1st extract} + \text{MMF in 2nd extract} + \text{MMF in 3rd extract})\text{in mg}}{\text{amount of CS}-\text{PNP in mg}} \times 100 \quad \text{Equation 3}$$

With the CS-PNP formulations made with HMWPLGA and HMWC, drug loading was found to increase with increase in drug to polymer ratio.

Table 7. Drug loading of MMF in CS-PNP formulations. Data represented as n=9, mean ± SD.

Formulation	% drug loading
MMF: HMWPLGA: HMWC=1:3:3	3.49 ± 0.71
MMF: HMWPLGA: HMWC=1:5:5	4.11 ± 0.58
MMF: HMWPLGA: HMWC=1:7:7	4.91 ± 0.42
MMF: PLA: MMWC= 1:3:3	2.95 ± 0.21
MMF: PLA: MMWC= 1:5:5	7.21 ± 0.42
MMF: PLA: MMWC= 1:7:7	6.12 ± 0.24

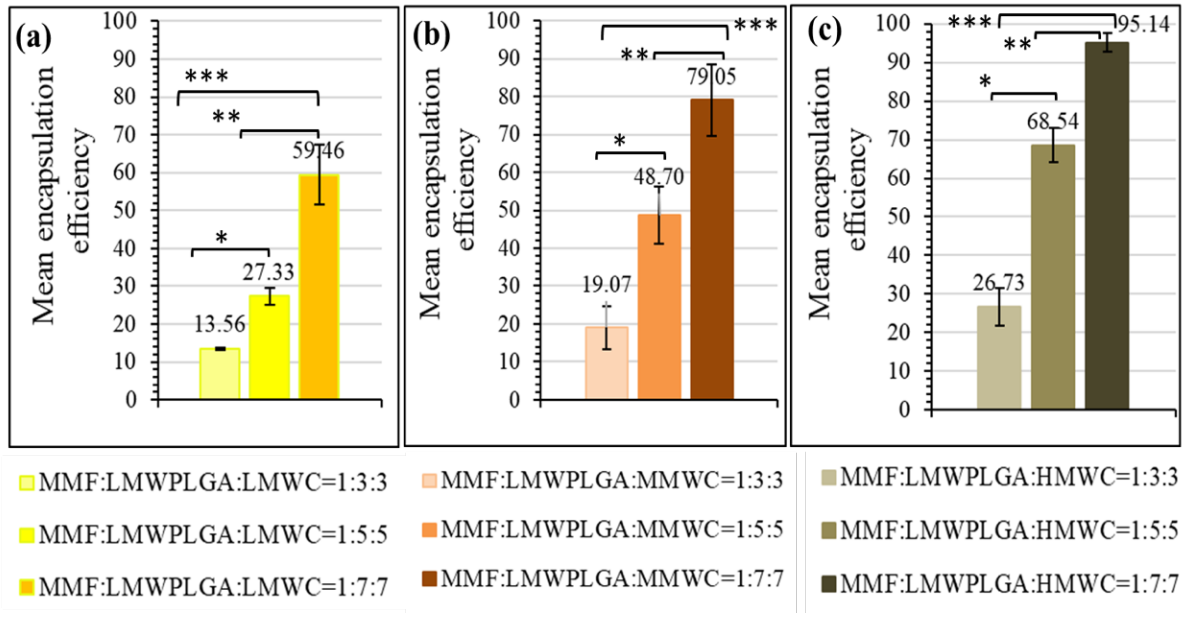


Figure 7. Graphical representation of encapsulation efficiency of CS-PNPs made with MMF, low MW PLGA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, LMWPLGA: low molecular weight poly lactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. EE was significantly different when the ratio of drug to polymer has changed within same types of polymer (*p=0.002, ** p=0.005, ***p<0.001).

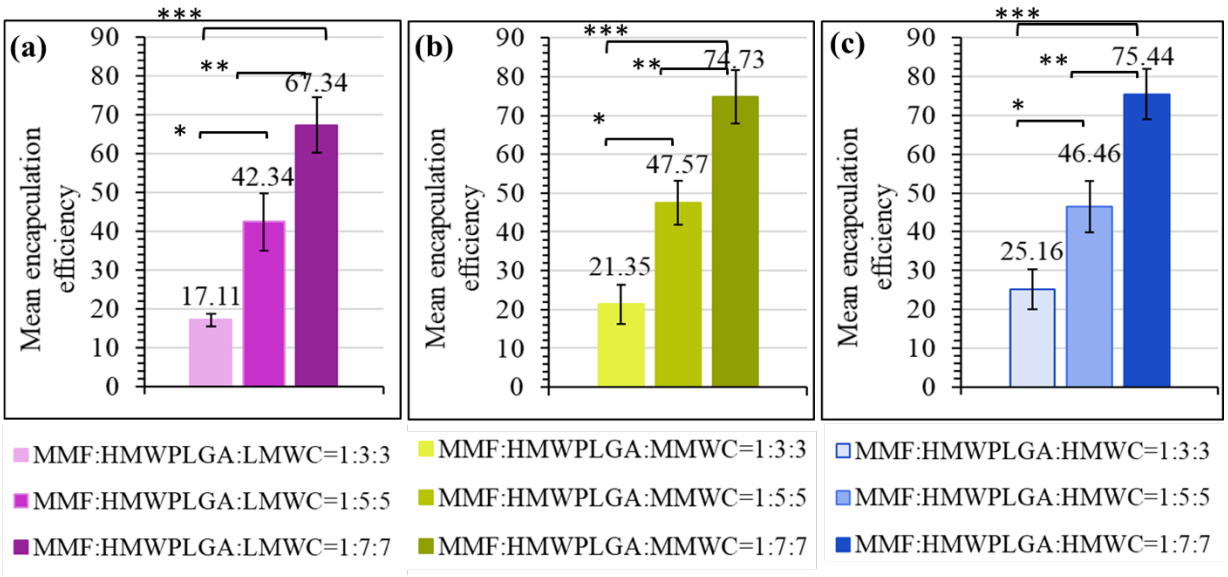


Figure 8. Graphical representation of encapsulation efficiency and i*n vitro* drug release of CS-PNPs made with MMF, high MW PLGA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, HMWPLGA: high molecular weight poly lactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. EE was significantly different when the ratio of drug to polymer has changed within same types of polymer (*p=0.007, ** p=0.002, ***p<0.001).

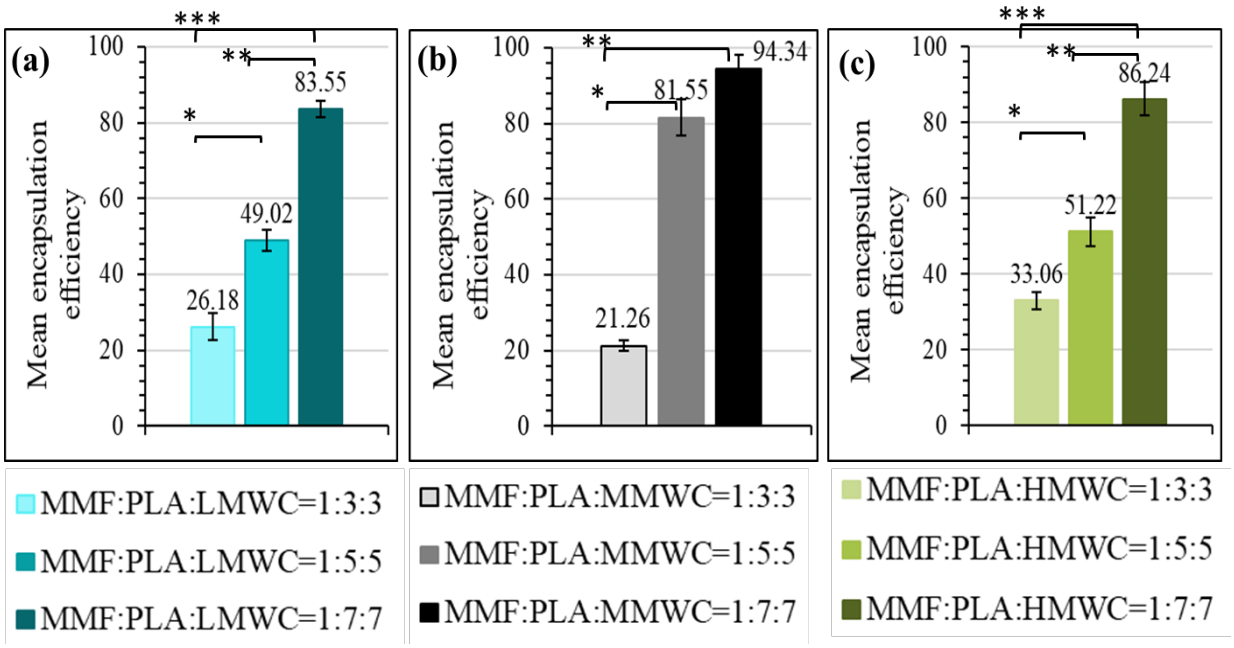


Figure 9. Graphical representation of encapsulation efficiency and i*n vitro* drug release of CS-PNPs made with MMF, PLA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, PLA: medium molecular weight poly(lactic) acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. EE was significantly different when the ratio of drug to polymer has changed within same types of polymer (*p=0.003, ** p=0.005, ***p<0.001).

4.4. In vitro drug release study.

In order to determine a composition most likely to achieve the desired slow release *in vivo*, drug release was evaluated in simulated gastric fluid, USP (SGF) over two hours followed by simulated intestinal fluid without enzymes, USP (SIF) over the next 22hrs. Both the media were enzyme-free because enzymatic action on PLGA/PLA is not the cause of its degradation (Makadia and Siegel 2011), which is rather due to hydrolysis. The objective was to determine if chitosan coating might delay burst release at acid pH, representing drug release in the stomach. At pH 5, the solubility of chitosan is 9.9 mg/mL but during our laboratory experiments, chitosan solubility in acidic solutions required time and energy in the form of bath sonication. Comparisons in release rate and extent were made between nanoparticles comprised of PLGA vs. PLA, and between two different molecular weight ranges of PLGA (low vs. high). The ratio of drug: polymer was also varied stepwise in the same manner as the drug loading studies, in order to determine the minimum of polymer needed not only for optimal encapsulation efficiency but also to retard drug release appropriately. The effect of chitosan coating on the core polymer nanoparticles (PLGA or PLA) was also investigated for impact on drug release rate for each core polymer composition, and also varying chitosan type (low, medium and high molecular weight range). The ratio of chitosan to PLGA or PLA was kept the same [e.g. 1:7:7 or 1:5:5 (w/w/w) refers to drug: polymer: chitosan ratio] as a reasonable limit on the number of combinations, although future studies may include a greater proportion of chitosan relative to the core polymer.

Several mechanisms are understood by which drug is released from nanoparticles; (i) diffusion through polymer matrix (ii) desorption of drug bound to surface (iii) nanoparticles matrix erosion and (iv) combines erosion-diffusion process (Danhier et al. 2012). Drug release from PLGA and PLA nanoparticles is generally governed by a diffusion-degradation process.

More specifically, in the early hours of drug release in media, a diffusion process dominates, which is then followed by degradation of the polymer matrix in the later hours (Mogi et al. 2000). A significant burst effect (>50% of drug) was seen in PNPs made with low MW PLGA or high MW PLGA within 2 h in SGF [Figure 10a and 10b], which has a pH of 2.0. With the PNPs made with low MW PLGA; at 1:3 drug: polymer ratio almost all the drug was released in 6h, while with 1:5 and 1:7 drug: polymer ratio, approximately 65% of the drug was released within 2h in SGF and the rest of the drug was more slowly released in SIF until 24 h, consistent with the concept of a longer time to erode the larger amount of polymer. The slight delay in the drug release with high MW PLGA compared to low MW PLGA at 1:7 ratio [Figure 10b and 10a respectively] was likely due to polymer length and lactic acid content (Mittal et al. 2007); high molecular weight corresponds to a greater hydrophobicity due to lactic acid, thereby theoretically delaying water ingress and therefore drug solubilization and release (Félix Lanao et al. 2013). This effect can be seen comparing Figures 10a and 10b (PLGA) 10c (PLA) where drug release is slower in PLA-based NPs. Overall, a significant burst release within first 2 h was observed in PNPs made with low MW PLGA and high MW PLGA likely due to PLGA autocatalysis in acid environment (Pamula and Menaszek 2008). The ratio of drug to polymer plays a key role in drug release, as is evident in Figure 10b. Since PLA is the most lipophilic of all the polymers used, drug release was further delayed and with 1:7 drug to PLA polymer ratio, a minimal burst release (20%) and a desirable sustained release profile until 24 h was observed [Figure 10c]. Overall, the PLA nanoparticles showed a sustained release of MMF over at least 12-15h and in some cases, up to 24 h with minimal burst release.

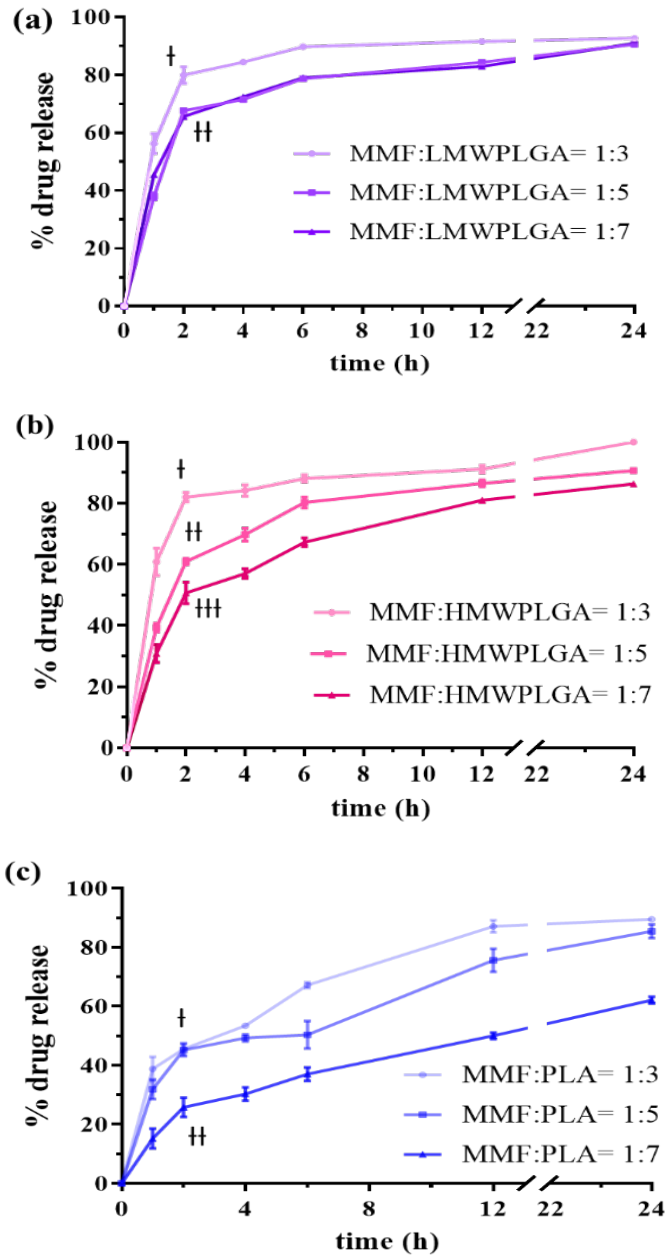


Figure 10. Graphical representation of *in vitro* drug release of PNPs made with MMF and low MW PLGA (a), high MW PLGA(b) and medium MW PLA (c). Data represented as mean±SD. MMF= mycophenolate mofetil, low MW PLGA= low molecular weight poly lactic-co-glycolic acid, high MW PLGA= high molecular weight poly lactic-co-glycolic acid and medium MW PLA= medium molecular weight poly(lactic) acid. Drug release at 2h was found to be different with different formulations prepared ($^{\dagger}p=0.004$, $^{\ddagger}p=0.006$, $^{\ddagger\ddagger}p<0.001$).

Around 60% of drug was released in the first 2h from CS-PNPs made with low MW PLGA plus LMWC or MMWC [Figure 11a and 11b] but when HMWC was substituted, only 40% of drug was released at this early timepoint [Figure 11c]. Almost 90% of the drug was released by the end of 12h [Figure 11]. Significant burst release from most the CS-PNP formulations made with HMW PLGA and all three types of chitosan could be due to the HMW PLGA [Figure 12]. The presence of 35% glycolic acid in HMW PLGA makes it more hydrophilic and degradable in SGF (Alexis 2005). With MMF: PLA: LMWC= 1:3:3, about 45% of drug released within 2 h, which may suggest that a lesser degree of chitosan coating was achieved, which was supported by zeta potential data (-22.1 mV). The CS-PNPs made with PLA and medium MW chitosan showed minimal burst release (<25%) within 2 h with the ratio 1:5:5 and 1:7:7 [Figure 13b], reflecting the low solubility of chitosan at this pH (Kumar et al. 2004). Although there was no significant difference at 1, 2 and 4h between CS-PNPs with PLA and MMWC at 1:5:5 and 1:7:7 ratios [Figures 13b], MMF: PLA: MMWC= 1:7:7 showed a sustained released of up to 24h. CS-PNPs made with PLA and HMWC showed a significant burst release. Even though there was burst release, the drug release after 2 h was well controlled with MMF: PLA: HMWC= 1:5:5 and 1:7:7.

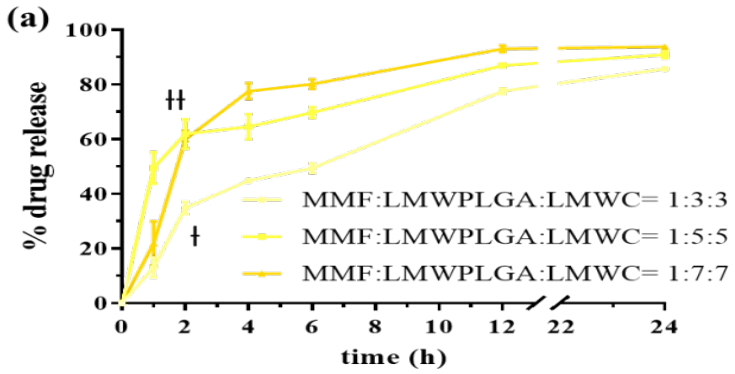
Table 8. Factors affecting burst release from the nanoparticles

Factors	**Cause**	**Effect**
Surface drug	Fabrication of nanoparticles, presence of	Rapid release

	media, absence of second coating	
Presence of pores (increased surface area)	Solvent evaporation, solvent type and temperature at which the solvent is evaporating.	Rapid release
Particle size	Mixing conditions while preparing nanoparticles, type of surfactant and surfactant concentration.	Large particle size- decreased surface area hence, slow release. Small particle size- increased surface area hence, faster release.
Drug crystals in pores	Differential solubility of the drug in the solvents while fabricating nanoparticles.	Faster release or dissolution limited formulation.
pH of the media	Polymer solubility, polymer degradation and drug pKa.	Rapid release at pH where drug solubility is maximum. Delayed release at all other pH(s).
Internal structure of nanoparticles	Fabrication method, choice of the solvent, solvent evaporation conditions and vapour pressure of the solvent	Variable drug release.

With the above results, MMF: HMW PLGA: HMWC= 1:7:7 and MMF: PLA: MMWC= 1:7:7 [Figures 12c and 13b respectively] were chosen as optimal formulations and were subjected to further characterization such as differential scanning calorimetry, surface

morphology and mucoadhesion potential via mucin binding studies. Moving forward, the oral pharmacokinetics of MMF will be assessed *in vivo* to understand the relationship between *in vitro* drug release and blood concentrations over time, and therefore whether this formulation approach will be suitable for achieving oral sustained release in the more rigorous environment of the gastrointestinal tract.



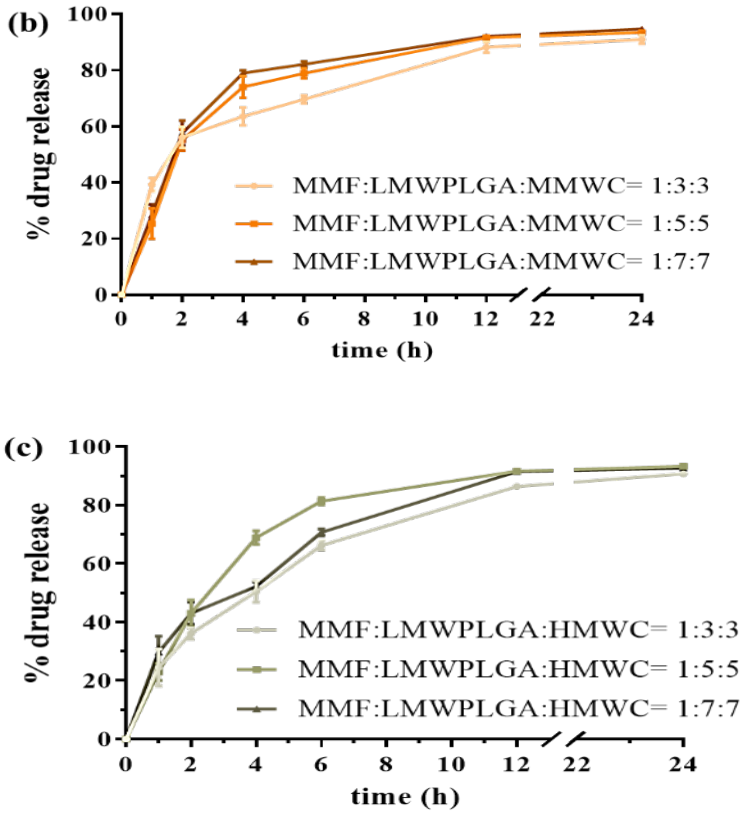


Figure 11. Graphical representation of i*n vitro* drug release of CS-PNPs made with MMF, low MW PLGA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, LMWPLGA: low molecular weight poly lactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. Drug release at 2h was found to be different with different formulations prepared ($^{I}p=0.004$, $^{II}p=0.002$).

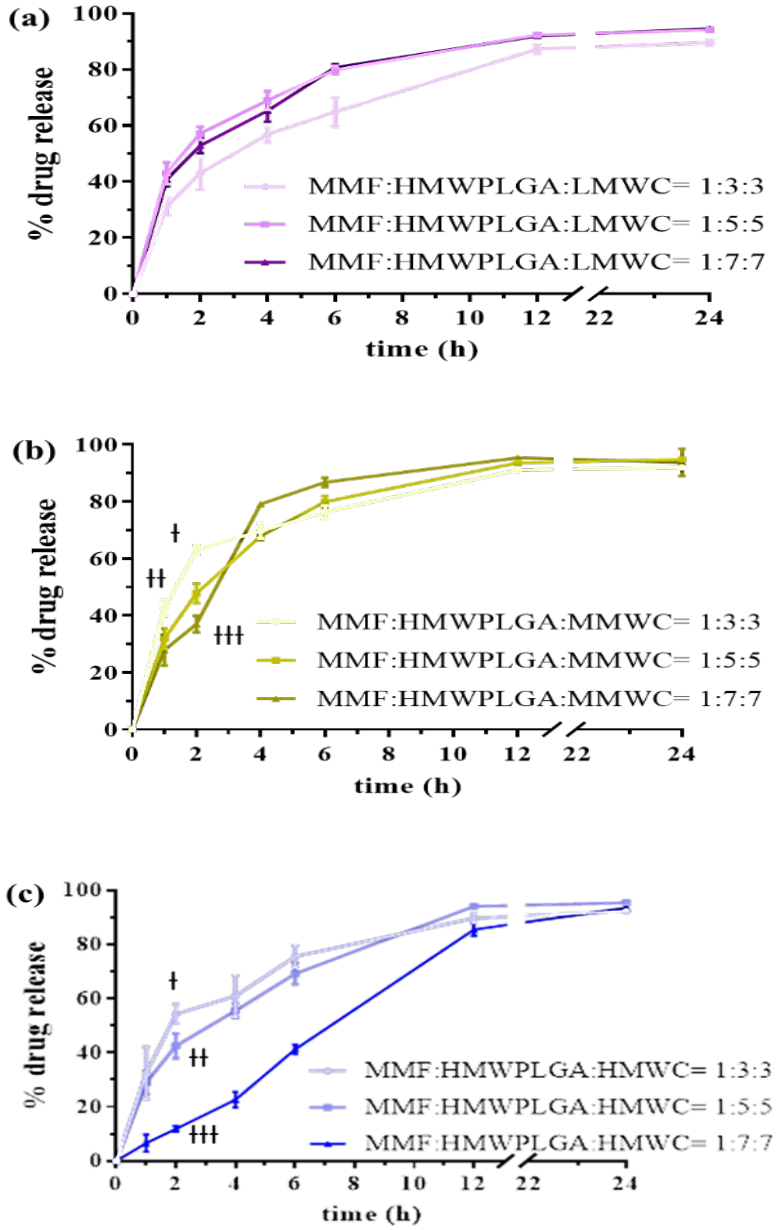


Figure 12. Graphical representation of i*n vitro* drug release of CS-PNPs made with MMF, high MW PLGA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, HMWPLGA: high molecular weight poly lactic-co-glycolic acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. Drug release at 2h was found to be different with different formulations prepared ($^{I}p=0.002$, $^{II}p=0.007$, $^{III}p<0.001$).

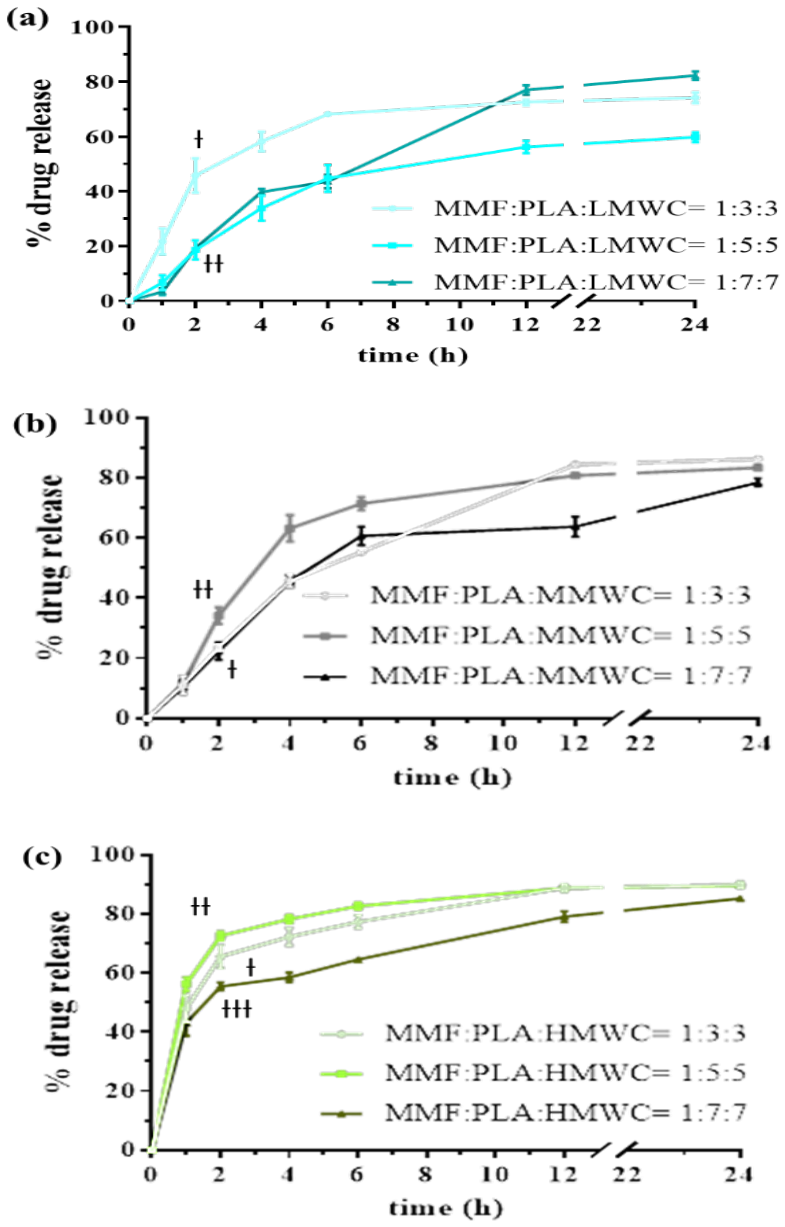


Figure 13. Graphical representation of *in vitro* drug release of CS-PNPs made with MMF, PLA and low (a), medium (b) and high (c) molecular weight chitosan. Data represented as mean±SD. MMF – mycophenolate mofetil, PLA: medium molecular weight poly(lactic) acid, LMWC: low molecular weight chitosan, MMWC: medium molecular weight chitosan, HMWC: high molecular weight chitosan. Drug release at 2h was found to be different with different formulations prepared ($^{\dagger}p=0.005$, $^{\dagger\dagger}p=0.003$, $^{\dagger\dagger\dagger}p<0.001$).

4.4. Scanning Electron Microscopy (SEM).

One of the advantage of analyzing nanoparticles with SEM rather than transmission electron microscopy (TEM) is the ability to look at the particle surface (Klang et al. 2012). SEM analysis revealed that the PNPs and CS-PNPs are spherical and correspond to the unimodal size distribution seen by dynamic light scattering (DLS) experiments. As mentioned above, SEM can help look at individual NPs unlike DLS. In DLS, average particle size of the sample is obtained. The nanoparticles exhibit smooth surface and minimal pores as indicated in Figure 14. Presence of pores can be evidently seen in the Figure 14C. Evaporation of the organic solvent while fabricating the nanoparticles is responsible for the formation of pores. Moreover, presence of pores increases the surface area of the particles, hence, rapid or burst release from the nanoparticles. Pore size depends on the rate of evaporation of the organic solvent and the temperature conditions. Elevated temperatures generally increase the rate of evaporation of organic solvent which further increases the pore size of nanoparticles(Liu et al. 2014). Absence of pores in the Figure 14D reflects chitosan coating when compared to the Figure 14C. Some of the SEM images (Figure 14C) also contain crystals which could either MMF or sucrose added before lyophilization. The bar on the SEM images help depict the size of nanoparticles. The optimal PNPs described above in the drug release studies are of 500 nm size while the optimal CS-PNPs are in between 500-1000 nm.

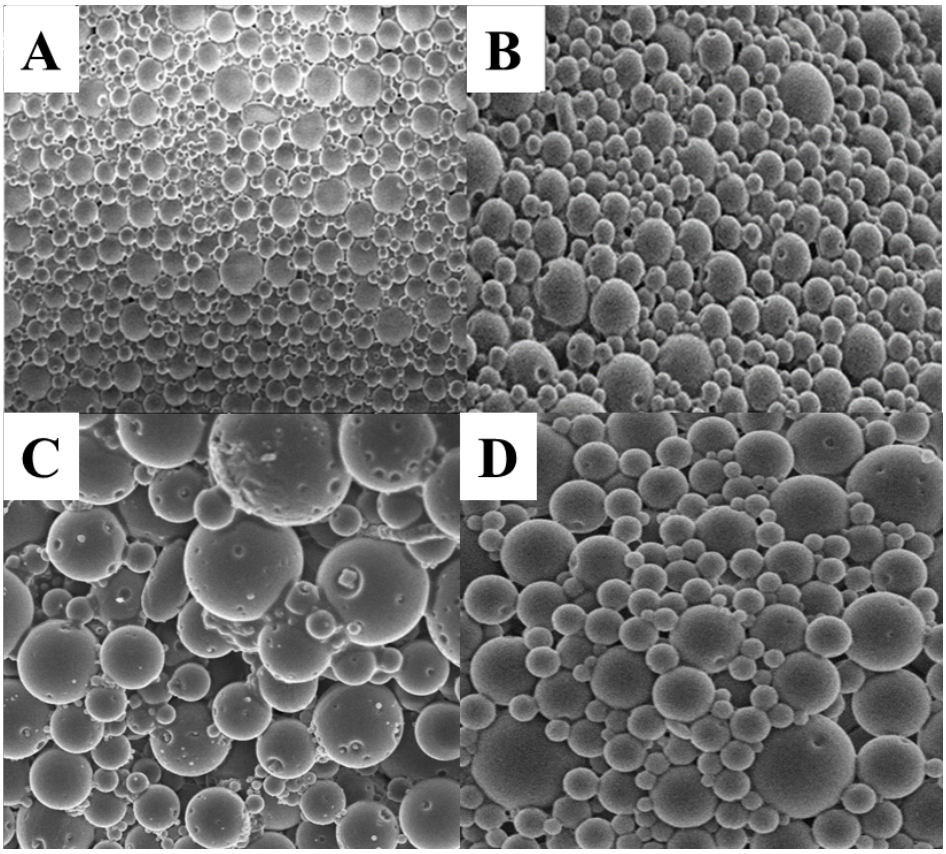


Figure 14. Scanning electron microscopy (SEM) images of MMF: PLA= 1:7 (A), MMF: PLA: MMWC= 1:7:7 (B), MMF: HMWPLGA= 1:7 (C) and MMF: HMWPLGA: HMWC= 1:7:7 (D). Sample magnification is 5000 (A) & 10000 (B, C and D).

4.5. Differential Scanning Calorimetry (DSC) analysis

DSC measures all the reactions where energy changes are involved. In particular, DSC can help analyze, specific heat, glass transition, melting, boiling, sublimation, decomposition and isomeration of a compound (Giron 1986). Apart from the above analysis, DSC can also help evaluate purity of a compound, quantification of crystallinity, polymorphism, stability and decomposition kinetics, presence of molecular water and hydrates (Sophie-Dorothée et al. 1999). As a result, DSC analysis is generally used as a

screening techniques in pharmaceutical formulations and dosage forms (Pyramides et al. 1995; Mora et al. 2006; Demetzos 2008). A typical DSC thermogram of any pharmaceutical dosage form would include analysis of the drug, excipients and the formulation. The changes in the DSC can help us understand physical and chemical transitions within the formulations. Physical interactions affect solubility, formation of eutectic mixture while chemical interactions are a serious concerns because it leads to drug degradation (Mathkar et al. 2009).

The thermotropic behavior and physical state of MMF in CS-PNPs was evaluated by DSC. Figure 15 illustrates the thermograms of MMF, PVA, MMWPLA and MMWC and the optimal formulations. The thermogram indicates an endothermic peak of medium MW PLA around 51^{o}C indicating glass transition temperature. The phase transition peak of pure MMF at 96^{o}C reveals the crystalline nature of the MMF starting material and the absence of this peak in the optimal formulation indicating that the amorphous form of MMF is present in the nanoparticles. The absence of MMF peak in the optimal formulation proves complete incorporation of drug into polymeric matrix (Joshi et al. 2014).

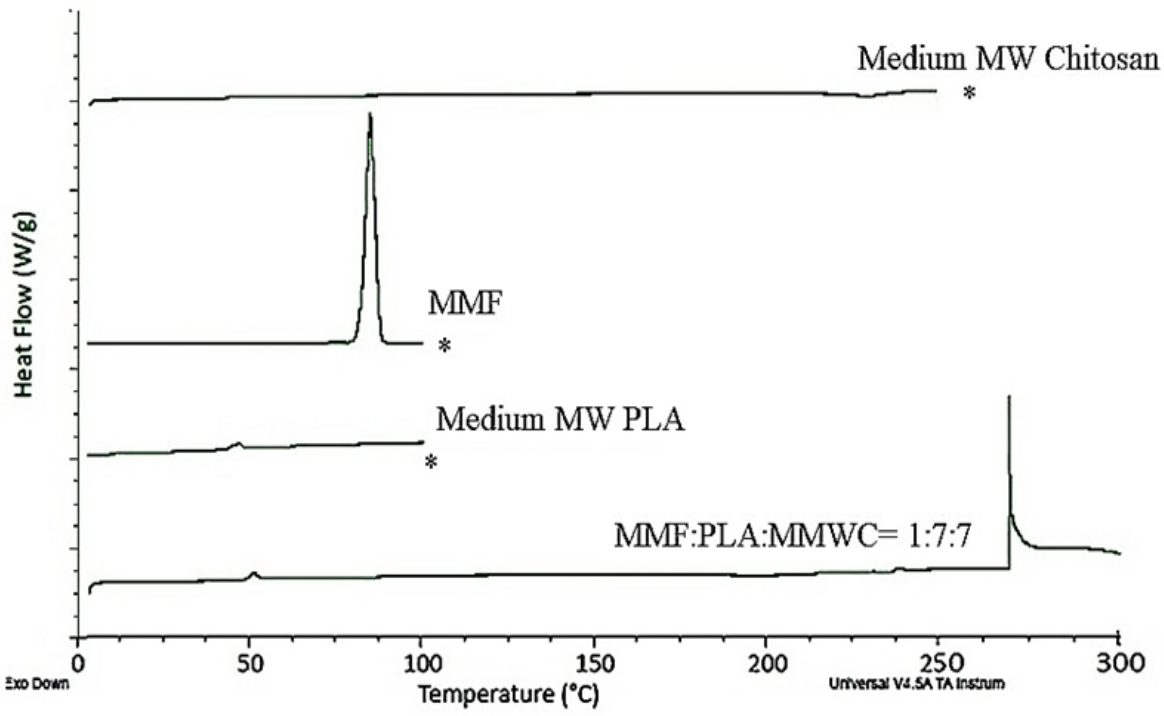


Figure 15. DSC thermograms of MMF, PVA, PLA, MMWC and MMF: PLA: MMWC= 1:7:7. *indicates sample degradation.

4.6. Potential mucoadhesive properties.

There are several methods of assessing mucin binding properties of nanoparticles which include, colorimetric assay (Yin et al. 2009; Pawar et al. 2013), mucoadhesive capacity (%) by spectrofluorimetry (Yin et al. 2009), detachment from porcine mucosa (Bernkop-Schnürch et al. 2003), force required to detach dosage form from mucosa using texture analyzer (Sogias et al. 2012), rheological method (Hassan and Gallo 1990), surface plasmon resonance imaging (Rupert et al. 2016) and *in vivo* imaging (Liu et al. 2017). In this project, zeta potential is evaluated upon incubating NPs in mucin. Although this method has a disadvantage such as determining gross mucoadhesive ability, it is simple and easy to

perform. Ideally, mucoadhesive properties can be confirmed by robust techniques like surface plasmon resonance or *in vivo* imaging. In surface plasmon resonance, molecular interactions can be evaluated yielding images.

Mucin alone in aqueous suspension (pH 7.2) exhibited a negative zeta potential, whereas freshly prepared nanoparticles comprised of MMF: HMW PLGA: HMWC= 1:7:7 and MMF: PLA: MMWC= 1:7:7 had a positive zeta potential. When the nanoparticles were incubated in mucin at 37° for 1h, there was a reversal to a negative zeta potential for both of these nanoparticle formulations [Figure 16a and 16b]. These changes in zeta potential indicate surface interaction of the nanoparticles with mucin but does not indicate affinity. Mucin binding is suggestive that in vivo the nanoparticles may be mucoadhesive, that is, stick to the mucosal surfaces of the GI tract, delaying transit through the GI tract. This would provide additional residence time in the gut during which the drug may be slowly released from the nanoparticles for systemic absorption of the free drug. The absorption of nanoparticles into the bloodstream is not anticipated due to their size. A mean diameter in the submicron range, however, may facilitate a deeper penetration into the mucosal layer, further enhancing retention. The base polymers, PLA and PLGA are subject to hydrolysis over time and would degrade, leaving lactic acid and glycolic acid, which are readily metabolized.

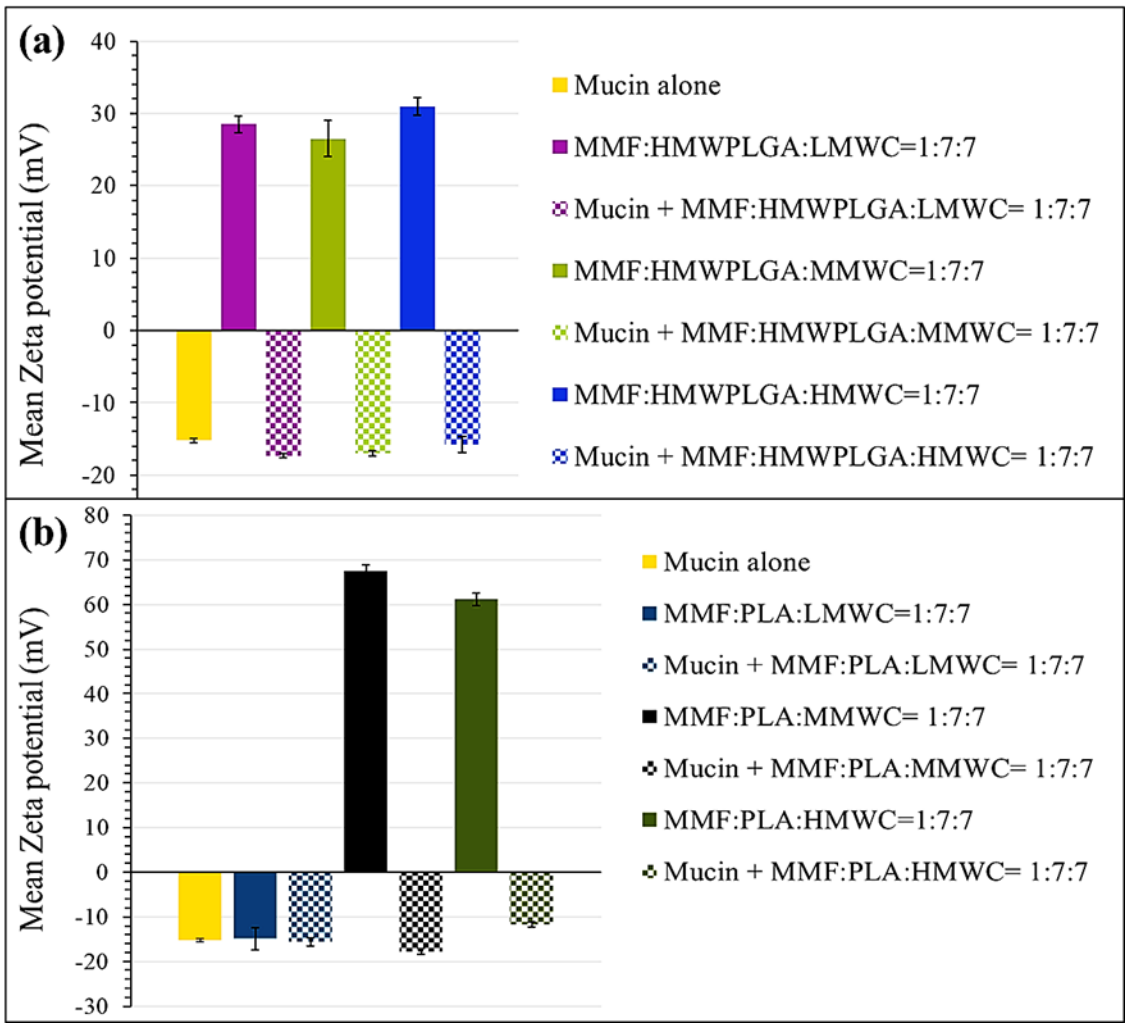


Figure 16. Potential mucoadhesive properties of CS-PNP formulations at 1:7:7 drug: polymer: chitosan ratio made with HMWPLGA (a) or PLA (b) and different grades of chitosan (n=3, mean± SD).

While testing the individual hypothesis, we were able to develop a formulation for improved therapy of MMF with *in vitro* characterization. The formulation developed could address the issues with current dosing of MMF however, further in-depth analysis has to be performed for some concrete conclusion.

Chapter V. Conclusion

Ideally, a sustained release formulation delivers drug over long time (24 h) when compared to an immediate release formulation. Formulating a sustained release dosage form has several advantages, of which, reducing the dosing frequency is a critical one for this project. Mycophenolate mofetil (MMF) is generally given to transplant patients as immediate release dosage form (Cellcept®) bid as 500mg tablets for the life of the organ transplant. A sustained release formulation would decrease the number of required doses per day, and potentially improve medication adherence with immunosuppressive therapy, thereby decreasing the risk of organ rejection.

Mucoadhesive polymeric nanoparticles were chosen as the formulation type to achieve sustained drug release and minimal burst release. During the first phase of formulation development, polymeric nanoparticles without chitosan were prepared to evaluate whether emulsion based solvent evaporation method is suitable to prepare nanoparticles in this study. However, some modifications were performed in the method to achieve nanoparticles suitable for sustained release and particle size. In this phase, the NPs were made with Cellcept®, ester-capped PLGA and vitamin-E TPGS as surfactant. The nanoparticles were within 300nm size, but encapsulation efficiency was less than 10%. In this study, several attempts were made to increase the encapsulation efficiency, but changing the polymer (acid-capped) and using MMF (pure drug) was successful.

During the next phase of formulation development, nanoparticles were prepared with just the core polymers to confirm the best core polymer suited for MMF. Of the three core polymers used which include; low MW PLGA, high MW PLGA and medium MW PLGA, NPs made with MMF, medium MW PLA at ratio of 1:7 (w: w) was chosen as the optimal

formulation. Nevertheless, for formulating chitosan coated polymeric nanoparticles all the core polymers at various molecular weights and chitosan at various molecular weights were chosen. Polyvinyl alcohol was chosen as the surfactant in fabricating the nanoparticles because it produced a stable emulsion while fabricating NPs. Several formulations were made in triplicates with different combination of core and/or coating polymer by emulsion based solvent evaporation method. All the formulations were evaluated for size analysis, encapsulation efficiency and *in vitro* drug release. The optimal formulations for any drug-excipient incompatibility were also evaluated with differential scanning calorimetry. The surface morphology of the nanoparticles was analyzed using scanning electron microscopy and the potential mucoadhesive properties were evaluated by charge reversal of NPs upon incubation in mucin.

The results envisage that mucoadhesive polymeric nanoparticle formulations containing MMF: HMW PLGA: HMWC= 1:7:7 and MMF: PLA: MMWC= 1:7:7 ratio may be most suitable for providing sustained drug delivery, with additional alternative candidates also identified. The particle size of these formulations was found to be 816nm and 1194nm with positive zeta potential (+31mV and +67.6mV) respectively. The encapsulation efficiency of the optimal formulations were 75.44% and 94.34% respectively. The positive zeta potential imparted by the chitosan coating may enable mucoadhesiveness of the nanoparticle formulation. The minimal burst release in SGF (<25%) and complete release over a prolonged time in SIF from the optimal formulations could be an advantage in maintaining a controlled rate of release from the nanoparticles in the gastrointestinal environment.

Chapter VI. Future Directions

Moving forward, a liquid chromatography- mass spectrometry (LC-MS) method for quantification of mycophenolate mofetil (MMF) and mycophenolic acid (MPA) will be developed (Maddela et al. 2017). This method is necessary for analyzing MMF and MPA in biological samples. Analyzing samples with LC-MS has advantages of specificity and sensitivity (Das and Kumar Pal 2014). Moreover, for *in vivo*, bioavailability and bioequivalence studies, it is necessary for analyzing the drug in biological samples with LC-MS because of the sensitivity, rapidity and resolution. Following are the details of the LC-MS method that will be used,

LC-MS instrument and conditions: A 4000 QTRAP® (AB Sciex, Ontario, Canada) mass spectrometer coupled with HPLC system (Agilent Technologies, USA) will be used for the study. Acetonitrile and 0.1% formic acid (80:20 v/v) mixture is chosen as mobile phase at 1 mL/min flow rate. The HPLC column for this study in Pursuit XRs C18 column (100 × 4.6 mm dimensions, Agilent). All the solvents used will be of LC-MS grade. Internal standards for this study include isotopic labelled compounds of MMF and MPA.

Sample preparation and conditions: All the plasma samples will be extracted by liquid-liquid extraction (LLE). The solvents generally used in LLE are methanol, acetone or acetonitrile. A solvent will be chosen depending on the extraction efficiency. Stock solutions, internal standards and quality control samples will be prepared in LC-MS grade solvents and stored in refrigerator until analysis.

Validation parameters such as selectivity, specificity, sensitivity, stability, linearity, accuracy, precision, recovery, dilution integrity and stability will be evaluated according to US-FDA guidelines.

The efficacy and performance of the optimal formulations will be assessed *in vivo*. Oral suspensions of two optimal formulations, MMF in carboxymethyl cellulose and Cellcept® will be evaluated in male Sprague-Dawley rats *in vivo* for pharmacokinetic parameters approved by animal ethics committee of the University of Saskatchewan. The proposed study will need male Sprague-Dawley rats as these are the commonly used rat species for pharmacokinetic studies with polymeric nanoparticles as per literature (Mittal et al. 2007; Wang et al. 2017). There are 16 animals which will be grouped into four groups, each with four animals. The test compounds will be randomly administered to the rats within each group. The animals will be administered all the test compounds by oral gavage at a dose of 28.5mg/kg × 1mL/dose. After the administration of dose, 0.5mL of blood will be collected from jugular vein cannula at predetermined time points; 0.5, 1, 2, 4, 6, 8, 12 and 24 h. Following completion of blood sampling, animals will be terminated by anesthetic overdose and exsanguination via cardiac puncture. As this is a pilot study and we are looking at optimizing a formulation whether it can release drug over time and not cause any significant effect on animals. Success in this animal work may further demand an animal trial with transplant organs.

Tacrolimus based nanoparticles were prepared by emulsion based solvent evaporation method for the treatment of inflammatory bowel disease (IBD) (Meissner et al. 2006). This research can be readdressed and checked for any possible application in formulating nanoparticles with sustained release properties. Similar to MMF nanoparticles, tacrolimus NPs will also be tested for effects of polymer molecular weight and type. Nanoparticle characterization such as particle size analysis, encapsulation efficiency, *in vitro* drug release, potential mucoadhesive properties and *in vivo* pharmacokinetics should be

performed. Ideally, a capsule dosage form containing nanoparticles of both tacrolimus and MMF can provide sustained drug release and reduce the dosage burden on transplant patients.

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