Formulation and Advantages of Furazolidone in Spray Dried and Liposomal Drug Delivery Systems

MUHAMMAD IRFAN ALAM

A thesis submitted in partial fulfillment of the requirements of the University of Sunderland for the degree of Doctor of Philosophy

February 2017

Abstract of Research

The current study has focused on the local delivery of furazolidone to the gut with the aim of generating alternative approaches for the treatment of Helicobacter pylori (H. pylori). Furazolidone has proven antibacterial activity against H. pylori, which has a unique niche in the stomach mucus. This drug was chosen as it is not currently used for the treatment of H. pylori and thus resistance is not expected to be a problem.

Chitosan micro-particles were formulated by the spray drying technique, followed by optimization of mucoadhesion and drug release profiles using glutaraldehyde crosslinking agent at two pH values (1.3 and 4.5). Results revealed that increasing glutaraldehyde decreased the mucin adsorption and at low pH drug release was increased. For liposomal formulations, the effects of furazolidone concentration, chitosan and cholesterol on encapsulation efficacy and in vitro drug release were evaluated. It was found that increasing the pH from 1.3 to 4.5 increased the mucoadhesive behaviour of chitosan coated liposomes from 42% to 60%. Also, increasing the furazolidone amount from 4mg to 5mg increased encapsulation efficiency. A combination of two antibiotics (including furazolidone) was prepared in muco-penetrative liposomal formulations; N-acetylcysteine was used for the muco-penetration effect with Pluronic F-127. These formulations were investigated for their charge effect on muco-penetration and drug encapsulation. The data showed that neutral liposomes easily diffuse through the mucus layer.

Escherichia coli was selected to establish the assay protocol for Helicobacter pylori. The microdilution approach was used for assaying the furazolidone minimum inhibitory concentration (MIC), which was found to be 16 µg/ml for E. coli and 4

µg/ml for H. pylori. In time-dependent killing studies, it was possible to observe

complete killing of the bacteria. Increasing furazolidone concentration by two fold of its MIC, reduced the time required to kill bacteria. The mucoadhesive drug formulations also increased the residence time of furazolidone in the stomach mucus from 2- 3 hours to 4-6 hours; this time period would then be appropriate for killing the bacteria in the stomach. For mucopenetration study complete killing was achieved in 2.5 hours when furazolidone with 1 % minimum inhibitory concentration of NAC which was used. Which was otherwise six hours when NAC was not added for augmentation.

To conclude, delivery of furazolidone was via application of novel liposomal and spray dried formulations to either increase movement across gastric mucosa (via a muco-penetration effect) or to increase binding to the mucus (via mucoadhesive action). Hence, the various approaches used in this research have showed success (to deliver effective amounts of furazolidone locally into the stomach mucus) and the co-encapsulation of furazolidone and N-acetylcysteine is a novel approach for the delivery of antimicrobial agents to the stomach.

Research activity

Conference presentation

Alam MI, Paget T, Elkordy AA. Local mucoadhesive drug delivery approach for furazolidone against *Helicobacter pylori*. Conference: **OMICS International 2016**. 8th International conference and exhibition on pharmaceutics and novel drug delivery system, 7-9 March, Madrid Spain.

Publications.

Alam MI, Paget T, Elkordy AA (2016). Characterization of furazolidone-chitosan based spray dried microparticles regarding their drug release and mucin adsorptive properties.*Powder Technology* 295:175–179.

Alam MI, Paget T, Elkordy AA (2016). Formulation and advantages of furazolidone in liposomal drug delivery systems. European Journal of Pharmaceutical Sciences. 84: 139–145.

Acknowledgements

Praise be to ***Allah***, Lord of the Worlds. The beneficent, the merciful, who is the entire source of knowledge and wisdom endowed to mankind; Who gave me courage and potential to pursue this goal Whom I believe that He never spoils any effort of good deeds.

It is a matter of great pleasure to express my sincere regards to my honorable supervisory team ***Dr. Amal Ali Elkordy and Dr. Timothy Paget*** for affectionate supervision, inspiring attitude, masterly advice and encouragement. Without their intellectual suggestion, it would have been impossible for me to complete this tedious work. I am also really thankful to ***Professor Roz Anderson*** for appreciable cooperation, kind help. At this occasion, I would also like to mention special thanks to ***Lina Hanry, Kayleigh Brannigan, Paul Neesam, Traver and Victoria Hann*** for their technical assistance

Words are inadequate to express my thanks to my honorable colleagues ***Mr. Baseer ur Rehman sheikh, Mr. Saad Moosa, Mr. Omer Abu Abid, Safwan Abdel Rahem, Amar Sulaiman and Huda*** for their help and cooperation during my research work.

I would like to express my great thanks to my parents and uncle (*Yussuf Moosa*) for their endless encouragement, prayer and support. and apple of my eyes *Haniya Irfan*

Table of Contents

List of Figures

List of Tables

List of abbreviations

NAC: N-acetyl cysteine

COPD: Chronic obstructive pulmonary disease

HEH: Hydroxyethylhydrazine

AOZ: Amino-2-oxazolidone

pbp1: Penicillin binding protein

POR: Pyruvate oxido-reductase

RND: Resistance-nodulation-division

PPI: Proton pump inhibitor

RBC: Ranitidine bismuth citrate

EGG-PC: Egg phosphatidylcholine

DSPC: 1, 2-Distearoyl-sn-glycero-3-phosphocholine

DOPC: 1, 2-Dioleoyl-sn-glycero-3-phosphocholine

DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

MLV: Multilamellar lipid vesicles

LUV: Large unilamellar vesicles

SUV: Smallunilamellar vesicles/Sonicated, Unilamellar Vesicles

CMC: Critical micelle concentration

HPMC: (Hydroxypropyl)methyl cellulose

MDa: Megadalton (one million daltons)

KDa: kilodalton

SGF: Simulated Gastric Fluid

PEG: Polyethylene Glycol

PSA: poly(sebacic acid)

PBS: Phosphate-buffered saline

CFU: Colony-forming unit

OD: Optical density

PLGA: Poly (lactic-co-glycolic acid)

LPS: Lipopolysaccharides

TEM: Transmission electron microscopy

CagA: Cytotoxin-associated gene A

VacA: Vacuolating cytotoxin A

Ppm: parts per million

NOD1: Nucleotide Binding Oligomerization Domain Containing

IL-6: Interleukin 6

LB: Luria broth

BHI: Brain-heart infusion medium

CLSI: Clinical & Laboratory Standards Institute

HPLC: High Performance Liquid Chromatography

LC-DAD: Liquid Chromatography with Diode-Array Detection

DDAB: Didecyldimethylammonium bromide

IPA: Isopropyl alcohol

MIC: Minimum inhibitory concentration

EE: Encapsulation Efficiency

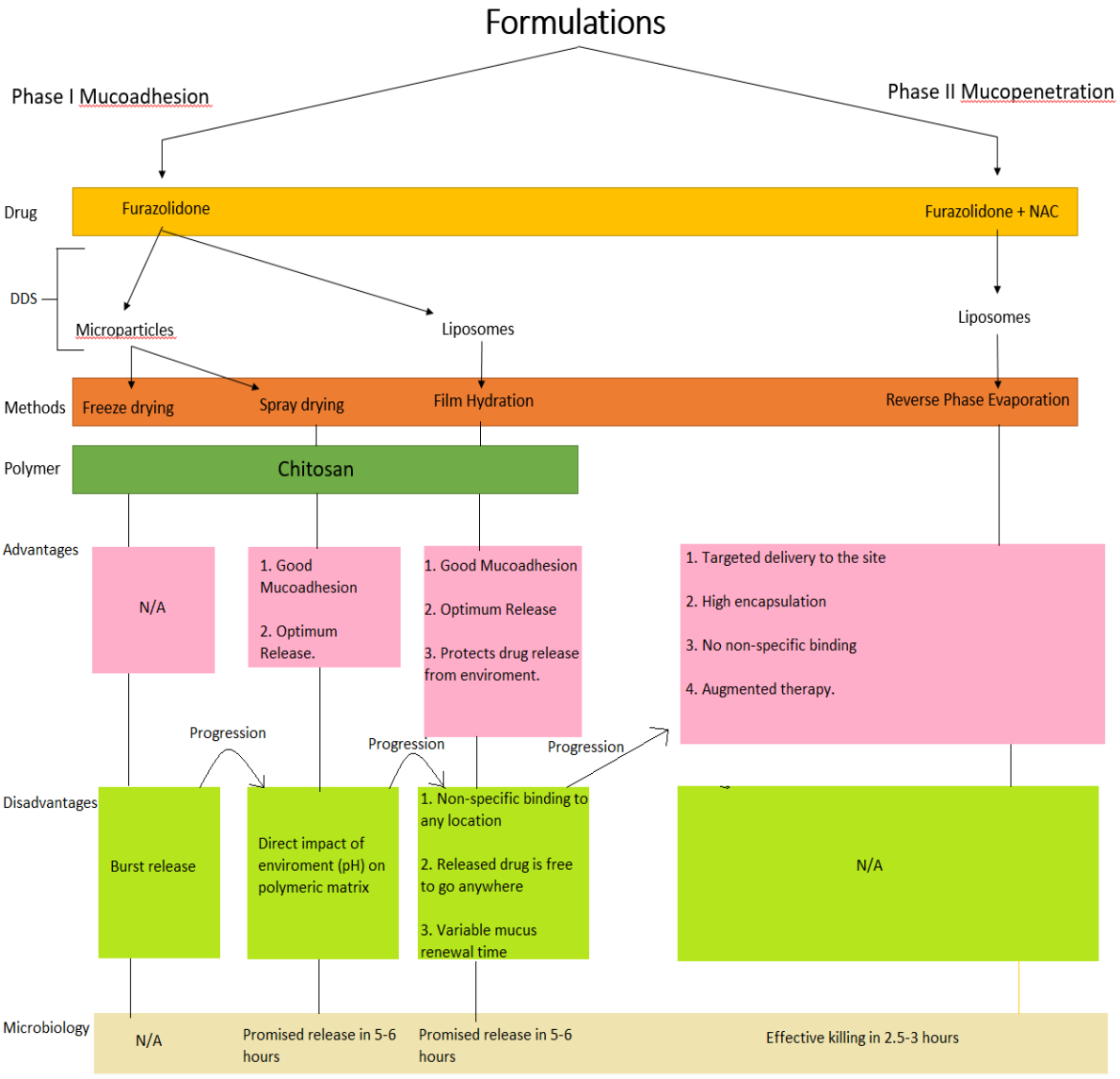
GTA: Glutaraldehyde

REV: Reverse phase evaporation

NCTC: National Collection of Type Cultures

AUC: Area Under t

CHAPTER ONE
INTRODUCTION


Formulations
Phase I Mucoadhesion
Phase II Mucopenetration
Drug
Furazolidone
Furazolidone + NAC
DDS
Microparticles
Liposomes
Liposomes
Methods
Freeze drying
Spray drying
Film Hydration
Reverse Phase Evaporation
Polymer
Chitosan
Advantages
N/A
1. Good Mucoadhesion
2. Optimum Release.
1. Good Mucoadhesion
2. Optimum Release
3. Protects drug release from enviroment.
1. Targeted delivery to the site
2. High encapsulation
3. No non-specific binding
4. Augmented therapy.
Progression
Progression
Progression
Disadvantages
Burst release
Direct impact of enviroment (pH) on polymeric matrix
1. Non-specific binding to any location
2. Released drug is free to go anywhere
3. Variable mucus renewal time
N/A
Microbiology
N/A
Promised release in 5-6 hours
Promised release in 5-6 hours
Effective killing in 2.5-3 hours

1. Introduction

In the current research furazolidone is used as model drug which was formulated in micro size particles and liposomal vesicles by using two different delivery approaches, mucoadhesion and mucopenetration, along with N-acetyl cysteine (NAC) with the aim to target *helicobacter pylori (H. pylori).*

1.1. Furazolidone

The principle drug under investigation in the current research project was furazolidone. It is yellow crystalline odourless drug with molecular weight of 225.158 g/mol. This drug is nitrofuran antibacterial agent (Vass *et al.,* 2008). Its chemical structure is shown in figure 1.1.

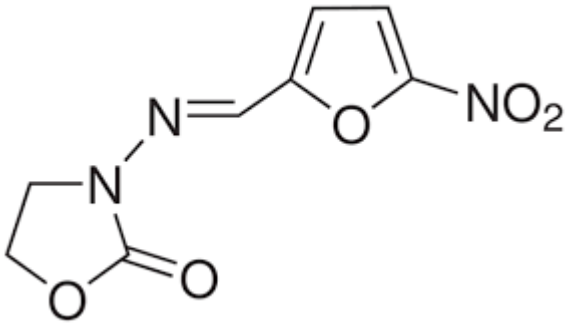


Figure 1.1. Chemical formula ($C_8H_7N_3O_5$) and structure of furazolidone (Pubchem open chemistry database ncbi)

It has limited water solubility of 4mg/100ml (O'Neil, 2001). However, it is readily soluble in acetonitrile. It is very effective broad spectrum antibiotic covering most of gram negative and gram positive microorganisms. It is also widely used against protozoal infections (Walzer *et al.,* 1991). It is marketed by GlaxoSmithKline and Robert laboratories under the name of Furoxone and Dependal-M in the UK. Its mechanism of action is not yet clear but it is proposed that it causes DNA damage by making the crosslinking in bacterial chromosomes and slow inhibition of monoamine oxidase and this bactericidal mechanism is beneficial because in this

way it can minimize the emergence of resistant strains (Dong *et al.,* 2001). Data suggested that it is well absorbed after oral administration, after single dose of 100mg per kg body weight in rats only 3% of unmetabolized drug was obtained in faeces (IARC, 1983). This drug is mainly metabolized by following two successive steps, the primary metabolic pathway starts with the reduction of nitro group to derivative followed by elimination by glutathione conjugation.

1.2. N-acetyl cysteine (NAC)

NAC is the sulphur containing derivative of amino acid cysteine. It is white crystalline odourless drug with molecular weight of 163.19486 g/mol. Its chemical structure is shown in figure 1.2.

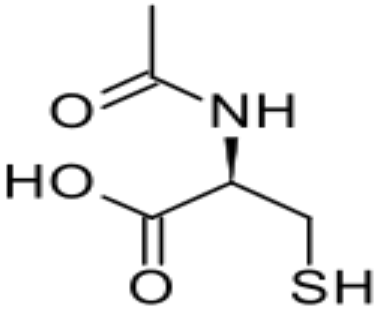


Figure 1.2. Chemical formula (C5H9NO3S) and structure of NAC. (Pubchem open chemistry database ncbi)

It is water soluble with solubility of 1g/5ml (Oslo,1980). However, it is partially soluble in chloroform and ether. It is very effective drug having wide range of therapeutic applications. It is mainly used as mucolytic agent in chronic obstructive pulmonary disease (COPD), and antidote of paracetamol toxicity (Zuin, 2005). According to the literature, it could be potentially used as an anti-psychiatric drug (Dean and Berk, 2011). This drug has good antioxidant properties and can also be used against both gram positive and negative bacteria including e.g. *pseudomonas aeruginosa* and *H. pylori* (Gurbuz *et al.,* 2005). Its mechanism of action against paracetamol poisoning is possibly due to formation of adduct directly with its

metabolite of N-acetyl-p-benzoquinoneimine. However, it reduces the viscosity of mucus in COPD and act as mucolytic agent by cleaving of disulphide bond in mucus which is mainly due to presence of its free sulfhydryl group and this action is enhanced by the increase of pH (Zuin, 2005). It can act as precursor of antioxidant enzyme glutathione (Moldeus and Cotgreave, 1994). Data suggested that it is well absorbed through oral route but presenting very low level of bioavailability (10-30%) as it follows first-pass metabolism. NAC has a small volume of distribution (0.5 L/kg). This drug is mainly metabolized via deacetylation in liver to cysteine, and subsequently metabolized (Ercal and Gurer-Orhan, 2012). Its anti-inflammatory effect is through inhibition NF-κß by redox activation of the nuclear factor kappa kinases thereby modulating cytokine synthesis (Pubmed.ncbi).

1.3. Resistance

In 1996 European gastroenterologists had a consensus of triple therapy against *H. pylori* with two antibiotics and one proton pump inhibitor (PPI), clarithromycin and amoxicillin / metronidazole (Francesco *et al.,* 1998). This triple therapy cannot achieve >80% intention to treat (ITT) (Palmas, 2002).

The global statistics shows that all the antibiotics used in first line of therapy against H. *pylori* failed to respond. Therefore, there is need to develop antibiotic and/or treatment that presents low resistance, effective eradication, low cost and safety. Number of studies suggested the use of furazolidone as rescue therapy against *H. pylori* due to increase resistance associated with other antibiotics (Dong *et al.,* 2001; Eisig *et al.,* 2005 and Jaime *et al.,* 2009). One study suggested the use of furazolidone in third line therapy (Suzuki, 2010). According to European guidelines, the failure in eradication of *H. pylori* after two cycles of therapy discourages the use of metronidazole and clarithromycin and recommends third line therapy after culture sensitivity that includes furazolidone, quinolones or tetracycline (Richard and

William, 2008). The use of furazolidone improved the eradication rate from 60% to 90% in the subjects after failure with first and second line therapy (Suzuki *et al.,* 2005). Both metronidazole and furazolidone are categorized as nitroheterocyclic and nitroaromatic drugs and it proposed in studies that they have the same mechanism of action via reduction of nitro groups. However, the mechanism of resistance of metronidazole for *H. pylori* is not similar to furazolidone (Goodwin *et al.,* 1998; Kwon *et al.,* 2000 and Whiteway *et al.,* 1998).

1.3.1. Resistance of amoxicillin against *H. pylori.*

Amoxicillin is the most commonly used antibiotic in first line therapy. The mechanism of action involves interference with penicillin binding protein in the bacterial cell wall and preventing the synthesis of peptidoglycan (Me´graud and Lehours, 2007). The molecular mechanism involved in the development of resistance is point mutation in penicillin binding protein (pbp1) which has rendered clarithromycin. (Nishizawa *et al.,* 2011a and Nishizawa *et al.,* 2014)

1.3.2. Resistance of metronidazole against *H. pylori.*

Metronidazole is the synthetic nitroimidazole derivative, and it is considered as primary choice in first line of therapy against *H. pylori* globally (Olokoba *et al.,* 2013). But this antibiotic is facing the resistance problem at different rates in different areas of the world. The highest resistance level reported in developing countries (50-80%) followed by Canada and America (22% and 21% respectively). However, in European countries the resistance varies between 20% to 31%. In Japan the resistance is 9-12% (Thung *et al.,* 2016). The mode of action of metronidazole involves intracellular electron transport chain of *H. pylori*. Briefly, free electrons are produced by Pyruvate Oxido Reductase complex (POR) and transferred to ferredoxin (frxB) or flavodoxin (frxA) enzyme system which in turn reduce the nitro

group in imidazole ring (Francesco *et al.,* 2011). This reduction leads to production of nitroso- and hydroxylamine containing compounds that damage bacterial DNA. However, mechanism of resistance of *H. pylori* involves insertion and deletion of transposons and frameshift mutation in genes of these enzymes (Smith and Edwards, 1995).

1.3.3. Resistance of Clarithromycin against *H. pylori*.

There is drastic decrease in the eradication rate of *H. pylori* using clarithromycin (Nishizawa *et al.,* 2014). The mechanism of action of clarithromycin involves inhibition of protein synthesis of bacterial cell after binding to 23S ribosomal subunit. The resistance in clarithromycin against *H. pylori* is broadly explained by two different mechanisms. First point mutation in peptidyl-transferase region in 23S ribosomal subunit which prevents binding of macrolide to ribosomal subunit (Versalovic *et al.,* 1996). This point mutation specific to positions 2142 (11.7%) and 2143 (69.8%) from adenine-to-guanine while adenine-to-cytosine at position 2142 (2.6%). Second mechanism that could be involved in resistance is efflux system. One of the four mechanism of efflux system known as resistance-nodulation-cell division (RND) has been found in *H. pylori* resistance strains (Bina *et al.,* 2000).

1.4. Eradication therapies.

Major factor responsible for the failure of the eradication of *H. pylori* is resistance against most of the antibiotics. However, the other factors are duration of treatment, cost effectiveness, compliance and side effects of therapeutics agent involved in therapy. The eradication therapy is therefore variable in different parts of the world depending upon the burden of disease, resistance to particular antibiotics and the cost. However, there are various choices for eradication therapies available with

different success rates and duration but none of them achieved more than 85% of eradication in any part of the world.

1.4.1. Dual therapy

Dual therapy involved the use of PPI and a single antibiotic which was either clarithromycin or metronidazole (Boer and Tytgat, 2000). This dual therapy was used in previous decades and now it is obsolete mainly due to development of resistance. It is sometimes considered with amoxicillin instead of clarithromycin or metronidazole because the resistance against amoxicillin is very low (Safavi *et al.,* 2016). The main disadvantage in using the dual therapy with amoxicillin and PPI is the penicillin sensitivity of number of people across the globe. Second major factor that is an obstacle for dual therapy is that amoxicillin works at pH above 5.5 and standard dose of PPI does not raise the pH to desired level in the short time required for amoxicillin to be effective (Almeida *et al.,* 2014 and De Francesco *et al.,* 2012).

1.4.2. First line triple therapy against *H. pylori*

According to guidelines first line triple therapy includes the combination of two antibiotics and PPI. Most commonly used antibiotics are clarithromycin with amoxicillin or metronidazole (alternate for penicillin allergies). It is very well established and has been used as primary treatment in developed as well as developing countries until recently (Urgesi *et al.,* 2012).

1.4.3. Second line therapy against *H. pylori*

Second line of therapy often referred as rescue therapy that is described in two different ways. First is known as quadruple therapy that includes the use of two antibiotics most commonly tetracycline and metronidazole with PPI and bismuth sub citrate (Malfertheiner *et al.,* 2007; Gisbert *et al.,* 2000a; Lam and Talley, 1998). The

main reason for the discontinuation of quadruple therapy is the side effects associated with bismuth salt (Suzuki *et al.,* 2010). There are some other studies have used ranitidine bismuth citrate (RBC) instead of PPI along with amoxicillin and metronidazole (Perri *et al.,* 2001b). The use of RBC improved the eradication rate from 5 -58% but it is no longer available due to its side effects. (Caselli *et al.,* 2007). Secondly, the use of levofloxacin is also recommended in second line therapy according to number of studies with eradication rate of 72-76% (Vaira et al. 2007; Gisbert and Morena, 2006c; Gisbert and Pajares, 2005; Saad *et al.,* 2006).

1.4.4. Third line therapy against *H. pylori*

According to Maastricht II–2000 Consensus Report culture sensitivity is not recommended after the failure of first line therapy (Gisbert and Pajares 2002). However, number of authors and clinicians recommend culture sensitivity after the successive failure of first and second line of therapy. Currently, there is no established third line therapy and according to European guidelines culture sensitivity is the determining factor for the selection of antibiotics. Third line therapy often requirs sensitivity testing before recommendation of antibiotics. Number of studies support the use of levofloxacin as major antibiotic for third line therapy (Tursi *et al.,* 2012). But the rise in resistance profile has been seen seen with the use of levofloxacin. Other studies suggested the use of levofloxacin to reduce the eradication rate but routine therapy may lead to development of resistance (Marzio *et al.,* 2006; Wong *et al.,* 2006). Therefore, it is more widely used as the rescue therapy to avoid the development of resistance, (Zullo *et al.,* 2010).

1.4.5. Adjunct therapeutic regimen for current study

1.4.5.1. Furazolidone

Management after the treatment failure from first and second lines of therapy has become a big challenge. There is a need to include any antimicrobial agent in therapy line that not only show low or no resistance but also having the ability to withstand the potential of developing resistance. Number of studies supported the use of furazolidone as antibiotic of choice against *H. pylori* in cases of therapeutic failure (Coelho *et al.,* 2005; Qasim *et al.,* 2005; Graham *et al.,* 2000). According to Hong Cheng and Fu-Lian Hu (2009), furazolidone could be used as first line therapy along with omeprazole with the eradication rate of 86%. (Hong Cheng and Fu-Lian, 2009). It is also used in second line quadruple therapy and rescue therapy (Eisig *et al.,* 2005). Furazolidone was also used in third-line treatment with eradication rate up to 90% (Richard and William, 2008). There are some other evidences that show the use of furazolidone in second line and rescue therapy (Sanches *et al.,* 2008). Excellent eradication rates associated with furazolidone in all stages of therapeutic lines is primarily due the low resistance and no cross resistance with the strains that are resistant to metronidazole (Mégraud and Lamouliatte 2003).

1.4.5.2. N-acetyl cysteine

The use of adjunct therapy is considered as a good therapeutic strategy for *H. pylori*. Number of additives such as vitamin C, probiotics, statins, aspirin and mucolytic agents like NAC have proven effectiveness in different capacities (Nseir et al., 2012; Chang and Gwang 2014). There is enough literature that shows the effectiveness of NAC in eradication of *H. pylori* by different mechanisms. *In vitro* study showed promising results upon inclusion of NAC to disrupt *H. pylori* biofilm and it is also hypothesized that NAC sensitize the bacterial cells for antibiotic (Cammarota *et al.,* 2012).

Another study showed that use of NAC not only detached the bacterial biofilm from stomach epithelium but also disaggregates the clumps of detached aggregates (Hall-Stoodley *et al.,* 2009). NAC was used previously in adjunct therapy that increased the eradication up to 70% when compare to the therapeutic regimen without NAC that showed 60% eradication rate (Karbasi *et al.,* 2013). According to Muhammad et al (2015) subjects pre-treated with NAC before culture selected antibiotic improves the eradication rate from 20% to 60%. In another study it was showen that NAC inhibits the growth of *H. pylori in vivo* and *in vitro* (Huynh *et al.,* 2004). Therefore, it can be concluded that NAC could be potential candidate for adjunct therapy in connection to first or second line therapeutic agents.

1.4.5.3. Omeprazole

The major factors underlying the challenges involved in triple therapy are acidic environment that is responsible for poor stability of antibiotics, poor permeation of antibiotics through mucus layer in stomach and side effects associated with antibiotics leads to patient noncompliance (Endo *et al.,* 2001; Endo *et al.,* 2002). However, the use of PPI offset these effects up to some extent by increasing the pH of stomach to enhance stability, absorption, and tissue penetration of antibiotic (Pedrazzoli *et al.,* 2001; Uygun *et al.,* 2007). PPI is the class of drugs that are used to treat acid related diseases in the stomach i.e. gastric ulcer and acid reflux diseases. It is also used in combination with antibiotics in treatment of *H. pylori* for the number of reasons. In the current research it is primarily selected to increase the pH of the stomach (> 4) and to treat the ulcer cause by *H. pylori* as a part of triple regimen. These are prodrugs that need acid for their activation. This drug is weak base with PK_a of 4 and the pH in the lumen of canaliculus is 1.0 therefore, the drugs of this class are transferred and accumulated into the canaliculus. Where it is converted into active form of sulfenic acids or sulfenamides due to presence of acid which in turns bind

irreversibly to the free accessible cysteine and blocks the proton pump that is terminal stage of acid secretion as shown in figure 1.14. (Shin and Sachs, 2008).

1.5. Drug Delivery systems

A major problem associated with the use of furazolidone are the side effects that are dose dependent and unfortunately no literature supports the dose optimization to create the fine balance between effective dose for eradication and triggering threshold to elicit side effects. However, it is postulated that use of low dose in controlled environment could only take up the benefits of furazolidone and could stop the side effects associated with it (Graham, 2012). Aliakbar et al (2015) in one study proved that lower dose of furazolidone is effective against *H. pylori*. One other study reported only minor side effects using low dose of furazolidone while maintaining the effective therapeutic outcomes (Cheng *et al.,* 2009). If the drug is delivered to the target site, the side effects could be controlled by releasing the drug at the target site. In 2006 a study claimed that cisplatin which is a chemotherapeutic agent targeted to treat tumour cells had eliminated the adverse effects associated with the drug (Katharine, 2006). According to Giuseppina and Agnese (2015) many drug have low therapeutic dose when compared to toxic dose, but their side effects could be controlled by encapsulating them in suitable carriers (liposomes) (Giuseppina and Agnese, 2015). Other studies in the clinical settings showed substantial decreased in the side effects of liposomal encapsulated drug when compared to free drug (Zamboni, 2008; Lasic, 1998).

1.5.1. Liposomes

The word liposome is derived from two Greek words **lipo** means fat and **soma** means body (Due, 2012). Liposomes consist of external lipid surface surrounding the aqueous cavity. Liposomes are small vesicles discovered in 1961 by Alec D

Bangham at the Babraham Institute Cambridge UK. Liposomes are composed of one or more phospholipid membranes that swell enclosing the aqueous cavity. Phospholipids are actually the long chain molecules with hydrophilic head region faced towards water cavity and lipophilic long hydrocarbon chain making the external surface (Akbarzahed *et al.,* 2013). The structure of phospholipids as shown in figure 1.3 consists of two distinct regions. The R group on the phosphate end is variable and mostly in case of liposomes it is choline that makes phosphatidylcholine (PC). (Why ? change)

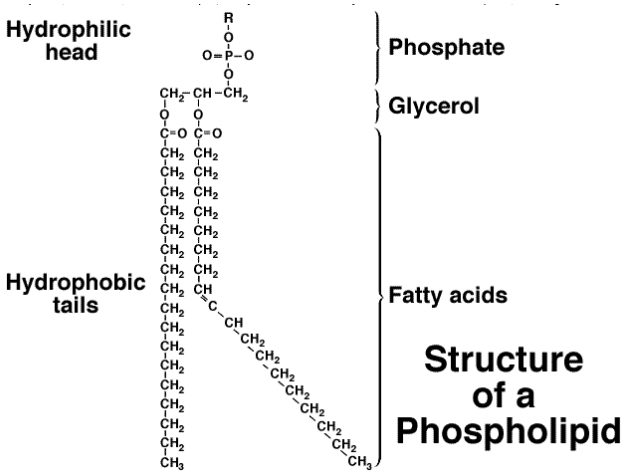
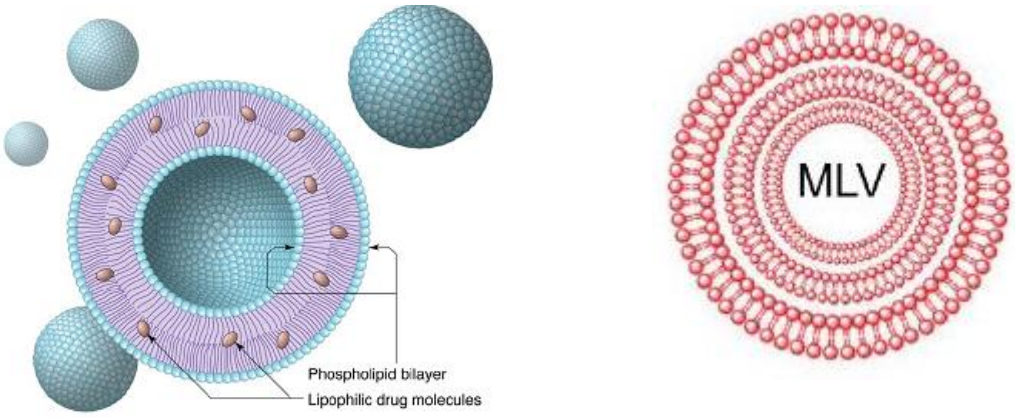


Figure 1.3. Phospholipid molecule with variable R group. (Riley 2015)

In the presence of water, the polar head region assembles themselves towards water and the non-polar hydrophobic chains orient themselves towards outer region and form single or multiple layer liposomal vesicles (Akbarzadeh *et al.,* 2013) as shown in figure 1.4 a and b. (Agarwal *et al.,* 2016)



a). b).

Figure 1.4. a). Single layer liposomal vesicles with lipophilic drug b). Multilayer liposomal vesicle (Agarwal *et al.,* 2016).

Different kinds of derivatives of phospholipids can be used for liposomes phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which are the two prime phospholipids in mammalian cells are commonly used. (Lie *et al.,* 2006). Lipids used in this research was egg PC that is phosphatidylcholine from egg source and DSPC that is 1,2-Distearoyl-sn-glycero-3-phosphocholine. The potential of liposomes to encapsulate both water soluble and insoluble drug makes them most appropriate means of drug delivery. Other characteristics like small size, no toxicity, biocompatibility, biodegradability and non-immunogenic for both oral and systemic administration also offers wide range of application in different areas (Giuseppina and Agnese 2015). The liposomes may or may not contain cholesterol depending upon the final use of the liposomes. However, in general they stabilize the lipid layer and impart the fluidity to the membrane (explained in Viva).

Liposomes are classified mostly on the basis of method of preparation and lamellarity (number of lipid bilayers). Each method used is associated with advantages and disadvantages. One the basis of number of layer they are categorized in two main sub categories as shown in figure 1.5.

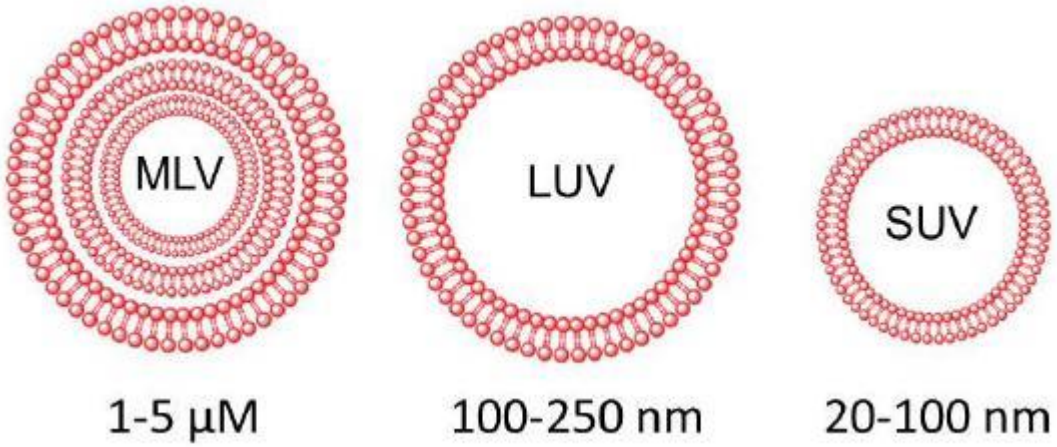


Figure 1.5. Different sizes of liposomes (Agarwal *et al.,* 2016).

Multilayer large vesicles liposomes (MLV) consist of more than one layer and size ranges from 1 to 5 µm. However, large unilamellar vesicles (LUV) ranges from 100 to 250nm and small unilamellar vesicles (SUV) 20-100nm.

The characteristics of the final liposomal formulations depend upon the amount of cholesterol present in the liposomal structure. Different amount of cholesterol is used in one study to optimize the characteristics of final formulation. (Briuglia *et al.,* 2015). However, size of liposome that ranges for 80nm to 1 µm depends upon method of preparation and their composition (Laouini *et al.,* 2013). Major advantage of using liposome as a drug carrier is biocompatibility, very little or no antigenicity and allergic reaction. (Siepmann *et al.,* 2012).

In the current study liposomes were used to co-encapsulate furazolidone and NAC in order to maintain i) the adjunct effect and ii). keeping the low concentration and controlled release of furazolidone to reduce the side effects. Several attempts were made in the past to co-encapsulate drugs in liposomes. Two anticancer drugs doxorubicin and irinotecan were co-encapsulated into liposome with encapsulation efficiencies of > 80% (Ishaque *et al.,* 2013). Similarly, resveratrol was co-encapsulated with paclitaxel in a PEGylated liposome with encapsulation efficiency more than 50% for tumor drug delivery (Jie *et al.,* 2016). In other study two anti-

tubercular drugs were successfully encapsulated with isoniazid encapsulated in aqueous core and rifampicin in lipid bilayer (Gürsoy *et al.,* 2004). Effective dose encapsulation in liposomes is another challenge especially when dealing with co-encapsulation.

The method of preparation and the selection of lipid mainly effect the encapsulation efficiency (Chrai *et al.,* 2002). Other contributing factors are charge of the lipids, cholesterol content and the nature of drug. Most commonly used lipid is phosphatidylcholine from egg or soya that is unsaturated lipid. Literature supports that use of egg PC is more beneficial as compared to soya PC because it contains long chain poly unsaturated fatty acids (Li *et al*., 2014). There are many synthetic lipids available in market with desired properties that have many advantages over natural lipids. They contain saturated long carbon chain lipids that could effectively increase encapsulation efficiency (Begum *et al.,* 2012; Monterio *et al.,* 2014). These lipids are relativity pure as compared to natural lipids because the natural lipids obtained from egg or soya could be contaminated with DNA or protein during extraction process. One of the major advantage of synthetic phospholipids is stability as they consist of saturated carbon chains they are less prone to oxidation and hydrolysis stress. Large number of literature support the used synthetically derived lipids that are available in market for liposomal preparation. These lipids include DSPC, DOPC and DPPC etc. all of them are phosphatidylcholines with different carbon chain lengths (Li *et al.,* 2014). (reference already present)

Method of preparation have also great impact on the encapsulation efficiency and reverse phase evaporation method is the method of choice to increase the encapsulation efficiency of hydrophilic drug. (Frézard *et al.,* 2000)

The rationale for the selection of this method is to increase the encapsulation of NAC in order to co-encapsulate the appropriate amount of NAC with furazolidone which could triggers the adjunct and modulation effect.

1.5.1.1. Methods of liposome's preparation

On the basis of method of preparation, liposomes are classified as shown in table 1.1.

Table.1.1. Classification of liposomes on the basis of methods of preparation

Mechanical Dispersion	Solvent Dispersion	Detergent Removal
Hand shaking	Ethanol injection	Dialysis
Sonication	Ether injection	
French pressure cell	Reverse phase evaporation	
Freeze-thawed liposomes		

1.5.1.1.1. Mechanical dispersion method

Hand shaking method, is one of the mechanical dispersion approach that is widely used to generate the MLV. These multi lamellar vesicles can be further processed by different means to transform them into LUV and SUV.

In the hand shaking method (figure 1.6) the lipid soluble drug is dissolved in organic solvent, which is then evaporated under vacuum usually by rotary evaporator until dry film appears on the walls of glass. Lipid film is then hydrated with buffer and left it for shaking above the transition temperature of lipid for half

an hour to hour.

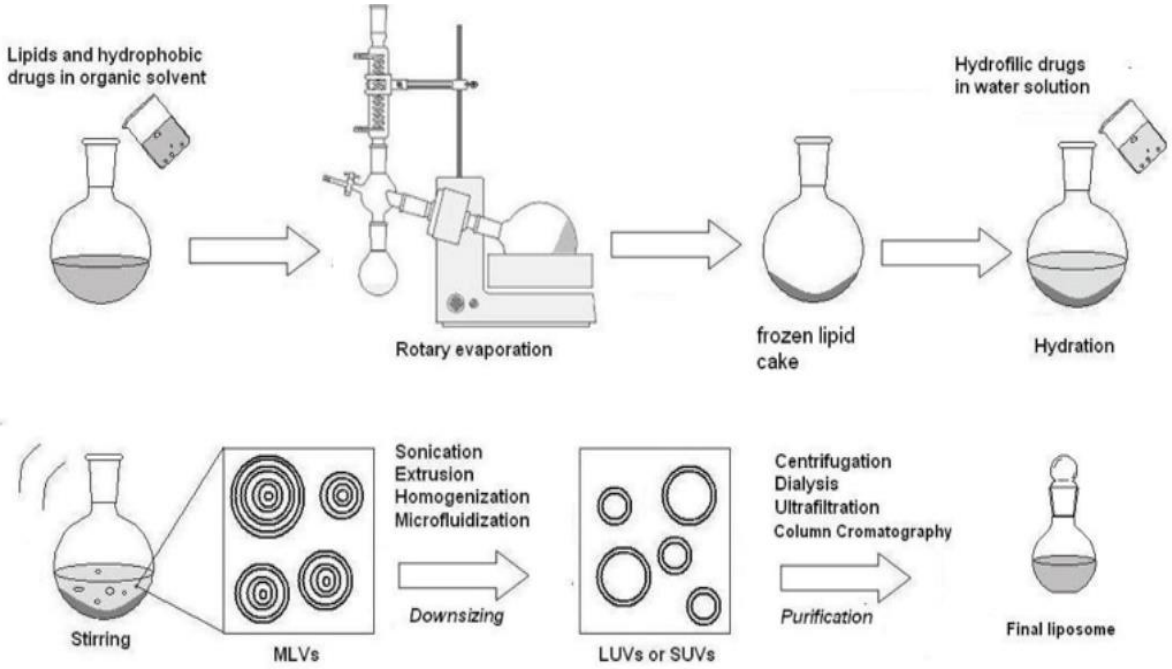


Figure 1.6. Schematic representation of different step involved in hand shaking method for the formation of MLV. (Deepthi and Kavitha, 2014)

Multi lamellar vesicles formed by handshaking method can be converted into LUV and SUV by following mechanical methods (Bhai *et al.,* 2012)

A. Sonication

Sonication could be done by probe sonication or bath sonication depending upon the drug molecule and lipids used to form the liposomes. The liposomal vesicles are converted from MLV to SUV by this method. Major disadvantage of probe sonication is contamination of liposomal vesicles with titanium from the probe and about 5% of lipid is de-esterified in one hour of sonication (Akbarzadeh *et al.,* 2012).

B. French pressure cell

In this method the MLV are extruded through small orifice at 4º C with 20,000psi pressure which converts them into SUV but the major difficulty using this method is attaining the temperature and handling of small volume (Akbarzadeh *et al.,* 2013).

C. Freeze-thaw method

In this method MLV suspension is frozen down to -196ºC in liquid nitrogen for 5 minutes followed by thawing for 5 minutes at 40ºC. The immediate high degree change in temperature creates MLV to LUV. Number of freeze and thaw cycles can vary between 5-10 cycles depends upon the required product. (Sriwongsitanont and Ueno, 2011).

1.5.1.1.2. Solvent dispersion method

There are three most common methods that follow the solvent dispersion approach. The approach used in this current study was reverse phase evaporation method.

A. Ether injection method.

Lipids dissolved in diethyl ether and gently injected into the aqueous phase that contained the molecules to be encapsulated at 65ºC under reduced pressure followed by the removal of organic phase that leads to formation of liposomes (Dua *et al.,* 2012).

B. Ethanol injection method

The only difference between ether injection and ethanol injection method is that lipid in this method is dissolved in ethanol and the second difference is that it is injected into large amount of buffer very rapidly that creates MLV. The disadvantages associated with this method is the final liposomes formed are very diluted as large volume of buffer is used and secondly the complete removal of ethanol is very difficult as it forms azeotrope in water (Dua *et al.,* 2012).

C. Reverse phase evaporation method

This method is advantageous as it can accommodate high amount of water soluble drug in the aqueous core. This method actually based on inverted micelles. In this

method water in oil emulsion is formed followed by the removal of organic phase that form the gel depending upon the concentration of cholesterol used. The gel then collapses and is converted into the liposomes under reduced pressure (Cortesi *et al.,* 1999).

1.5.1.1.3. Detergent removal method

At critical micelle concertation (CMC) the detergents solubilise the lipids and when the detergents are removed the resulting micelles become saturated with lipids and combine to form large unilamellar vesicles.

1.5.1.2. Liposomal/Nano particulate therapy against *H. pylori*

Number of attempts were made in order to devise targeted drug delivery system containing two or more antibiotic that deliver drugs in close proximity to *H. pylori*. Ramteke et al (2008) designed triple therapy based gliadin nanoparticles containing amoxicillin, clarithromycin and omeprazole. The system used was mucoadhesive that released the drugs locally in stomach. However, the use of omeprazole to increase the pH of stomach gives better results through systemic route via canaculies of partial cells because omeprazole is prodrug which is activated in partial cells. Chitosan- glutamate nanoparticles were also designed to deliver triple therapy (amoxilline, clyrythromycin and omeprazole) locally in stomach (Ramteke *et al.,* 2009). Therapeutic approaches against *H. pylori* is faces two impeding factors. One of them is access of antibiotics to the organism in human body and other is resistance as discussed previously in this chapter. Previous studies have focussed to overcome the accessibility problem but the antibiotics used could still lead to develop resistance problems. There is one study that co-encapsulated ampicillin and metronidazole in aqueous core and lipid layer respectively in connection with *H. pylori* therapy. This study was more focused on the interaction

of liposomes with bacterial cells directly but doesn't provide any evidence of access of drug to the bacterial cell in its niche (Pierre-Louis, 2008)

No literature is available that includes low resistant antimicrobial agents delivered to niche of *H. pylori* with augmented therapy that resolves both the issues of antibiotic resistance as well as overcome the accessibility problem at the same time.

1.5.2. Microparticles

Another approach used in the current research is the formulation of furazolidone into micro sized particles with mucoadhesive property to increase the residence time of drug in the stomach. Microparticles were formed by using spray draying technique with crosslinking agent glutaraldehyde to optimise the size and mucoadhesion properties of microparticles. Microencapsulation is widely studied area in the field of drug delivery, major advantages include rapid distribution over the large surface area, high bioavailability and no toxicity by dose dumping (Bhalekar *et al.,* 2013; Beck *et al.,* 2008). Microencapsulation can be achieved by number of ways including emulsification/separation, milling, and polymer phase separation but most commonly used is spray drying as it is easy, straight forward, less time consuming and controlled approach (Tewes *et al.,* 2006). Polymeric microparticles is another approach that provide the wide area of application in the field of drug delivery. The studies have shown the use of different polymer like Eudragit S 100, chitosan and carbapol for preparation of polymeric microparticles. (Venkateswaramurthy *et al.,* 2010).

1.5.2.1. Microencapsulation against *H. pylori*

Microencapsulation has been extensively investigated for the alternate treatment of *H. pylori* by different researches. Large literature supports different approaches for the microencapsulation of antibiotics in polymers. In connection with novel approach

to eradicate *H. pylori* via microencapsulation some studies focused on designing of floating microspheres with clarithromycin as model drug (Paruvathanahalli *et al.,* 2008; Bathini *et al.,* 2011). Floating microspheres have showed promising results but the main disadvantage associated with floating system are the maintenance of certain amount stomach fluid to provide buoyancy which is altered by gastric emptying and presence of food in stomach (Vishal *et al.,* 2013).

Therefore, mucoadhesion provides a platform for polymeric microparticles against *H. pylori*. In another, study modified chitosan microparticles were used with glycan for targeted delivery. These microparticles bind specifically to *H. pylori* adhesins (Inês *et al.,* 2016). Similar kind of work presented in another study where bacterial binding chitosan microparticles were prepared by ionotropic gelation method (Ines *et al.,* 2013). Both the studies have shown very promising results in terms of binding and subsequent detachment of *H. pylori* form stomach surface but major concern is the accessibility of microparticles themselves through mucus barrier. However, Liu et al (2005) prepared amoxicillin microspheres that demonstrated improved eradication than non-encapsulated drug. Emulsification/evaporation method was used in two different studies for the preparation of amoxicillin microspheres in polymeric system with aim of delivering drug locally in the stomach (Shiva *et al.,* 2010; Faizi *et al.,* 2010). There are some other studies which support microencapsulation of amoxicillin for controlled release therapy against *H. pylori*. However, Majithiya et al (2016) microencapsulated clarithromycin as a model drug in chitosan microparticles by using glutaraldehyde as cross linking agent following emulsification technique (Majithiya *et al.,* 2005). Only limited number of literature sources are available for microencapsulation of furazolidone for delivery aim to target H*. pylori*. Solvent evaporation method was used in one study for microspheres

loaded with furazolidone by using three different polymers namely Eudrigit, carbapol and HPMC (Venkateswaramurthy *et al.,* 2010)

Commonly used methods for microencapsulation are solvent evaporation, coacervation, emulsification/internal gelation as mentioned above. However, literature also shows the preparation of sustained release microparticles by using spray drying approach. (Al-Zoubi *et al.,* 2008). Using spray drying methods adds the benefits to microencapsulation in terms of reproducibility, uniformity of size, ease of use and controlled parameters, high encapsulation, speed of process and high sphericity (Rege *et al.,*2003). Microparticles for sustained release drug delivery presented by Maa and Prestrelski (2000). Similarly, erythromycin and clarithromycin microparticles were also prepared by using spray drying approach (Zgoulli *et al.,* 1999).

Spray dried chitosan microspheres were designed in different studies have shown non uniform or burst release (He *et al.,* 1999; Huang *et al.,* 2003). However, addition of crosslinking agent and compatibility of drug to polymeric matrix could control the drug release from spray dried polymeric microparticles (He *et al.,* 1999).

1.6. Drug delivery approaches

Number of advanced technologies are being investigated and are currently used to enhance the delivery of drugs to the target site to improve effectiveness of various therapies (Rosen and Abribat, 2005; Shmulewitz *et al.,* 2006). Currently, different nano and micro particulate based drug delivery systems are most commonly used approaches for targeted and localized drug delivery. One of the major advantages of using micro or nano size particulate system is that they can be modified to attain required features in order to achieve some of the desired therapeutic goals including sustained release of drugs (Langer, 1998; Farokhzad and Langer, 2006) and site penetration (Prego *et al.,* 2005; MacKay *et al.,* 2005). Conventional non modified

particle based system are eliminated from number of sites in the body by mucociliary clearance e.g. orally administered nanoparticles may come across three different possibilities in gastrointestinal tract (GIT) that is lined with mucus layer. They may come in contact with chyme and excreted through fecal elimination, second possibility is their adhesion to mucin fibers and clearance with mucus wash off or lastly they can penetrate through the mucus (Hanes *et al.,* 2003; Ponchel and Irache, 1998; Kreuter, 1989). Due to rapid elimination, the transit time is not long enough for nano/microparticles to release effective amount of encapsulated drug which results in low bioavailability and poor efficacy (Samuel *et al.,* 2009). In order to resolve these concerns number of novel scientific approaches are currently under course of development and investigation for example, mucoadhesive or bioadhesive particulate system. The ultimate aim of using all these strategies is prolonged gastric residence time and delivery of drug to site of *H. pylori*.

The current research was focused on delivery of furazolidone locally to the stomach epithelium to treat *H. pylori*. To obtain the desired aims two different approaches were used i). mucoadhesion; to increase the residence time of the drug in stomach and ii). Mucopenetration; to deliver the combination of two antibiotics in close proximity to *H. pylori*.

1.6.1. Mucoadhesion

Mucoadhesion is the approach in which different formulations can be combined with one or more polymeric systems in order to remain adherent to mucus at different anatomical locations of body and maintained the controlled release of drug over prolonged period of time. The advantages of this system include local bioavailability of the drug at the site of action, controlled release, biocompatibility and increased resident time. Mucoadhesive system rests on mucus layer that is sticky mesh work of glycoproteins.

1.6.1.1. Mucus

It is a viscoelastic layer that is present in different parts of the body including eye, GIT, female genital tract, oral and nasal cavity. Basic function of mucus is to protect the epithelial tissue from chemical and physical injury, bacterial infections and dryness. Major component of mucus is mucin fibres that are crosslinked with each other to form meshwork. These mucin fibres belong to high molecular weight glycosylated protein family (0.5 to 40 MDa) secreted by goblet cells and submucosal glands (Carlstedt and Sheenhan 1989; Wickstrom *et al.,* 1998). Mucin is present in different organ with different sizes depending on the location of the body ranges few hundred to thousands of amino acids.

There are three kinds of mucin in humans one kind is membrane bound that remain adherent to plasma membrane because they bear additional hydrophobic membrane-spanning domain and ranges from 100–500 nm in length (MUC1 and MUC4) and other class is secretory mucin (MUC2, MUC5AC, MUC5B, and MUC7) and the third class is unclassified yet (MUC3, MUC6, and MUC8). Mucin genes encode mucin as rod shaped monomers of 0.3 to 0.5 million Dalton which subsequently undergoes post translational modification of glycosylation (Bansil *et al.,* 1995; Gendler and Spicer 1995).

Two different areas are found in mature mucins:

1. The amino and carboxyl terminal regions that are slightly glycosylated, with cysteines that contribute towards formation of inter and intra monomer disulfide linkages.
2. A long central region consists of multiple tandem repeats of 8 to 100 residue that are rich in serine or threonine amino acid.

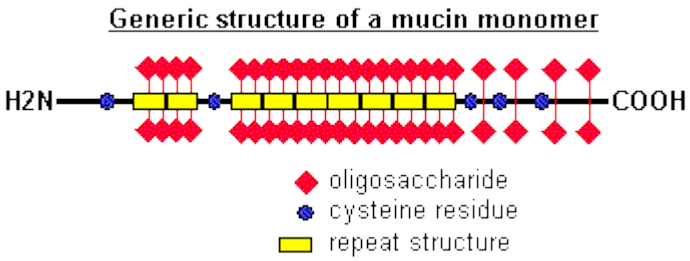


Figure. 1.7. structural assembly of mucin monomers (Bowen, 1998)

The pH of the mucus depends upon the anatomical location. Highly acidic environments like in stomach causes the aggregation of mucin fibres that increase its viscoelasticity (Bhaskar *et al.,* 1992; Celli *et al.,* 2007). pH of the gastric mucus changes from lumen (pH 1-2) to epithelial surface where it reaches up to pH 7.0 (Schreiber and Scheid, 1997). Like the pH the thickness of mucus layer depends upon the mucosal surface. The thickest mucus layer is present in human GIT particularly in stomach and colon (Hansson, 2012). Other important characteristic of mucin is that it is constantly secreted and then washed off in a cyclic manner. It lasts from minutes to hours depends upon the thickness of mucus with gastric mucosal clearance time of 4-6 hours (Monika Schäfer-Korting, 2010).

1.6.1.2. Mechanism of mucoadhesion

The mechanism of mucoadhesion is not yet completely understood but number of theories exist to postulate about the proposed mechanism. Basically, two consecutive steps are involved. Contact stage followed by consolidation phase. In first phase contact is established between mucoadhesive formulation and the mucosal surface, the factors involves in spreading over the mucus surface after contact depends upon the location of mucosal surface in human body. In gastrointestinal tract the factors that promotes the contact and spreading are peristaltic movement, the flow rate of fluids in the cavity and the Brownian motion of the particulate system.

One other important factor is the fine balance between attractive and repulsive forces. As the particles approach to the mucosal surface they come across with repulsive forces i.e. electrostatic repulsion and osmotic pressure, therefore in order to establish the strong contact the attractive forces like Van der waals and electrostatic attraction must overcome repulsive forces (Smart, 2005). In consolidation step the molecules of mucoadhesive formulation penetrates its chains into mucin and form the secondary bonds (Bindu *et al.,* 2010) The prerequisite for this chemical and mechanical interaction is building group of hydrogen bond (-OH, -COOH), flexible chain and surface-active properties that leads to mucus adhesion (Mathiowitz *et al.,* 1999). One other mechanism proposed is dehydration theory that is based on hypothesis when mucoadhesive formulation comes in contact with mucus dehydration occurs due to the difference in osmotic pressure. This osmotic difference is concentration dependent and diffuses the water from mucus into the formulation and this movement of water leads to consolidation of the adhesive forces (Carvalho *et al.,* 2010).

1.6.1.3. Theories of mucoadhesion

There are four major theories (Sharma *et al.,* 2011) proposed for mucoadhesion mechanism based on mechanical forces, electrostatic interaction, adsorption phenomena and spreading.

A. Wetting theory.

This is mainly related to spreading of liquid formulation over the mucus surface and it is primarily based on the contact angle of the liquid molecule with the mucus surface. Generally, lower the contact angle, more will be the mucoadhesion.

B. Fracture theory

This theory doesn't actually support the mechanism of mucoadhesion However, it works indirectly by taking into consideration the force needed to break the attraction after adhesion is established between two surfaces. This theory is mainly concerned with rigid and semi-rigid adhesive materials.

C. Diffusion theory

In this theory it is postulated that the polymer and mucin chain interpenetrate into each other and the degree of mucoadhesion depends upon the degree of penetration of polymeric chain. According to the literature the optimum length ranges from 0.2 to 0.5 µm can provide efferent adhesion.

D. Adsorption theory

This theory involves two types of interaction when the polymers come in contact with mucin. One of the them is kind of permanent adsorption that involves covalent, ionic and metallic bonds that is not the desired. However other involve the weak electrostatic bonds that is temporary setup and could be removed easily.

E. Electron theory

This theory only applies if the incoming polymer is electrically different in terms of charge from the mucus layer. This difference leads to flow of electron that cause the formation of double electronic layer on the interface (Bindu *et al.,* 2010)

1.6.1.4. Factors affecting mucoadhesion

There are many factors that are involved in the mucoadhesion that must be taken into view before designing the polymeric mucoadhesive formulation. The minimum molecular weight for effective mucoadhesion is 100,000 g/mol however the optimum

range is 200,000 to 7000,000 g/mol (Roy and Prabhakar, 2010). For the desired entanglement the polymer chains must be flexible enough and the density of the cross linking is another determining factor. Greater the density of crosslinking lesser will be the rate of flow of water that leads to lesser degree of swelling of polymer chain and the adhesion becomes week, therefore density of crosslinking agent contributes towards the adhesion up to certain limit but beyond that it poses negative impact on the adhesion (Shaikh *et al.,* 2011). Hydration of the mucoadhesive formulation influences the macromolecular mesh size, optimum swelling and pore size which in turns control mucoadhesion. Secondly, hydration also expose the bio adhesive sites of hydrogen bonding and electrostatic interaction. There is not enough data to support the exact effect of charge on mucus adhesion however it is presumed that neutral polymers loosely bound to mucus as compare to charged polymers. Literature support adhesion of both cationic and anionic polymers depending upon the pH of medium because it regulates the degree of ionization (Roy and Prabhakar, 2010).

1.6.1.5. Chitosan

The selection of polymeric materials is very important and in current research chitosan is used as polymer. It is considered as a choice material because it is biocompatible, non-toxic, low cost, hydrophilic in nature and biodegradable. It is long sugar (polysaccharide) molecule composed of β-(1-4) glycosidic linkage -of D-glucosamine and N-acetyl-D-glucosamine as shown in figure 1.8. (a). It is a semi crystalline polymer produced by deacetylation of chitin. The degree of deacetylation varies from 60% to 100%. The average molecular weight of commercially produced chitosan ranges from 3800-20000 Dalton. It is categorized as pseudo natural cationic polymer that is soluble in aqueous solution therefore it finds its way in various of drug delivery applications. The solubility of chitosan depends upon the

degree of deacetylation and the molecular weight. Around 50% deacetylation makes it soluble in acidic aqueous media (Younes and Rinaudo 2015). The solubilization takes place due to protonation of the amino group on position C_2 of the D-glucosamine shown in figure 1.8 (b).

The solubility depends upon the pH of the medium, acid used for the protonation, percentage of the acetylation. It is soluble in acidic pH but the number of proton should be equal to the number of amino group present in the chain of chitosan (Rinaudo, 2006). This amount can be controlled while designing the polymeric system. However, in situ protonation depends upon the strength of HCl in stomach but in regard to this research project the aim is to make it less soluble so it can retain its mucus adhesion assembly which is favoured at high pH. Commercially available chitosan by Sigma–Aldrich comes in two grades one with high and other with low molecular weight. Low molecular weight chitosan ranges from 20 kDa and 190 kilo Dalton with Degree of deacetylation (DD) < 75% while high molecular weight lies between 190 kDa and 375 kDa having DD > 75%.

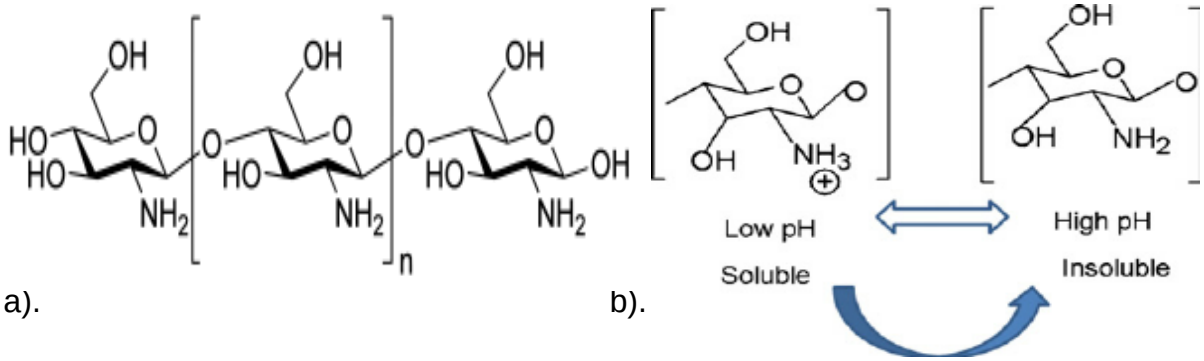


Figure 1.8. a). chemical structure of polymeric repeat of chitosan, b). the site of protonation at lower pH on amine group (Dasha et al., 2011)

1.6.2. Muco-adhesive drug delivery systems

Micropartciles are also considered as worthwhile particulate drug delivery systems because of their small size and drug loading capacity. The size of microparticles ranges from (1000-3000 μm) having drug and layers of polymers as coating material. These microspheres can exhibit short residence time at site of absorption. However, bioadhesive microspheres can be used as good alternative having number of advantages over conventional microspheres like high surface to volume ratio and prolonged time of contact with mucus layer. These factors leads to enhanced absorption and bioavailability of the drugs (Lehr *et al.,* 1992; Chowdary and Rao, 2003).

Mucoadhesive microspheres loaded with amoxicillin were prepared with high efficiency of drug entrapment demonstrated 50% mucoadhesion after 12 hours in stomach (Patel and Chavda, 2009).

A study conducted in 1999 co-encapsulated amoxicillin and metronidazole in chitosan based mucoadhesive microspheres that demonstrated burst release in two hours at pH 1.2 because chitosan does not withstand low pH (Shah *et al.,* 1999). The use of PPI could increase the pH of the stomach that could be helpful while using the chitosan as a polymer. Similar results were find in this study that increase in pH of the medium favours more uniform and sustained release. Chitosan based tetracycline microparticles demonstrated rapid release from pH 1.2 to 2.0 that is physiological pH of the stomach. However, with increase in pH up to 5.0 the release was controlled up to 3 hours (Hejazi and Amiji 2002). However, this issue was further investigated by Portero et al (2002) postulated that reacetylated chitosan could be used to control the release of drug by decreasing the solubility of chitosan. But on the other hand reacetylation reduces the encapsulation efficiency of

particulate formulation. Another disadvantage is that prolonged reacetylation of also effect antimicrobial properties of the drug encapsulation (Portero *et al.,* 2002).

One of the study developed microspheres with glutaraldehyde as cross linking agent for amoxicillin by using spray drying approach (Patel and Patel 2007).

Drug release pattern from microparticles depends up on number of factors like presence of cross linking agent, pH of the medium and size of the particles. When the dissolution, media comes in contact with micro particles it erodes the polymeric shell and the drug adsorbed on the surface as well as in the shell immediately release and shows burst release pattern. (He *et al.,* 1999). Therefore, increasing the cross linking agent could prevent initial burst release. (Analava and Baishakhi 2011).

In comparison to mucoadhesive microparticles, polymer coated liposomes also established reliable means for oral delivery because of either increased retention time in GIT or high penetration into the mucus layer (Takeuchi, 2001). Variety of mucoadhesive polymers can be used for coating of liposomes like chitosan, carbapol and edrigid etc. (Werle and Takeuchi, 2009). The use of chitosan is more appropriate for gastro retentive mucoadhesive system especially in connection with *H. pylori*. Most of therapeutic regimen is favoured by high pH and with the influence of PPI and chitosan as a polymer give the good results in terms of release and mucoadhesion at higher pH discussed previously in this chapter.

Chitosan coated liposomes were prepared in another study for insulin by using reverse phase evaporation with satisfactory results in terms of mucoadhesion and encapsulation activity (Zheng-hong *et al.,* 2004). In 2001 study recommended that coating of liposomes with chitosan increase the stability of liposomes and hence the drug entrapped in gastric fluid (Filipović-Grcić *et al.,* 2001). In the other study, it is proposed that chitosan coating effects drug release profile and therefore selection

of chitosan ratio to liposomes could effectively yield the controlled release system with desired properties (Mohsen *et al.,* 2010).

No literature resources are available that are specifically designed for chitosan coated liposomes for *H. pylori*. However, one study conducted in 2016 that demonstrated the coating of amoxicillin loaded liposomes against *Staphylococcus aureus* but these liposomes were non intended for use in gastric environment (Menikarachchi *et al.,* 2016). However, in general the results of these study suggested that by coating with chitosan the release time was increased. Therefore, it was concluded that chitosan coating can increase the release time in addition to delayed retention when in contact with mucus membrane

However, overall absorption and penetration of micro/nanosized mucoadhesive particulate system is compromised due to rapid mucus turnover, gastric motility and proteolytic activity. All these factors contribute towards shorter resident time of mucoadhesive particulate formulation in stomach. Efficient adherence of mucoadhesive particulate system to mucus is another factor that limits the penetration and retards transport of these particles across the mucus layer and subsequently underlying epithelia. (Lin *et al.,* 2009).

1.6.3. Mucopenetration

Second approach used in this research was mucopenetration to deliver the drug across the mucin barrier. In this approach two antibiotics were used i.e. furazolidone and NAC. However, this NAC was used to perform three major function. One of them is modulating effect on activity of furazolidone and secondly it was used to enhance mucopenetration because it can act as mucolytic agent and finally as anti-inflammatory. The mucopenetrative effect of the formulations was further boosted up by the concurrent use of pluronic F-127.

1.6.3.1. Pluronic F-127

“F” in F-127 represents flakes in the products and it is non-ionic surfactant consists of polyoxyethylene-polyoxypropylene copolymers. (Yeon *et al.,* 2000). Lengths of the two PEG blocks is 101 repeat units while the approximate length of the propylene gycol block is 56 repeat units shown in figure 1.9. F-127. It has very low toxicity, it is colourless and odourless, free-flowing granules and waxy in appearance. Its molecular weight is approximately 12500 Daltons. It is a thermoreversible gel (3-6) that makes it suitable for carrier in different drug delivery system (Loyds and Allen, 1994). It is used in delivery system that are compatible with almost all route of administration including oral, nasal, viginal, parenteral and topical.

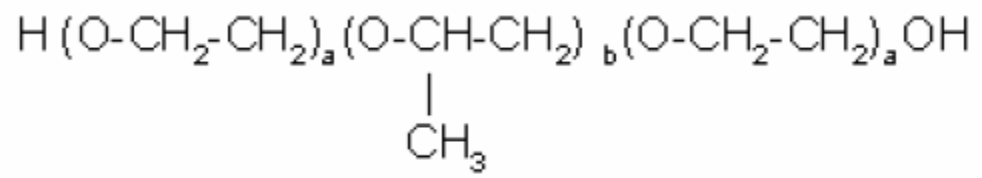


Figure 1.9. Chemical formula of pluronic F-127 (Escobar-Chávez, 2006).

In recent years pluronic-127 has gained interest in penetration studies including transdermal penetration and mucus penetration studies. Pluronic when used in high concentration forms macromolecular aggregates with hydrophilic polyoxyethylene chain arranged towards external surface that is enhance the mucus penetration (Guzman, 1994).

1.6.3.2. Muco-penetrative drug delivery systems

In order to overcome shortcomings associated with mucoadhesive systems, there is a need to develop particulate systems that penetrates the mucus membrane and deliver the drug in close proximity to the site of *H. pylori*. Inefficient penetration of nanoparticles across mucus layers leads to elimination of nanoparticles with

washing off mucus (Xiuying *et al.,* 2011). Mucus-penetration is therefore considered as a major determining factor for transport, uniform distribution and prolonged drug stay at the site of infection across mucus surface (Roger *et al.,* 2010; Lai *et al.,* 2009; Romero *et al.,* 2011).

Number of different studies have been carried out in this regard. Recently used PEG-PSA (poly sebacic acid)-based biodegradable nanoparticles demonstrate deep penetration across human mucus barrier (Tang *et al.,* 2009). Polystyrene and polylactic- co-glycolic acid (PLGA) nanoparticles coated with Pluronic® polymers show lower degrees of mucoadhesion. Proposed mechanism for muco penetration is decrease of hydrophobic and/or electrostatic interactions, therefore leading to efficient penetration of the nanoparticles across mucus barrier (Yang, Lai, and Wang *et al.,* 2010; Hanes *et al.,* 2011).

In one study nanoparticles consist of diblock copolymers of poly (lactic-co-glycolic acid) and poly (ethylene glycol) (PLGA-PEG) were engineered. They were prepared by following solvent diffusion method. One other group fabricate biodegradable diblock copolymer of poly (sebacic acid) and poly (ethylene glycol) (PSA-PEG). These nanoparticles efficiently penetrate through cervicovaginal mucus (CVM). When compared with water, diffusion rate of these two type of particles were reported only 8 to 12 times lower than theoretical speed in water. Based on these calculations it is predicted that 75% of total PLGA-PEG nanoparticles can penetrate across 10-μm-thick mucus in 0.5-hour duration time. These results can lead to foundation for the development of mucus penetrating nanoparticles for improved drug and gene delivery at mucosal surfaces. (Tang *et al.,* 2009).

1.7. Biological aspects of the study

The study is focused on different strategies to deliver the drug against *H. pylori* that survive in alkaline conditions inside deep mucosal layer of the stomach (Scott *et al.,* 1998).

1.7.1. Biology of *H. pylori*.

H. pylori is gram negative microaerophilic bacteria, helical in shape that survive under limited oxygen concentration. It was first discovered in 1983 by Warren and Marshal.

1.7.1.1. Diseases and epidemiology of *H. pylori*

It is mainly involved in gastric ulcer, acute and chronic gastritis and in some cases it progresses to non-cardia gastric cancers and MALT lymphoma and around 50% of world population is infected with *H. pylori* (Mitchell *et al.,* 2016). It is most common in developing countries up to 70%. Approximately 40% of people living in the UK are having *H. pylori* (Cancer research UK, 2016) whereas 23-30% reported in USA (William *et al.,* 2007).

1.7.1.2. Morphology

It is gram negative spiral shaped microorganism with length of 3µm and 0.5 µm diameter. It is motile with 4-6 flagella at one end that are approximately 30nm in diameter and 12-15 nm in length as shown in figure 1.10 ('Rourke *et al.,* 2001). It represents overall negative charge and hydrophilicity in vitro that may contribute towards easy penetration into mucus (smith *et al.,* 1990). It contains outer membrane proteins that comprise of five different protein families. These proteins help in adhesion to host surface, colonization and immune response. The major is putative adhesins family. *H. pylori* binds with the carbohydrate moieties in epithelial region by these adhesins proteins. Porins are another class of OMP that helps in ionic transport across the bacterial membrane. It is also believed that these proteins contribute towards the resistance of *H. pylori* against hydrophilic antibiotics. Recently,

discovered another type of outer membrane proteins called iron binding proteins (Paul *et al.,* 2001). Other two protein families are flagellar proteins and hemagglutinins. Outer membrane of *H. pylori* consists of cholesterol glucosides, phospholipids and lipopolysaccharide (LPS). Lipopolysaccharide consists of high percentage of muropeptides, having pentapeptide side chain ending in glycine and containing (1–6)-anhydro-N-acetylmuramic acid. It consists of three major components O side chain, a core oligosaccharide, and lipid A**.** Sometime “O” antigen of LPS may be fucosylated or nonfucosylated *N*-acetyllactosamine units. Fucosylated mimics type 2 Lewis blood group antigens (Le^x and Le^y) present on the host epithelium (Aspinal *et al.,* 1996). Lipid A of *H. pylori* presents low pyrogenicity and low toxicity due to distinctive phosphorylation and acetylation in outer membrane. The hydrophilic backbone of the lipid A consist of β-(1 to 6)-linked D-glucosamine (GlcN) disaccharide 1-phosphate (O'Toole *et al.,* 2001)

In addition to OMP *H. pylori* also secrete some proteins that are classified as secretory proteins mainly involve in pathogenies. These include vaculating cytotoxin (VAC A), cytotoxic associated gene antigen (cag A), urease, heat shock protein (Hsp) and superoxide dismutase. Urease is major enzyme derived by autolysis of bacterial cell followed by the adsorption on intact neighbouring cell (Suhas *et al.,* 1996) based on secretory proteins they are further classified into two categories type I (secretes cag A and vac A) and type II (secretes vac A).

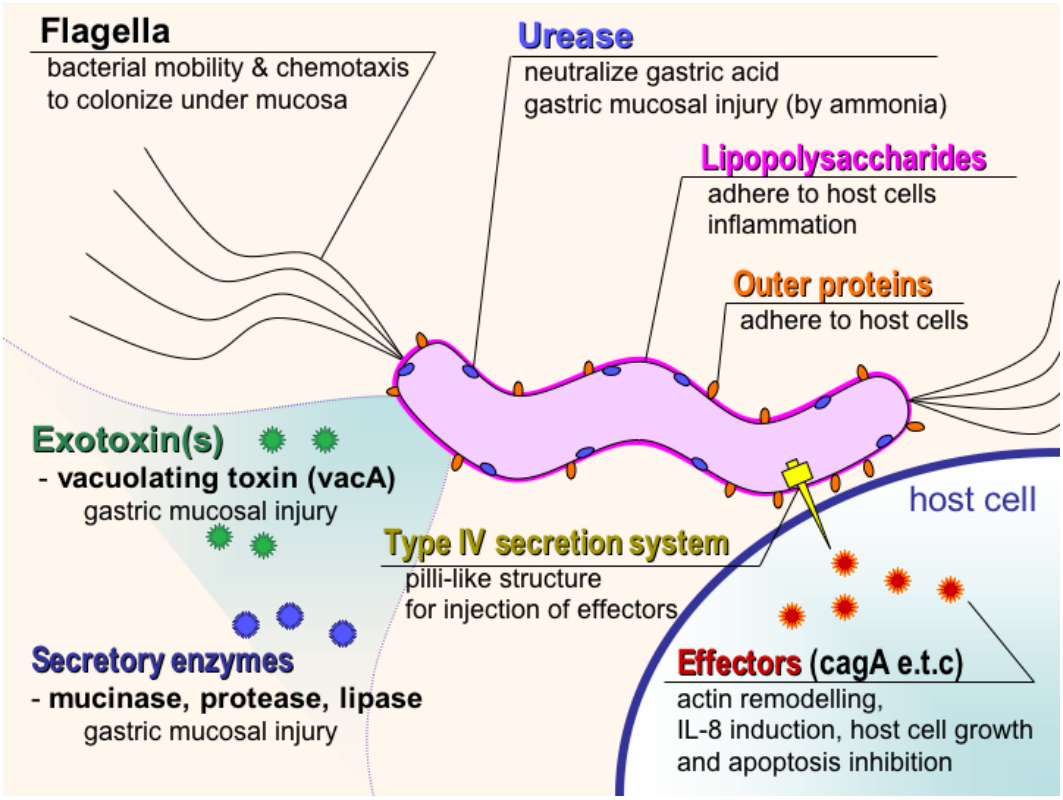


Figure 1.10. cellular structure of *H. pylori* (Tambe, 2012)

1.7.1.3. Mode of Transmission

Its exact mode of transmission is still not clear but most of the scientists agree oral faecal route is the most probable route of its transmission mainly due to contaminated drinking water. It travels through the digestive system, and infects the stomach or the first part of the small intestine. The factor that increase the likelihood of acquisition of infection are poor hygiene, crowded area share the same resources, poor sanitation, infected family member and faecal contamination water supply. Humans are considered as main reservoir of *H. pylori*. Person to person saliva could also be considered as secondary source of transmission (Linda, 2000).

The progression of infection is documented into five different stages and take ages to reach the final stage and may not complete all the stages as shown in table 1.2 (Kuster *et al.,* 2006).

Table. 1.2. Stages of progression of *H. pylori* pathogenesis.

Stage	Description of stages of infection progression	Pathology
1	Normal stomach lining	(Normal mucosa)
2	Inflammation of the stomach lining	(chronic gastritis)
3	Loss of stomach cells and impaired digestive system	(atrophic gastritis)
4	Transformation of the stomach lining	(intestinal metaplasia)
5	Beginning stages of stomach cancer	(dysplasia)
6	Stomach cancer	(gastric adenocarcinoma)

1.7.1.4. Pathogenesis

The pathogenesis involves two major steps i). penetration into mucus from lumen of stomach and ii). adaptation and adherence to the stomach epithelium. It moves from region of low acidic pH (lumen) where pH is 1.2 to the area of high pH (epithelium) with pH of 6-7. First of all, it moves from lumen to epithelium to avoid low gastric pH that ranges from 1.2 to 2 in the lumen. At the interface of epithelium, it secretes the adhesin proteins Bab A that binds to Lewis antigen present on the surface of epithelium and SabA that binds to sialyl-Lewis x antigen expressed on gastric mucosa (Ilver *et al.,* 1998).

A. Gastric ulcer and gastritis.

Bacteria comes in contact with gastric epithelium and after establishing the firm contact it secretes the enzyme called urease that converts urea into carbon dioxide and ammonia. The ammonia helps in regulating the pH for bacteria weakens the epithelial lining and as a results the pepsin enters into the site of damage and cause the tissue damages gastric ulcer that leads to gastritis and gastric ulcer (Sokic-Milutinovic *et al.,* 2015)

B. Inflammation

The mechanism responsible for inflammation relies on type IV secretion system. As shown in figure 1.10, this injection system injects cagA and peptidoglycan from bacterial cell to the epithelial cell. Once inside the epithelial cell peptidoglycan in recognised by the immune sensor Nod 1 that encounter peptidoglycan by stimulating the release of cytokines. The release of cytokines cause inflammation (Testerman and Moris 2014).

C. Cancer

Two different theories exist that explains the mechanism of cancer caused by *H. pylori.* First theory proposes the increased production of reactive oxygen species that may lead to the mutation of host epithelium cell and leads to cancer (Kim *et al.,* 2011). The other theory called perigenetic pathway postulates that secretion and injection of vacA and cagA directly effects the cell lining and cause inflammation as a result the immune response assaults by stimulating the expression of TNF-α and/or interleukin 6 (IL-6). This tumour necrotic factor (TNF) alter gastric epithelial cell to mutated epithelial cells and leads to cancer (Ku *et al.,* 2013).

1.7.2. Stomach

The Stomach is the muscular and dilated portion of digestive tract and it is present between oesophagus and intestine in the left side and upper part of abdomen. Anatomically it is divided into four portions (Ross and Wilson,2010). Cardiac region which is adjacent to oesophagus. Oesophagus empties its contents into this region by oesophageal sphincter. This portion of stomach contains cardiac glands. Fundus is slightly raised portion of stomach that lies against diaphragm and mostly the gases are stored in this region. Body it is the major region of the stomach mainly contains gastric fluid and consist of cardiac glands. However, Pylorus is the terminal end if the stomach and empties into small intestine by pyloric sphincter. This region

is important current studies because *H. pylori* resides in this area of stomach and sometimes extends its pathological effects into duodenum of small intestine.

1.7.2.1. Histology of the stomach

Histology of the stomach describes different layers of stomach. Layers of stomach walls are similar in all four parts of stomach with only difference in mucosal appearance that is specific for particular part of stomach as shown in figure 1.12.

Mainly stomach walls consist of four layers.

- Mucosa
- Submucosa
- Muscularis propria
- Serosa

A. Mucosa

Mucosal layer is further subdivided into three layers (Junqueira, 2005) The surface epithelial layer that consist of simple columnar epithelial cells called surface mucous cells that secretes mucus. There is invagination widely distributed in this layer called pits. These pit extends down to the next layer called lamina propria. The third sub layer of mucosa is muscularis mucosa that comprise of smooth muscles cells.

Sometime it is divided into two major components.

- Superficial foveolar compartment consists of epithelium, pits and mucus secretions that renews after every 3-4 days.
- Deep glandular compartment, having different thickness depends upon the part of stomach. It consists of glands and muscular layers.

Mucus it is alkaline viscous gel secreted by surface epithelium cells to protect the underlying mucosa from acidic environment of stomach. The thickness of mucus is approximately 1 mm and it is washed off after every 4-6 hours. This layer is important because *H. pylori* lives underneath the mucosal secretion on the surface of

epithelial cells and sometime in the surface cell of the pits and inside of lamina propria of pyloric part of stomach as shown in figure 1.11.

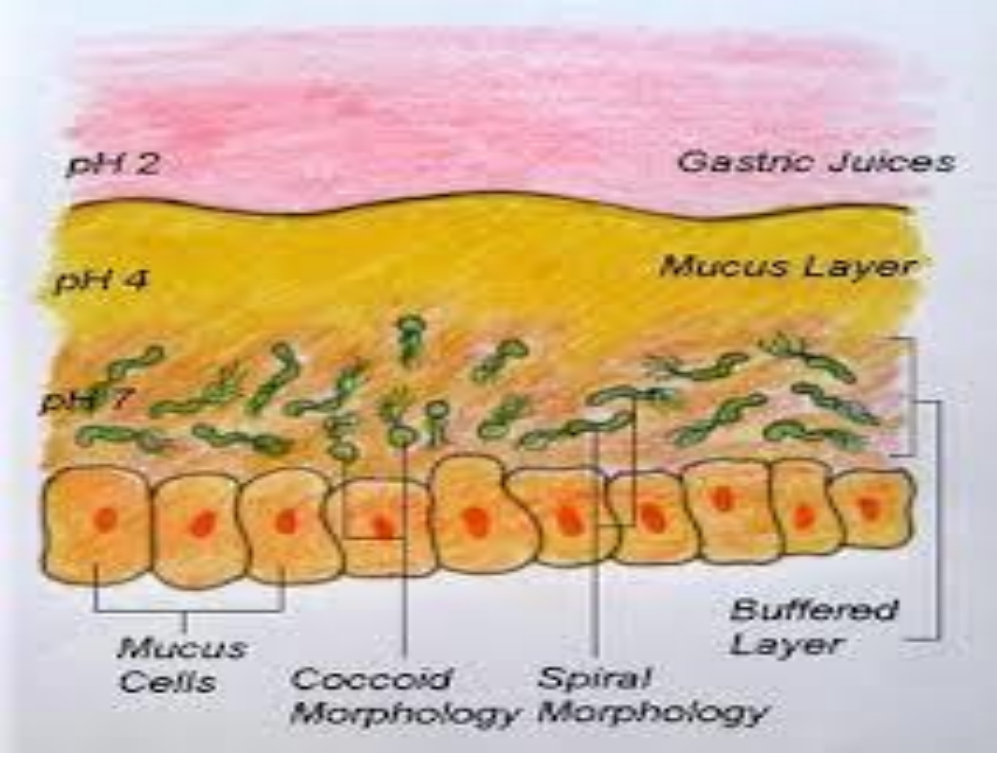


Figure 1.11: Mucosa layer of stomach showing the mucus secretion layer of 1mm thickness and the surface epithelium cells without pits and the attachment of *H. pylori* onto surface mucous cell with different pH values from lumen to epithelium. (Michelle Wiepjes,2008)

B. Submucosa

It consists of dense irregular loose connective tissues. blood vessels, lymphatics and nerves are mainly present in this layer.

C. Muscularis propria

It consists of two layers the first one is circular smooth muscular layer and the second one is longitudinal smooth muscular layer. This layer is primarily responsible for the peristalsis.

D. Serosa

This is the outermost layer of loose connective tissue

Figure. 1.12. Diagrammatic representation of layers of the stomach (Michelle Peckham,2004)

1.7.2.2. Parietal gland of stomach.

These are the glands present in the corpus and fundus of stomach and they are responsible for the secretion of acid (HCl) into the lumen of stomach. they are bound by special type of membrane bound protein called hydrogen/potassium adenosine triphosphatase enzyme (H+/K+ ATPase) or normally called proton pumps. this pump is responsible for the exchange of acid into lumen from the canaliculi of parietal glands in exchange with K^+ from the lumen as shown in figure 1.14. However, the source of Cl^- ions is through exchange of bicarbonate from blood on the other side of the gland 1.13.

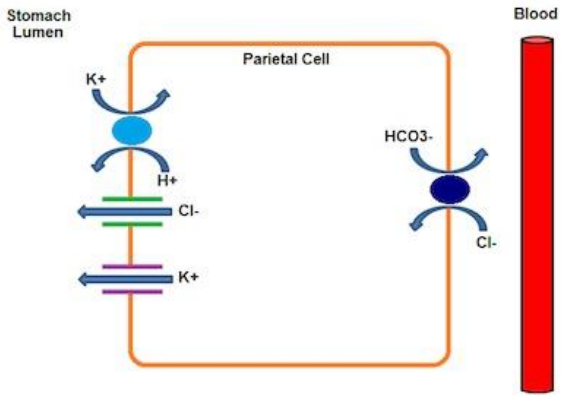


Figure 1.13. Parietal gland exchange of H^+ /Cl^- ions into lumen and exchange of Cl^- ion form blood to parietal gland. (Eric Leung, 2011).

Two different drug delivery systems were used in this research. Liposomes were prepared by using two different approaches one of them was hand shaking method (first phase) and the second was reverse phase evaporation methods (second phase) to encapsulate furazolidone alone in the first phase and furazolidone with NAC in the second phase. However, polymeric microparticles of furazolidone with chitosan were formulated by using the spray drying technique.

Liposomal encapsulation of furazolidone along with augmenting agent NAC could be possible option for the effective treatment when drug is delivered locally to stomach in order to prevent systemic adverse effect. Only limited literature is available for liposomal encapsulation of furazolidone or NAC. Study conducted in 2010 successfully encapsulated furazolidone in liposomes against leishmaniasis (Tempone *et al.,* 2010). Hasanin and co-workers (2014) successfully encapsulated NAC in liposomal formulation designed for the treatment of *P. aeruginosa* (Hasanin *et al.,* 2014).

1.8. AIMS AND OBJECTIVES

1. To use the antimicrobial agent that presents no or very low resistance. Furazolidone was selected for this purpose but the side effect associated with furazolidone limits the use of this drug against *H. pylori*. Therefore, the study was aimed to produce drug delivery system that can reduce the amount of furazolidone used against *H. pylori* by maintaining the minimum effective dose.

2. To develop different drug delivery approaches that can avoid the systemic route for furazolidone in order to reduce the associated side effects and achieve the effective killing of *H. pylori*. No literature is available that includes furazolidone with low resistance delivered to niche of *H. pylori* that resolves both the issues of antibiotic resistance as well as overcome the accessibility problem at the same time.

3. To investigate the delivery system that carries the adjunct therapy with the use of furazolidone as an alternate antibiotic to those commonly in practice coupled with NAC to overcome the developing resistance issue.

CHAPTER TWO
MATERIALS AND METHODS

2.1. Materials.

Furazolidone and NAC, chitosan, cholesterol and Schiff reagent periodic acid and pepsin (partially purified from porcine stomach) were purchased from sigma Aldrich. Glutaraldehyde, coumarin-6, reconstituted mucin type III, didodecyldimethylammonium bromide 98% (DDAB) and dihexadecyl phosphate (DCP) were purchased from fisher scientific, UK. Defibrinated horse blood, fetal calf serum and CampyGen gas generating packs were purchased from thermofisher UK. Columbia blood agar base and DENT selective supplement for *H. pylori* was purchased from oxoid, UK. Tris buffer and calibration particles CPC (300, 400, 1000 and 2000) were purchased from Izon Science, Oxford, UK. OCT compound for cryosectioning was purchased from Agro Scientific UK.

H. pylori stain NCTC 12455 was purchased from culture collection public health England. Freshly excised sheep stomach tissue was obtained from Green Marshal Abattoir Bishop Auckland UK.

2.1.1. Lipids

Phosphatidylcholine (Egg PC 80 E S) was given as a gift sample from Lipoid Switzerland. However, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar, USA.

2.1.2 Reagents and solvents

Distilled water was used in all the experiments where necessary throughout the project. Table 2.1 includes all the reagents and solvents used in this project.

Table.2.1. List of chemical reagents used in the project

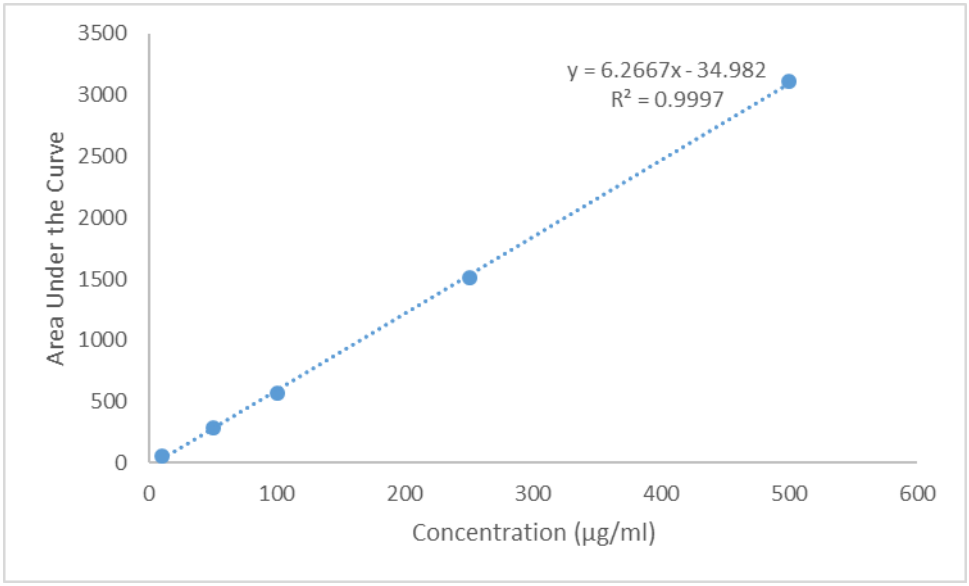
Compound	**Supplier**
Acetonitrile	Thermofisher. U.K.
Chloroform	Thermofisher. U.K.
Disodium hydrogen orthophosphate	Sigma Aldrich. U.K.
Acetic acid	Sigma Aldrich. U.K.
Hydrochloric acid	Sigma Aldrich. U.K.
Isopropanol	Sigma Aldrich. U.K.
Pluronic F-127	Sigma Aldrich. U.K.
Orthophosphoric acid	Thermofisher. U.K.

2.2. Calibration curves

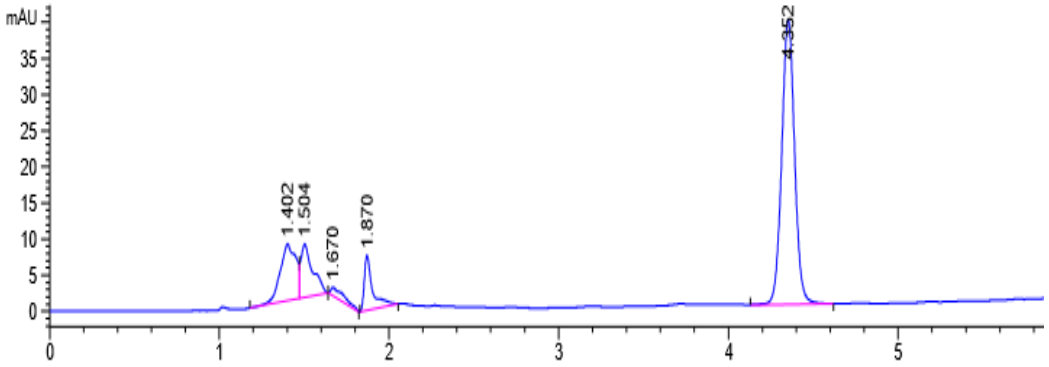
Four different calibration curves were used in this study in different analysis. This section of the chapter describes compounds, medium of dissolution (solvent), dilution used and the method followed for making of calibration curves.

2.2.1. Calibration curve of Furazolidone.

Due to low solubility of furazolidone in water, stock solution was prepared in acetonitrile by adding 50 mg of drug in 100ml. All dilutions (10, 50, 100, 250 and 500 µg/ml) were made in acetonitrile. The experiment was conducted in triplicate and all the dilutions were prepared separately for each set of experiment. HPLC (method description in section 2.6.3.3) was used to generate calibration curve and 0.5ml of sample was injected each time. Area under the curve were plotted against concentration used as shown in figure 2.1.



a).



b).

Figure 2.1: a). Calibration curve of furazolidone b). HPLC chromatogram of furazolidone.

2.2.2. Calibration curve of Mucin

Standard solution for calibration curves (0.1, 0.25, 0.5, 0.75 and 1 mg/2ml) were made in 5M NaOH and absorbance was measured by UV/visible spectrophotometer at 555nm. The experiment was performed three times and separate dilutions were made for each experiment (Figure 2.2).

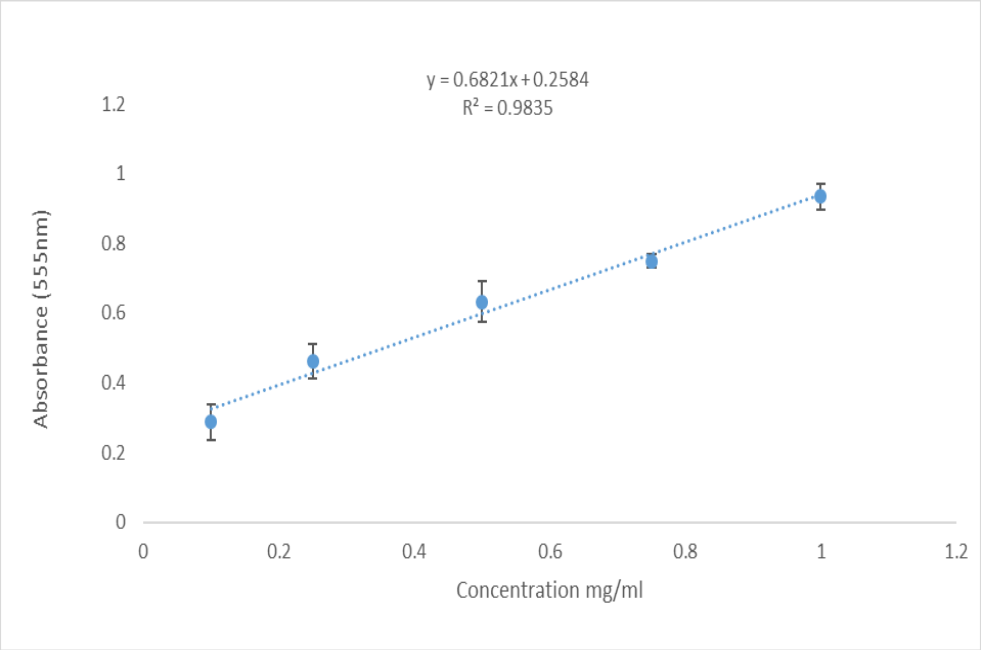
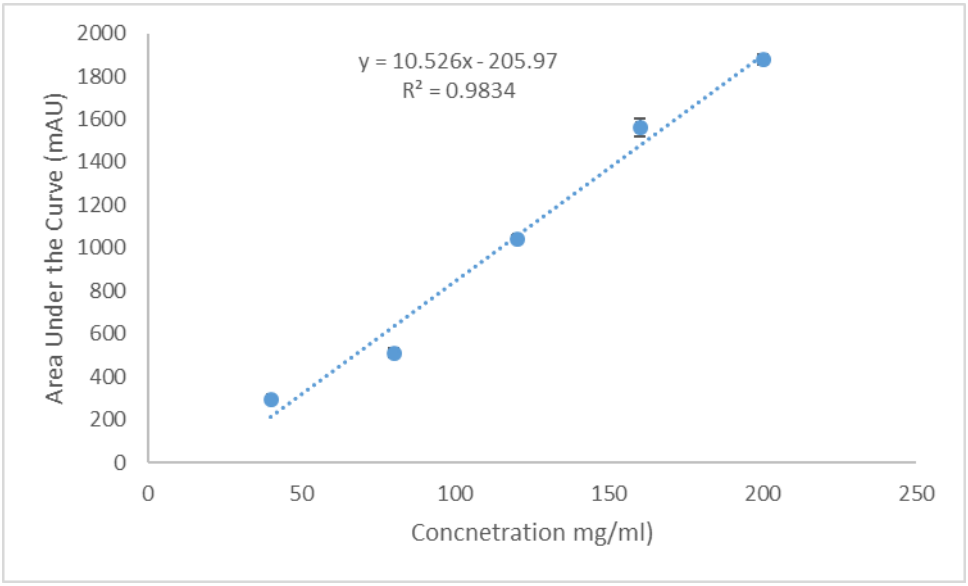


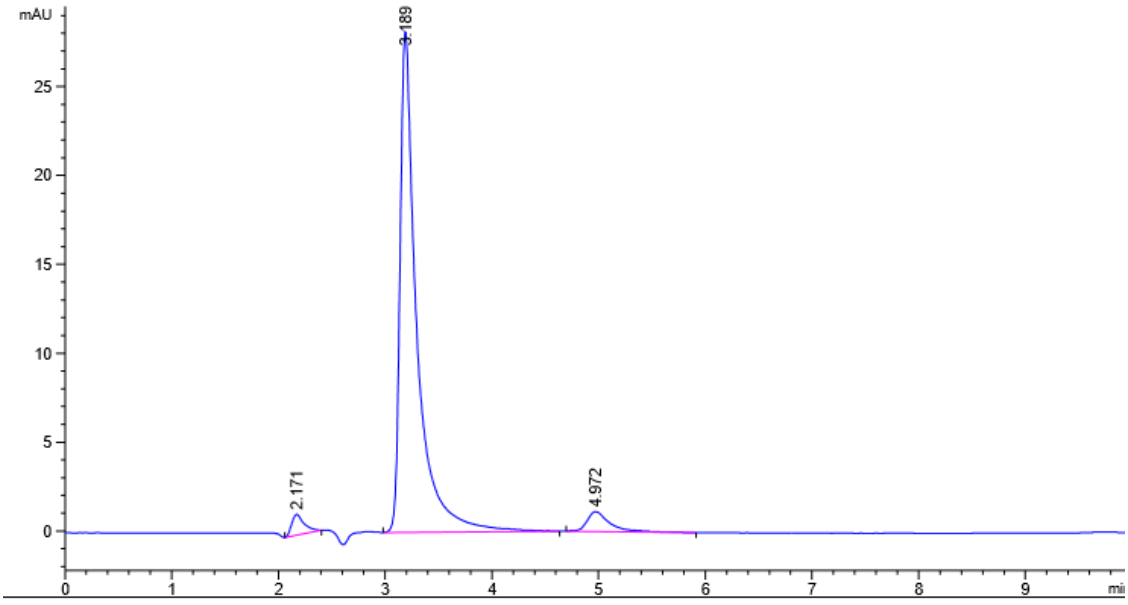
Figure 2.2: Calibration curve of Mucin type I in 5M NaOH

2.2.3. Calibration curve of NAC

NAC was readily soluble in water therefore stock solution and dilutions were made in distilled water. Accurately weighed 200 grams of NAC was dissolved in 5ml distilled water in 10 ml volumetric flask. After continues stirring the volume was made up to the mark by adding distilled water. 100µl of each dilution was injected into HPLC (detailed method is in section 2.8.3.1). Dilutions used were (40, 80, 120, 160 and 200 mg/ml). The experiment was conducted in triplicate and all the dilutions were prepared separately for each set of experiment. Area under the curve were plotted against concentration used as shown in figure 2.3.



a).



b).

Figure 2.3: a). Calibration curve of NAC b). HPLC chromatogram of NAC

2.2.4. Calibration curve of Coumarine-6

The concentration of coumarine-6 was used in parts per millions (PPM). Stock solution and the dilutions were made in chloroform. The calibration curve was constructed by measuring intensity in fluorimetry at emission wavelength of 455 nm and excitation wavelength of 508 nm. The dilutions were 0.05, 0.1, 0.25, 0.5, 0.75

and 1 PPM. The experiment was conducted in triplicate and all the dilutions were prepared separately for each set of experiment. Intensity were plotted against concentration used as shown in figure 2.4.

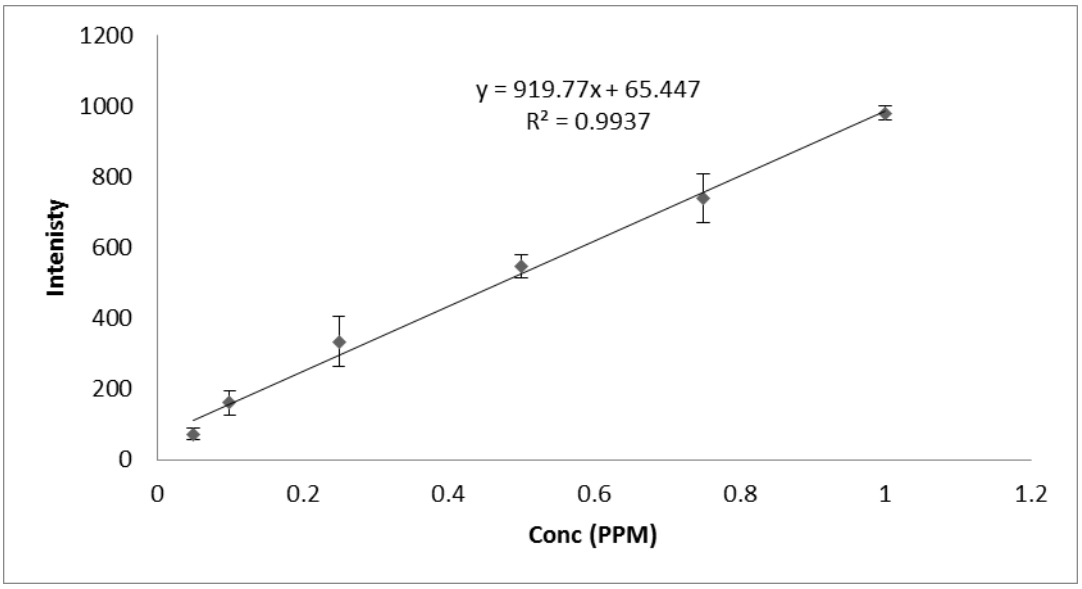


Figure 2.4: Calibration curve of Coumarine-6.

2.3. Preparation of polymeric microparticles

2.3.1. Preparation of spray dried microparticles

Chitosan micro-particles were prepared by using spray drying (Buchi B-290 Switzerland). 0.1 % w/v of chitosan solution (1mg per ml) in 15ml of 1% acetic acid solution was prepared by continues stirring for six hours. Different volumes of 1% glutaraldehyde as crosslinking agent were added to make different formulations listed in Table 2.2. For drug loading 10 mg or 15mg of furazolidone was dissolved in water or water and acetonitrile mixture in different formulations as shown in table 2.2.

The experimental conditions were, inlet temperature was 160^0C, pressure was 40 bars, flow rate was 2ml/min, aspirator 100% and pump was 65%. The resultant dry powder was collected in a dry collection bottle.

Table 2.2: Composition of microparticles prepared by spray and freeze drying methods.

Formulation	Chitosan (0.1%)	Drug (mg)	Glutaraldehyde (1%)	Solvent
F1	15 mg	10	2 mg (0.2ml)	Acetonitrile/distilled water (3:82)
F2	15 mg	15	4 mg (0.4ml)	Acetonitrile/distilled water (3:82)
F3	15 mg	15	6 mg (0.6ml)	Water
F*f*1	10 mg	10	-	Acetonitrile/distilled water (3:82)
F*f*2	10 mg	10	4 mg (0.4ml)	Acetonitrile/distilled water (3:82)
F*f*3	15 mg	10	6 mg (0.6ml)	Acetonitrile/distilled water (3:82)

2.3.2. Preparation of Freeze dried microparticles

Chitosan micro-particles were prepared by using bench top freeze dryer. Briefly, required volume 0.1 % w/v of chitosan solution (1mg per ml) in 10ml of 1% acetic acid solution was prepared for formulation F*f*1 and F*f*2. Whereas for F*f*3 15mg of chitosan was dissolved in 10ml of 1% acetic acid solution by continues stirring for six hours. 25% w/v of commercially available glutaraldehyde solution (Sigma Aldrich) was diluted down to 1% (1g/100ml) and different volumes of 1% glutaraldehyde as crosslinking agent were added to make different formulations listed in table 2.2. For drug loading 10 mg of furazolidone was dissolved in water and acetonitrile mixture.

Vials containing polymeric drug suspension were frozen at−20ºC for 2 h. After primary freezing vials were stored at −80ºC for 24 h. The samples were then placed on the storage shelf in the vacuum chamber of the freeze dryer. The pressure during

lyophilisation was maintained at 40 mbar. Sample were lyophilized for 48h followed by nitrogen purging to ensure the removal of the residual moisture.

2.4. Characterization of chitosan microsphere

2.4.1. Percentage yield of spray dried samples.

The yield of the process was determined by weighing the spray dried particles using Precisa analytical balance (405M-200A Swiss quality Switzerland) by using following equation

$$\frac{Powder\ recovered}{Total\ drug\ and\ chitosan}\ X\ 100\text{---------Equation. 1}$$

2.4.2. Drug Content determination

The powder collected contains drug and polymer was dissolved in specified amount of distilled water to make suspension. 1ml of suspension was centrifuged. Acetonitrile and acetic acid was added to pellets to dissolve the drug and chitosan and then injected 0.5ml into HPLC. The concentration was calculated from AUC by using standard calibration curve of furazolidone.

2.4.3. Particle Size Analysis

Particle size was determined by Izon qNano particle sizer (Izon Science Ltd. NewZealand) by using tris buffer. Reference calibration particles (CPC) were selected on the basis of expected size of formulation particles and then suspended in tris buffer provided by Izon company which was filtered through syringe by using syringe filter 0.2µm and put into Izon qnano by using micro pipette. By maintaining the steady stable current flow on screen CPC particles were replaced with particulate formulations in the same tris buffer and particles size was measured in terms of current block that is proportional to the diameter of particle size. Two

different nanopores (1000 and 2000) were selected for each formulation for the measurement of microparticles. However, for mucoadhesive liposomes nanopores 100, 200 and 400 nm were used for each formulation to get more reliable data. For mucopenetration liposomes calibration particles CPC 500 and nanopore 400 nm were used. Selection of nanopores for each formula was based on the expected size and particle size distribution.

2.4.4. Muco-adsorption of spray dried microparticles

For mucin adsorption calorimetric method was used in which periodic acid and Schiff reagent were used for the determination of remaining free mucin after its adsorption on the chitosan micro-particles. 0.1 g of Sodium metabisulphate was added to every 6ml of Schiff reagent and incubated at 37 °C till it turned into pale yellow color. 10µl of 50% periodic acid was added to 7 ml of 7% acetic acid to make periodic acid reagent. Periodic acid reagent (0.2ml) was added to sample and incubated for 2 hours followed by addition of 0.2ml of Schiff reagent room temperature and kept it for 30 minutes. Absorbance was measured at 555nm by UV spectrophotometer (Talaei *et al.,* 2011).

Mucin solution was prepared and 2 ml of micro-particle suspensions (F1 to F3) containing different amount of chitosan and crosslinking agent were centrifuged. Supernatant was discarded and pellets were dispersed into standard mucin solution separately and vortexed for 5 minutes and analyzed for free mucin concertation at predetermined time intervals (1.5, 3, 4.5 and 6 hours). Separate Eppendorf with mucin and micro-particles formulation was used for each time interval. The dispersion was centrifuged at 4000 rpm for 5minutes, supernatant was used for the measurement of free mucin by using the method stated above.

The concentration of free mucin was determined by using standard calibration curve of mucin in section 2.2.2. (Type-I with bound salicylic acid 9-17% from sigma Aldrich).

2.4.5. *In-vitro* drug release

Modified dispersion method stated by (Shazly *et al.,* 2008) was used to perform in vitro dissolution. Release was determined for all formulations (spray dried) (0, 0.5, 1, 1.5, 2, 3, 4 and 5 hours) at pH 1.3 and pH 4.5. However, *in vitro* release of freeze dried microparticles was determined at pH 4.5 only. In this method spray dried microparticles equal to 2 mg of drug was centrifuged at 4000 rpm for 5min. However, in case of freeze dried microparticles 2ml of suspension was used that contained different amount of drug corresponding to each formulation. Supernatant was discarded and pellets were transferred to new vials without disturbing the yellow colored drug at the bottom. Pellets were washed with water three times and suspended in 5ml of SGF at desired pH. After specified time 0.5ml of sample was withdrawn from and replaced by equal volume of fresh SGF which was filtered by 0.2 µm filter and then analyzed by HPLC analysis.

2.4.6. Particle morphology

Scanning electron photomicrographs of all microspheres were taken by microscope (Hitachi S3000N, Hitachi High-Technologies UK-Electron Microscopes, Wokingham Berkshire, UK). Small amount of each sample was attached to a 15mmdiameter aluminum specimen stub using double sided carbon adhesive tabs (Mikrostik adhesive, Agar Scientific), and the powder samples were sputter-coated with a thin layer of gold/palladium mixture to allow them to be electrically conductive. This was carried out using a Quorum Technology (Polaron range) SC760, whereby the samples are exposed to argon atmosphere at 10 Pa. The samples are coated at a process current of 18–20mAfor 2 × 105 s,with a turning through 180° in between.

2.5. Preparation of liposomes.

Liposomes were prepared by thin film hydration method using different ratio of cholesterol. Accurately weighed phosphatidylcholine, cholesterol (table 2.3) and cremophore ELP as co-surfactant were dissolved in 8 ml of chloroform. However, furazolidone (table. 2.3) was dissolved in 3ml of acetonitrile. After dissolving, chloroform-acetonitrile mixture containing drug and other constituents was transferred in 100 round bottom flask and organic solvents were evaporated by rotary evaporator (Buchi RE 121 Switzerland) at 60°C. After evaporation thin film on inner surface of flask was flushed with nitrogen gas for 10 min to remove the traces of organic solvent followed by rehydration of film by 5 ml tris buffer pH 7.4 at 53°C for half an hour.

Table 2.3. Composition of mucoadhesive liposomal formulations

Formulation	Drug (mg)	Lipid: Cholesterol (weight/mg)
L1	4	106:53
L2	5	106:53
L3	8	106:53
L4	4	106:10.6
L5	5	106:10.6
L6	8	106:10.6

2.5.1. Preparation of Mucoadhesive liposomes

Liposomes were prepared by conventional film hydration method previous section with coumarin-6 as fluorescent dye instead of furazolidone and then coated by chitosan for mucoadhesion. Equal volumes of liposomal suspension and 0.6% w/v solution of chitosan in 0.1% v/v glacial acetic acid were mixed at rate of 1 ml per

minute by continuous stirring at 25°C as shown in table 2.4. Resulting suspension was kept in a refrigerator overnight.

Table 2.4. Composition of fluorescence labelled liposomes

Formulation	**Composition** Coumarin-6: Lipid: Cholesterol	**Mucoadhesive liposomes** Chitosan
LC_1	2.5 µg:26.5mg: 2.5mg	0.6% (W/V)
NLC_1	2.5 µg:26.5mg: 2.5mg	0

LC_1: Liposomes containing coumarin-6 with chitosan; NLC_1: Liposomes containing coumarin-6 without chitosan.

2.6. Characterization of mucoadhesive liposomes

2.6.1. Mucoadhesion analysis by fluorimetry

Mucoadhesion analysis was performed at two different pH i.e. 1.3 and 4.5. Freshly excised stomach of sheep was cut into 2x2 cm slices. 100 µl of liposomal suspension (LC_1 and NLC_1, Table 2.4) was spread onto each tissue specimen separately. Each tissue specimen was placed in 5ml vial separately containing simulated gastric fluid (SGF) contained 0/1% pepsin (sigma P-700), 20.5mmol NaCl and 2.7mmol KCl, and 0.1M HCl adjusted at the required pH. Vials were put on a shaker incubator (50 rpm) at 37^0C. Tissue specimens were taken out at predetermined time intervals (0, 1, 1.5, 3, 4.5, 6 hours) and rinsed with 10ml of phosphate buffer saline (PBS) to remove un-adsorbed liposomes. Mucus was removed carefully and put in 5ml of 5M NaOH solution for 12 hours to dissolve mucus or any traces of tissue completely. Isopropyl alcohol (IPA) and acetic acid was added to samples to disrupt the lipid membrane and dissolve chitosan followed by centrifugation at 6000rpm for 10 min to extract coumarin-6 from liposomes. Supernatant was removed and intensity was measured by fluorimeter and percentage of dye recovered from stomach tissue was determined by using

calibration curve of coumarine-6. Non mucoadhesive liposomal suspension (NLC_1, Table 2.4) was used as a control for comparison and results of both formulations were compared.

2.6.2. Mucoadhesion analysis by fluorescence microscopy

2.6.2.1. Theory and Principles of fluorescent microscopy

The commonly known equation of energy and wavelength shows that energy is inversely proportional to wavelength (Max Plank's equation).

$$E = \frac{hc}{\lambda}$$ ---------- Equation 2 (Hellmut Fritzsche, 2007)

There are number of chemical substances that absorb the light with higher energy and low wavelength and emits the low energy and shorter wavelength light, such substances are known as florescent substances. These substances are irradiated with light of high intensity (photon), the electron in the outer shell excited to higher energy level where they dissipate the part of energy in molecular collision and after short time when they drop again to ground level they lose their energy in the form of photon with less energy and longer wavelength. The phenomenon is called Stork's shift (Kenneth, 2003) This lead to the basis of florescence. Number of compound and dyes are used for florescence and in current study coumarin-6 was used as a florescent dye to confirm mucoadhesion of liposomes on gastric mucus.

Coumarins have remarkable physicochemical properties which make them suitable for the use as florescent probes in the field of nano science, micelles and polymeric systems (Wagner, 2009) Coumarin dye has very poor florescent quantum yield therefore substitution of coumarin results in strong florescent coumarin compound emitting in blue green region (Acar et al., 2015). In florescent microscope these dyes absorb the blue light with short wavelength and release green light with longer wavelength (Valeur, 2001).

Working principles of florescent microscopy

Basic working of florescent microscope starts when the light with high energy usually mercury lamp put on to the material stained with florescent light through the filter. The filter separates blue light from rest of the components of white light. After filtration only blue light enters into system and reflected by dichroic mirror mounted in such angle that it bends the blue light to the specimen (figure 2.5). when this blue light falls on the specimen with florescent dye it excites the electron and when electron comes to ground state it emits green light. the portion where the dye is more confined is more prominent than rest of the area. In a current study the dye is accumulated in liposomes and appears as well defined small vesicles on stomach slices. Once the specimen emits green light it goes upward and pass through dichroic mirror that further filters blue light and only allow the green light to pass through. After this light pass through barrier filter that blocks any residual blue light and finally reaches to eye piece as shown in figure 2.5. Where the image is perceived as green specimen (Article library, 2016).

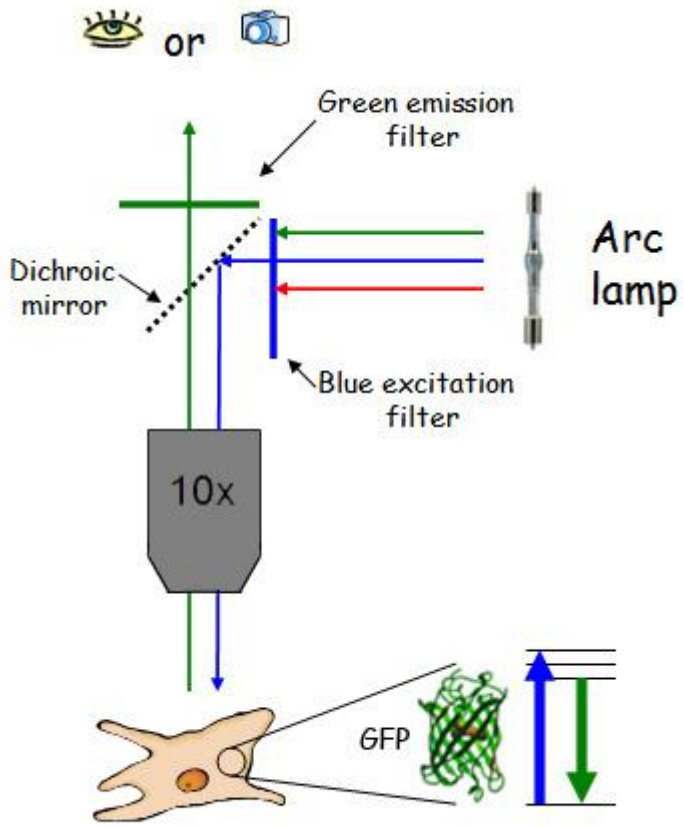


Figure 2.5. Line ray diagram of florescent microscopy (Microscopy and imaging department of Duke University and medical Centre).

2.6.2.2. Preparation of microscopic slides

Freshly excised sheep stomach was cut into 0.5 x 0.5 cm slices. Each slice was coated with 0.5 ml of mucoadhesive liposomal suspension contained coumarin-6 as florescence dye. Slices were incubated in 5ml SGF at required pH i.e. 1.3 and 4.5. Vials were put on a shaker incubator (50 rpm) at 37^0C. Tissue specimens were taken out at different time intervals over 6 hours. Then, tissue specimens were immediately snap freeze by using liquid nitrogen and OCT into a block. Each specimen was cut in 10µm slices by using cryostat (LEICA, Germany) and observed under fluorescence microscope.

2.6.2.3. Snap freezing and cryo-sectioning

Unfixed tissue of 2 cm diameter was put into tissue mold made up of aluminium foil before putting the tissue a drop of OCT (consisting of poly ethylene glycol and polyvinyl alcohol) was placed in the bottom of the mold and the tissue was fixed on top of OCT. Proper orientation was adjusted by using forceps ad tweezers. Tissues were than covered in OCT for few minutes. Special care was taken and the Aluminium molds were inverted before putting OCT to avoid air bubbles in labelled Aluminium molds. The molds were put on to the special scoop and immersed into the Dewar of liquid nitrogen at -190ºC for 2 minutes for snap freezing. OCT is viscous at room temperature, but freezes into a solid below −20°C. This embedding protects specimen from heating and also helps protect the tissue from drying during storage and supports the tissue during sectioning. Cryostat was the instrument used for the slicing of stomach embed in OCT. Cryostat is actually the microtome inside specialized freezing chamber where tempura can be kept at -20°C. The microtome is capable of cutting the tissue up to 1 micrometre thin slice but for this experiment 10µm was selected and the sections were picked up on a glass slide and viewed under florescent microscope.

2.6.2.4. Microscopic examination of slides

The slides and cover slips were washed with PBS before fixing. After washing prepared slides from cryostat were fixed with the mount and coverslip were mounted on top. A piece of filter paper was placed around the edge of the coverslip to absorb excess mounting medium. The slides with specimen and mount were kept overnight before viewing to fix the specimen onto the slides. For fixing regular nail polish was used on the sides of cover slip and kept for three minutes. Most common problem associated with fluorescent microscopic specimens is the fluorophores readily loses fluorescence upon excitation during viewing. This could be overcome by fixing the

slides with mount. Fixed slides were viewed under microscope. The microscope was turned on and the slide was place in inverted position with the specimen side facing towards the source of light. Ordinary light was used to focus the specimen to desired area of the specimen by using the 40X objective. Once it was focused the ordinary light was changed into fluorescent light by switching the shutter. Objective was also changed suitable for oil immersion. The slides were viewed and images were taken with the camera mounted on the microscope.

2.6.3. Encapsulation efficiency of mucoadhesive liposomes

Encapsulation efficiency of liposomes was determined by using two different approaches to compare the results of both methods. First method was refrigerated centrifugation and second the method was sephadex gel filtration.

2.6.3.1. Sephadex column elution method

For determination of encapsulation efficiency of liposomes 0.5ml of liposomal suspension was poured into sephadex column. After pouring 0.5 ml of liposomal suspension, 15 ml of liposomes were collected till the turbidity diminished and then 25 ml of free drug were collected into separate flasks. Liposomes were then treated with isopropyl alcohol (IPA) to disrupt the membrane. Both samples were then injected into HPLC for analysis

2.6.3.2. Refrigerated centrifugation method

The yellow colour of furazolidone was taken advantage of during refrigerated centrifugation. As furazolidone is lipophilic and have very low solubility in water i.e. 8-10mg/100ml, washing of supernatant three times with water after centrifugation could only remove few micrograms of drug. Liposomal suspension containing liposome bounded as well as free drug was centrifuged at 4°C for 10 minutes at 15000rpm and three layers were generated. Supernatant in first layer was discarded

and second layer of liposomes were removed by using micropipette without disturbing third yellow coloured layer of the un-entrapped drug. Liposomes were transferred into new Eppendorf and washed with distilled water followed by re-centrifugation. The cycle of washing and centrifugation was repeated three times and liposomal pellets without any free drug were disrupted with IPA and concentration was determined by HPLC

2.6.3.3. HPLC analysis of furazolidone

The HPLC analysis was performed on Agilent chem station LC-DAD, with UV spectrophotometer (USA). The column (4.60 mm×150 cm) was used for analysis. Gradient system of mobile phases was used that consists of 0.5% phosphoric acid water with pH adjusted at 7.4 mobile phase A and acetonitrile mobile phase B. The flow was maintained at 1 ml/min. Temperature of the column was maintained at 30°C and wavelength was kept at 320 nm. The gradient was run from 20 % to 80 % mobile phase B within 2 min and maintained for 7 min at 80%. Then, the mobile phase B retained to 80% aiming to go back to the initial concentration (80/20, A/B ratio) within 5 min. The HPLC System was maintained for 10 min prior to the next injection. Agilent ChemStation software was utilised to process peak areas and retention time. All the experiments were conducted in triplicates.

2.6.4. *In vitro* drug release of mucoadhesive liposomes

For *in vitro* drug release the same modified dispersion method stated by (Shazly *et al.,* 2008) was used described in section 2.4.5. The only difference was the time intervals 0, 0.5, 1, 1.5, 2, 3, 4 and 6 hours. The readings were taken in triplicate at each time point separately.

2.6.5. Microscopic examination of liposomes by using negative staining Transmission Electron Microscopy (TEM)

Morphology of the liposomes was observed by a TEM (Hitachi H7000 transmission electron microscope, Japan) using negative staining technique employing 1% (w/v) of sodium silicotungstate solution. A drop of the niosome suspension was applied on 400 mesh formvar copper grid (supplied by Agar Scientific, UK) on paraffin and the sample was allowed to adhere to on the formvar at room temperature (21 ± 1 °C) for 15 min. The excess suspension was removed and a drop of 1% (w/v) of sodium silicotungstate solution was applied for 5 min. The remaining solution was then removed. The obtained specimen was later observed under the TEM.

2.7. Preparation of mucopenetrative liposomes

Cationic, anionic and neutral liposomal formulations were prepared by using modified reverse phase evaporation technique (REV) stated by (szoka *et al* 1978). For cationic liposomal preparation didodecyldimethylammonium bromide 98% (DDAB) was used in the lipid mixture and for negatively charged liposomes dihexadecyl phosphate (DCP) was used. However, for neutral liposomes only 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were used. The composition and weight of lipid, cholesterol, charged moieties, furazolidone and NAC is given in table 2.5.

Table 2.5. Composition of mucopenetrative liposomal preparation. All components were weighed in milligrams

Liposomes		Lipid	Cholesterol	DDAB	DCP	Furazolidone	NAC	Pluronic
Positive	MP3	65	6.5	1	-	6	14	+
	MP6	65	6.5	1	-	6	14	-
Negative	MP2	65	6.5	-	1	6	14	+
	MP5	65	6.5	-	1	6	14	-
Neutral	MP1	65	6.5	-	-	6	14	+
	MP4	65	6.5	-	-	6	144	-

Calculated amount of lipid component DSPC and cholesterol along with charged moieties for anion and cationic liposomes were weighed and added in 50 ml round bottom flask and dissolved in chloroform. Furazolidone was separately dissolved in minimum amount of acetonitrile (6mg in 2ml) which was then added into lipid mixture. Solvent was then evaporated by rotary evaporator under reduced pressure for 15 min. At this stage the system was purged with nitrogen and the lipid layer was re dissolved into solvent for reverse phase vesicles. The solvent selected was the mixture of isopropyl ether and chloroform 2:1 v/v. The aqueous phase was added to the system already contained dissolved NAC and pluronic in required formulation with organic to aqueous phase ratio of 3:1.

The flask was sealed in nitrogen and the mixture was subjected to sonication for 20 minutes in sonicator water bath until the mixture became clear one phase dispersion. The mixture was kept for 30 min to check whether the system separates upon standing. Organic solvent was than evaporated under vacuum until the odour disappeared. Froth was not formed because low amount of cholesterol was used. 5-10 ml of additional buffer was added to evaporate the traces of organic solvent.

The liposomes generated were subjected to 5 freeze-thaw cycle by thawing the liposomes above transition temperature of DSPC to decrease the size and lamellarity of liposomes. However mostly the liposomal population is expected to be LUV when using REV approach. The suspension of the liposomes was frozen in -80ºC for 2 hours and then five cycles of freezing and thaw were performed. In a single round of freeze and thaw the suspension was kept into liquid nitrogen (-190 ºC) for 5 min followed by sudden thaw in water bath maintained at 40 ºC.

2.8. Characterization of mucopenetrative liposomes

2.8.1. Mucopenetration assay.

For mucopenetration six formulations were prepared by using the same approach of REV but instead of encapsulation the of drug, coumarin-6 was encapsulated in liposomes. Free dye was separated from entrapped dye by using refrigerated centrifugation followed by separating the green coloured layer from the white pellets. However, this time additional step of separation through sephadex G-50 column was performed by the use of eluting buffer in order to avoid any traces of free dye because very thin layer of 1mm mucin was used in analysis.

2.8.1.1. Rotating silicon method

Dowsen *et al* 1998 used reconstituted sigma porcine gastric mucin type I for diffusion assay of cationic nanoparticle at concentration of 60mg/ml. Similar concentration was used in this experiment. The suspension of 60mg/ml of mucin was prepared in distilled water.

Modified method of rotating silicon tube was used for mucus diffusion assay. Briefly, silicon tube of 8mm diameter and 4mm length was used. The tube was inclined and mucin suspension was added by micropipette carefully to avoid any air bubble. Three tubes were used for each formulation to estimate the diffusion after each hour.

Once it is filled with mucin then freshly prepared 20µl liposomal suspension contained entrapped florescent dye was added and the tube was incubated at 37ºC on shaker incubator at 50 rpm from 1 to 3 hours depends upon time of estimation.

At predetermined time interval (1, 2, and 3 hours) the tube was removed and snap freeze at -80º C for two hours. After freezing the tube was cut into 1 mm slice by precision cutter and Vernier calliper. Mucin (approximately 10µl in 1 mm slices) along with liposomal suspension (20µl in 1 mm slice) was removed and diluted down with 450 µl of 5M NaOH (to dissolve the mucin) and 20 µl Isopropyl alcohol (to disrupt the liposomal layer). The clear solution was analysed by fluorimetery and the intensity was used to calculate the percentage of diffusion in each mm slice by using calibration cure of coumarin-6 described in chapter 2 section 2.2.4. The experiment for all the formulations were performed in triplicate

2.8.2. Particle size determination and zeta potential of mucopenetrative liposomes

Particle size and zeta potential was determined by izon q-nano particle sizer and zeta analyser. (Izon q-nano, New Zealand)

2.8.2.1. Particle size analysis.

After 5 cycles of freeze and thaw the particles expected were less than 1 micron, therefore the nanopore membrane of 500nm was used that covers the range of particles size from 300 to 800nm. For both size determination and zeta potential calibration particles CPC 500 were used. For particle size analysis only one calibration was performed and the current blockage magnitude was measured which was directly proportional to the size of the particles. The detailed steps of maintaining the steady current and the use of calibration particles and particulate samples was discussed in section 2.4.3.

2.8.2.2. Zeta potential measurement

For zeta potential determination control suite V3.1 software was used that combine and analyse the balance between i). **Convection** which is the flow of carrier through the pore due to gravity and any applied pressure. ii) **Electro-osmotic flow** – the "pumping" of fluid through the nanopore due to the applied voltage and the surface charge of the pore and iii). **Electrophoretic mobility** that is movement of the particle through the liquid due to its attraction to the oppositely charged electrode and finally give the single value of surface charge. For charge analysis four different calibration runs were performed by using same nanopore and CPC particles but at pressure zero and 2 with the applied low voltage. The RMS noise was kept low throughout the run to generate the reliable data.

2.8.3. Encapsulation efficiency of mucopenetrative liposomes.

Encapsulation efficacy was performed by using refrigerated centrifugation technique. The liposomal suspension was centrifuged at 4 ºC for 10min at 10000 rpm. The supernatant was removes and pellets were washed with water to remove the non-encapsulated NAC. However, for separating the non- encapsulated furazolidone being yellow coloured compound was separated from the white pellet. Once the liposomal suspension was free from un-encapsulated drugs the pellets were suspended into 5ml of buffer at pH 7.4. After that 0.5ml was diluted down in 10ml and 1 ml out of that was mixed with IPA.

After disrupting the lipid layer sample was injected into two separate HPLC system of to determine the concentration of furazolidone and NAC. Once the concentration was determined the percent encapsulation of both drugs was calculated based on the original amount of the drug used.

2.8.3.1. HPLC analysis of N-acetyl cysteine.

The method used to determine the concentration of furazolidone throughout HPLC is already described in section 2.6.3.3. However, the method for NAC was explained by Ourique and co-workers in 2013. This method needs no derivatization which was the additional step for analysing NAC before discovery of this method. Briefly, the sample contain NAC and lipids were dissolved in mobile phase which was 0.05 M KH_2PO_4 and acetonitrile (95:5v/v) and the addition of 0.095% phosphoric acid v/v. 5 % acetonitrile stabilize the elution was injected into stationary phase column which was C -18 (250 x 4.6 mm; 5 µm, 100 Aº). The system used was Agilent chem station LC-DAD, with UV spectrophotometer (USA). 100µl of sample was injected and measurement was performed at 214 nm.

2.8.4. *In vitro drug* release of mucopenetrative liposomes

In vitro release of all formulations by using modified dispersion method stated in previously in this chapter section 3.2.1.3. The analysis in this chapter was performed up to 3 hours instead of six hours at predetermined time intervals (15, 30,45,60,90, 120 180 minutes). The percentage drug release calculated according to drug encapsulated.

2.9. Microbiological assay of mucoadhesive liposomal formulation (L5) against *E. coli*

2.9.1. Antimicrobial assay of furazolidone against *E. coli*

2.9.1.1. Inoculum size determination

For performing antimicrobial assay, the most important step is to optimize the size of inoculum which is recommended in CLSI guidelines. In order to get appropriate inoculum size there are two different approaches that are commonly used, i). McFarland turbidity method and ii). Spectrophotometer turbidity. For using the

second approach the growth calibration curve of turbidity v/s cfu/ml for every organism involved in assay is strongly recommended. The calibration involves following steps in general. Making of five to eight working dilutions of bacterial cell suspensions followed by absorbance reading by using pre-selected wavelength mostly 550 to 650 nm. After checking the absorbance, the cells are plated out from the suspension for viable bacterial count and the cell count are plotted against the absorbance reading (Sutton, 2006).

i). McFarland Turbidity Standards

This method is based on the visual estimation of the bacterial cell culture on the pre-defined scales of turbidity in comparison with McFarland Standard. McFarland Standard solution ranges from 0.5 through 10 mainly composed of Barium salts. Rapid approach and no incubation are the major advantage of this method. In contrast its disadvantage is manual interpretation of the turbidity and there is not fixed guideline to give the exact estimation. secondly, this approach is not universal and only applicable to specific microorganism (Sutton, 2011)

ii). Spectrophotometer

This method measures turbidity directly. But the use of this method is subject to certain limitation and need the calibration for each instrument before use. Because the results could be effected by width of slit, detector, condition of the filter, lamp and light source. Most commonly used wavelength ranges from 420 – 660 nm but it depends upon the type of microorganism. For using this approach, it is necessary that cell must be in physiological state of growth. This could be done by taking single well-isolated colony from the refrigerated stock followed by harvesting for overnight before analysis (Koch, 1994). Secondly, the instrument should be blanked with the broth before measuring the turbidity and the proper orientation of cuvette in the path of light is also important (Alupoaei ,2004). Light scattering techniques or turbidity method to estimate the biomass is rapid easy and non-destructive method. The

theory of this method is based on the scattering of light when bacterial culture comes in path of the light that is passed through bacterial suspension. The amount of the light that pass through the suspension is not considered however, all the light that is not absorbed is scattered and the amount of scatter is proportional to biomass (Gilbert, 1987).

There are two different approaches of making calibration curve for bacterial growth which depends on the type of bacteria. Making the dilution of overnight grown cell cultures and plating them out at the same time but because in overnight cultures, there are a lot of dead cells that increase OD but not the cfu/ml, and it is better to work with live cells. Therefore, appropriate way of making OD_{600} Vs cfu/ml curve is to grow the cells and measure while they grow.

For growth calibration curve of OD_{600} vs cfu/ml of *E. coli* was prepared from freshly growing cells. 100 µl of broth was inoculated with overnight culture and OD_{600} was adjusted to 0.05 for E. coli. Then, 200 µl of media was put into each well of the first row and absorbance at 600nm was recorded. At zero time point the $OD6_{00}$ of first cell was considered and the rest were disregarded. After that OD_{600} was recorded for the second well of first row after 30 min and then absorbance of 3^{rd} 4^{th}, ^{5th}, and 6^{th} well of the first row was measured after every hour till the reading became stationary.

At the same time all the wells of first column was filled with 180ul of media and the inoculum was serially diluted by putting 20 µl from first well to second well and so on all the way down to the sixth well. Then 20 µl from each well was plated onto LB agar plates in triplicate and incubated overnight and the colonies were counted after 24 hours of incubation. Same steps were repeated for each column after every hour. OD of 6^{th} column was greater than 0.5 nm therefore it was diluted 10 times and $OD_{600\ was}$ recorded, then the value was multiplied with 10 to get the original value. Finally, graph was plotted between OD and cful/ml after 24 hours of incubation.

To get the cfu/ml, colonies on each plate were counted, then multiplied with dilution factor and the amount plated was converted into ml.

For example, 124 colonies on the 10^{-5} plate: 124 (number of colonies) * 100000 (dilution factor) * 5 * 10 (because we plated 20 µl).

2.9.1.2. Antimicrobial susceptibility Testing

In order to check the efficacy of antimicrobial compound the most appropriate way is to check its minimum inhibitory concentration (MIC). It is a quantitative method to check the susceptibility of microorganism for the drug in question. This approach is very precise which tells the specific concentration of antibiotic needed to inhibit growth of bacteria. Antimicrobial susceptibility is commonly used to select the best antibiotic but in this study this approach was used to check the antimicrobial efficacy of furazolidone against *E. coli* and furazolidone and/or NAC against *H. pylori*. Number of method for antimicrobial assay has been developed but all of these methods are established on standard guidelines outlined by the European Helicobacter Study (Megraud *et al.,* 2013). The approach used in this study was broth microdilution method. It is one of the earliest and reliable method for checking the antimicrobial activity. The procedure involves two-fold dilutions of antimicrobial in broth (liquid medium) in the tubes that are inoculated with bacterial suspension of $1–5\times10^5$ CFU/mL (according to CLSI guidelines) overnight at 35°C. After incubation the tubes are examined for visible turbidity and the antibiotic concentration that shows no growth is concede red as MIC value. The major disadvantage is the use of excessive material and the visual interpretation of the MIC value. Therefore, microbroth dilution method is derived that is based on same principle but it is carried out in 96 well microtitter plate and the results are interpreted by micro plate reader. This method is applicable to all of the antimicrobial agent which is the advantage over other methods and therefore it could be used to identify the potential of novel antimicrobial agents.

According to calibration curve OD_{600} = 0.9 gives 1.75 x 10^8 cfu/ml.

(A laboratory Manual" says OD (600) of 1 is approx. 5x10^8 to 1x10^9 cells/ml in LB or other rich medium)

At this OD, adjusted inoculum was diluted down to 1:100 to achieve a bacterial load of 1.7 x 10^6 cfu/ml. From here 100 µl was picked that contained 1.7 x 10^5 cfu/ml in it and out of that only 50 µl was dispensed in microtiter plate so the inoculum of 50 µl contained 8.7 x 10 ^4 cfu/ml that satisfy CLSI recommendations. According to CLSI inoculum should be ~10^4cfu/ml for MIC.

First MIC of free furazolidone was calculated without liposomal formulations to calculate the cut-off point of minimum concentration of drug that inhibit bacteria after incubation of 24 hours. Dilutions of drug used was16µg/ml. According to drug solubility in water, stock solution was prepared by adding 18mg of drug in 500 ml and put on magnetic stirrer overnight followed by three consecutive episodes of sonication for 10 min in sonication water bath and then put on magnetic stirrer for 30 min on 60^0C to get the drug completely dissolved. 1 ml of stock solution contained 32 µg and 100 µl was dispensed into first well so it contained 3.2 µg /100µl. Remaining wells of first row were filled with 50 µl of broth. Then 50 µl from the first well was added into the next well containing 50 µl broth. Further 1:1 dilution were made until 6^{th} well. Remaining 50 µl was discarded from the 6^{th} well. 50µl of inoculum that contained 8.7 x 10 ^4 cfu/ml was added into each well of first row of 96 well plate up to 7^{th} and 8^{th} well that doesn't contain antibiotic and considered as +ve control Mix of 50 µl distilled water and 50µl of broth was added in 11^{th} well as blank. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm. The plate was incubated for 24 hours at 37^0C on shaker incubator as represented by table 2.6.

The absorbance was read again in the reader and the values was subtracted to check the effect of drug**.** All tests were performed in triplicate. The value that gave the sharp decline of absorbance was considered as MIC (Karina *et al.,* 2002)

Note:-

So, in the first well it was 1.6µg in 50 µl and when 50µl of inoculum was added it became 1.6µg in 100 µl or 16µg/ml.

Table 2.6. Representing the 96 well plate arrangement

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
D+I	D+I	D+I	D+I	D+I	I+M	I+M	M	M	M+D	M+D	B
D+I	D+I	D+I	D+I	D+I	I+M	I+M	M	M	M+D	M+D	B
D+I	D+I	D+I	D+I	D+I	I+M	I+M	M	M	M+D	M+D	B
B	B	B	B	B	B	B	B	B	B	B	B

D=drug, I=Inoculum, M=Media, B=Blank Cell

2.9.1.3. Cut off point and incubation time for liposomal preparation of Furazolidone against *E. coli*.

MIC of the drug was calculated as 16 µg/ml for free drug therefore this concentration was used as reference and the experiment was performed by using MIC (16µg/ml) and 2 fold dilutions of MIC (8 µg/ml) for liposomal formulation that was incubated at 1, 2, 4, 6 hours to pinpoint the least incubation time required by the minimum concentration of drug to inhibit the bacteria.

Encapsulation efficiency of liposomal formulation (L5) was 47% and initial drug added was 5mg in 5ml liposomal suspension (table 4.1) which implies; 0.47 X 5 = 2.35mg in 5ml of suspension was encapsulated. 2.5 ml of suspension was centrifuged and free drug was discarded (2.35/5 = 0.47mg/ml, therefore 2.5ml contained 1.175mg of encapsulated drug)**.** Liposomal pellets were suspended in

3.6ml of buffer (1.175mg/3.6ml buffer, therefore 0.326 mg/ml or 326µg/ml). Out of this suspension 1ml was diluted in 9ml buffer (326µg/10ml, that was equal to 32.6µg/ml).

1 ml of stock solution contained 32.6 µg and 100 µl was dispensed into first well so it contained 3.2 µg /100µl. Remaining wells of first row were filled with 50 µl of broth. Then 50 µl from the first well was added into the next well containing 50 µl broth. Further 1:1 dilution were made until 3rd well. Remaining 50 µl was discarded from the 3rd well. 50µl of inoculum that contained 8.7 x 10 ^4 cfu/ml was added into each well of first row of 96 well plate up to 4th and 5th well that doesn't contain antibiotic and considered as positive control. At the same time all the wells of column 1 to 5 except the top most row was filled with 90µl of media and then 10-fold serial dilution were made by putting 10 µl. From first well to second well and so on all the way down to the sixth well. Then 20 µl from each well was plated onto LB agar plates in duplicate and incubated overnight and the colonies were counted after 24 hours of incubation. Four different set of microtittre plates were used for four different time intervals to get incubation data at each time point.

2.10. Antimicrobial assay of mucopenetrative liposomal formulation MP1 against *H. pylori*

2.10.1. Growing of *H. pylori*

Direct plating of the culture from the stock is highly recommended because of difficulty of growth of *H. pylori* in broth. Basic requirement in the growing media are agar base, growth supplements like horse or sheep blood and selective supplements like antibiotics. Commonly used agar base are brain heart infusion and columbia agar. Concerning the growth supplement, it is mandatory to add blood or serum, which includes numerous nutrients (vitamins and oligoelements, etc.) which

enhance *H. pylori* growth. However, for broth culture fetal calf or bovine serum albumin are commonly used. Selective supplements used to inhibit the bacterial and fungal contamination and two commonly used selective media are for *H. pylori* are Skirrow's or Dent. Both of them are more or less similar contains, vancomycin to cover gram-positive cocci, trimethoprim and cefsulodin for gram-negative rods; and amphotericin B to prevent fungal contamination (Dent JC and McNulty CA 1988).

Xia et al. claimed that *H. pylori* can grow under aerobic condition but it generates low cell count and small colonies (Xia *et al.,* 1994). However, anaerobic or microaerophilic conditions are ideal for growing H. pylori (Bury-Moné *et al.,* 2006) Number of systems are available that produce microaerophilic atmosphere. One of them is anaerobic chamber that mix three gases N2, Co2 and oxygen in appropriate proportion but this lab facility is very expensive (Anoxomat, MART Microbiology BV, Lichtenwoorde, The Netherlands). However, the use of anaerobic jar with gas generating packs is alternate cost effective and reliable method but this approach requires the gas pack changing after every 24 hours. the other key factor is the temperature of incubation and 37°C is considered optimum for its growth. primary culture. Primary culture with optimum conditions produce colonies from 4 – days (Zwet *et al.,*1994) but for sub culturing only 2-3 days are enough to produce colonies. For broth culture BHI is preferable with fetal calf and to obtained active bacterial cell mass subculture with incremental increase of media from 5 ml to 500 ml is recommended. Number of methods commonly used in practice for the isolation, in vitro growth and antimicrobial culture sensitivity for this bacteria has been established. However, the approach of in vitro assay against *H. pylori* depends upon the antibiotic used. There is lack of internationally agreed consensus for the method of antimicrobial susceptibly of *H. pylori*. But commonly used approach is phenotypic approach.

2.10.1.1. Reconstitution of bacterial strain

To reconstitute 0.5 ml of BHI broth supplemented with 5% fetal calf serum was added to ampule followed by dissolving the content carefully without producing any aerosol. The broth was kept for 10 minutes to allow the bacteria to rehydrate. Then sub-cultured on blood and chocolate agar plates to obtained solid agar plates supplemented with DENT supplement. For broth culture the fresh BHI broth contained 5% calf serum and DENT was inoculated with reconstituted strain. All the plates and tubes were incubated in 2.5L oxoid anaerobic jar with campyGen gas pack at 35ºC for 7 days shown in figure 2.6.

Figure 2.6: Oxoid Anaerobic jar with campyGen gas packs and agar plate

2.10.1.2. Lawn growth of *H. pylori*

Stored blood plates were removed from cold room and allowed them to cool at room temperature. 10ml of fresh BHI broth was taken into test tube. Patched *H. pylori* grown in previous section was suspended into BHI by using cotton swab. Then the re-suspended cells were evenly spread onto surface of agar plate by cotton swab shown in figure 2.7. Plates were allowed to dry and then incubated at 35ºC for three days in anaerobic jar.

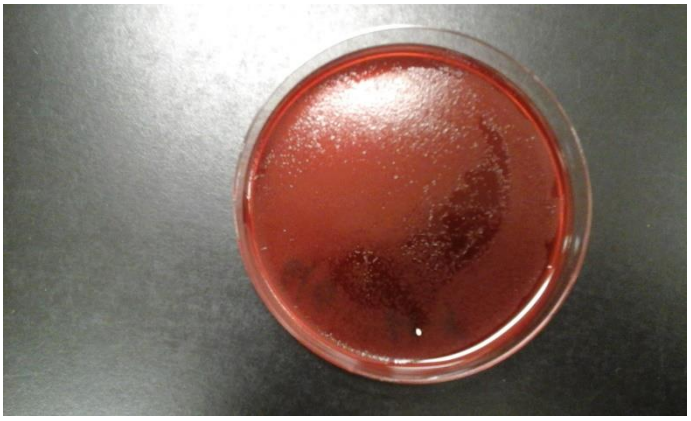


Figure 2.7: Lawn growth of *H. pylori*

2.10.1.3. Individual colonies of *H. pylori*

Removed lawn growth by using sterile cotton swab and suspended into 1ml BHI broth. Took 100µl of the bacterial suspension by using pipette on blood and chocolate agar and spread entirely across the surface by using glass spreader as shown in figure 2.8. The plates were incubated at 35ºC for three days.

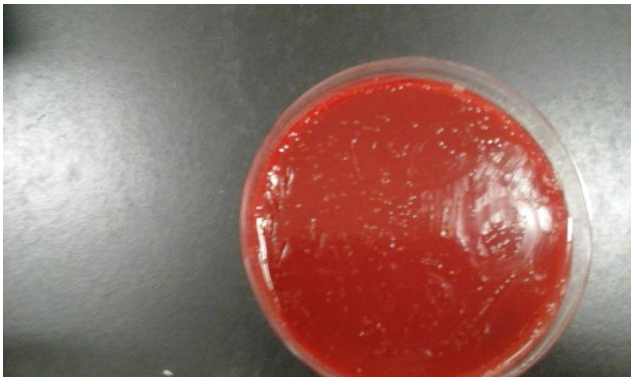


Figure 2.8: Individual colonies of *H. pylori* on blood agar plates

2.10.1.4. Quantification of bacterial growth

In order to check the activity of antimicrobial agent the predetermined concentration of bacterial suspension is the most important requirement e.g. in broth dilution method according to CLSI guidelines the bacterial suspension should be 1-5 x 10^4 cfu/0.1 ml. Therefore, it is necessary to quantify the bacterial suspension. Number of methods are available to quantify the bacterial growth. In this chapter turbidity method and plate count method was used. Bacterial cell growing in a broth make it

turbid, that directly effects the amount of the light absorbed which can be used to estimate the number of bacterial cells. Spectrophotometer contains the photocell in which the light absorbed by bacterial suspension is measured. Light absorbed is directly proportional to the cell concentration.

2.10.1.5. Standard liquid growth of *H. pylori*

The cells from lawn growth were taken by using sterile cotton swab and suspended in 1 ml of BHI broth. Pipetted 100µl into fresh test tube contained 10ml of BHI supplemented with FCS and DENT. The liquid media was swirled to distribute the cell uniformly and incubated at standard growth conditions for 2 days.

2.10.1.6. OD controlled growth and calibration curve

For making growth quantification calibration different $OD6_{00}$ were selected and colonies were counted at each OD. The aliquot from the liquid culture prepared in previous section 2.10.1.6 was removed and obtained the OD 6_{00}nm. The value obtained was adjusted for pre-determined $OD6_{00}$ values (0.05, 0.1, 0.3 and 0.5) by using the formula.

$$\#\ \text{of ml} = \frac{target\ OD(600) x\ final\ culture\ volume}{OD(600) of\ starting\ culture}$$

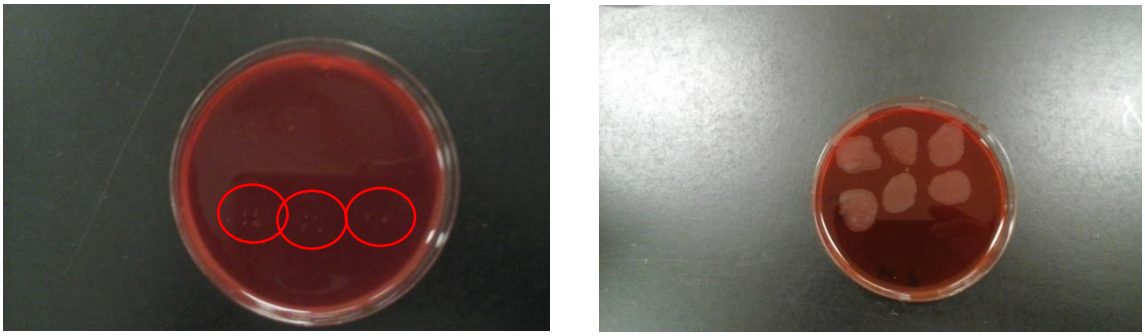
1. # of ml = amount of liquid taken from original culture
2. Final culture volume was always kept 2 ml.
3. $OD6_{00}$ of starting culture was 0.53

For example, to make OD600 at 0.05

$$\#\ \text{of ml} = \frac{0.05\ x\ 2}{0.53} = 200\ \mu l$$ of start culture in 1800µl of fresh media adjusted OD to 0.05.

By adjusting the $OD6_{00}$ at each point the culture tubes were put in anaerobic jar with campyGen gas packs to avoid the oxygen. The amount of broth for serial dilutions

was calculated (0.9ml) and put in to blank vials. Jar was opened and put each OD adjusted culture into blank vial and 4 serial dilutions were made to obtained 10^{-1} through 10^{-4}. 10µl from each vial was plated onto specified position of the plate and plates were incubated in anaerobic jar for 3-5 days at 35ºC. Spot plating technique was used therefore two plates were used for 4 dilutions of each OD as shown in figure 2.9. (Jeannette and Scott, 2004)



a). b).

Figure 2.9. Spot plating technique for different dilutions from a). 10^{-1} through 10^{-6} and b). from 10^{-1} to 10^{-3}

2.10.2. Minimum inhibitory concentration of furazolidone and NAC against *H. pylori*

Minimum inhibitory concentration (MIC) is the gold standard method to check the susceptibility of antimicrobial agent against microorganisms. It is defined as the minimum concentration of antimicrobial agent that inhibits the visible growth of bacteria after overnight incubation. However, in micro dilution method by using plate reader the concentration that gives substantial drop in absorbance is considered as MIC. First MIC of free furazolidone and NAC was calculated without liposomal formulations to determine the cut-off point of minimum concentration of drug that inhibit bacteria after incubation of 24 hours. Dilutions of drug used was 32µg/ml for furazolidone and 28mg/ml for NAC but in MIC assay concentrations of both drugs were prepared in 100µl of broth separately as shown in table 2.7.

From growth calibration of *H. pylori* OD_{600} = 0.5 gives 1.1 x 10^7 cfu/ml. At this OD, adjusted inoculum was diluted down to 1:10 to achieve a bacterial load of 1.1 x 10^6 cfu/ml. From here 50 µl was picked that contained 5.5 x 10^4 cfu in that dispensed in microtiter plate so the inoculum of 50 µl contained 5.5 x 10^4 cfu

NOTE: By adding 50 µl of drug, the final volume became 100 µl, therefore it will be 5.5x 10^4 cfu/0.1ml

According to CLSI inoculum should be ~10^4 cfu/0.1 ml for MIC. (Marie. B Coyle, 2005). According to furazolidone solubility in water, stock solution was prepared by adding 16 mg of drug in 500 ml and put on magnetic stirrer overnight followed by three consecutive episodes of sonication for 10 min in sonication water bath and then put on magnetic stirrer for 30 min on 60^0C to get the drug completely dissolved. However, 280 mg of NAC was dissolved in 10 ml of water simple stirring. 100 µl of furazolidone was dispensed into first well of row A. 100 µl of NAC was dispensed into first well of row B therefore it contained 3.2 µg /100µl for furazolidone in row A and 2.8 mg /100µl of NAC in row B. Remaining wells of first and second were filled with 50 µl of broth. Then 50 µl from the first well was added into the next well containing 50 µl broth. Further 1:1 dilution were made until 5^{th} well in each row**.** Remaining 50 µl was discarded from the 5^{th} well. 50µl of inoculum that contained 5.5 x 10 ^4 cfu/ml was added into each well of first and second row of 96 well plate up to 5^{th}. However, 6^{th} well that doesn't contain antibiotic and considered as +ve control. Mix of 50 µl distilled water and 50µl of broth was added in 7^{th} well as blank. The plate was incubated for 24 hours at 37^0C on shaker incubator under microaerophillic conditions in anaerobic jar. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm. All tests were *performed in triplicate.* The value that gave the sharp decline of absorbance was considered as MIC (Karina *et al.,* 2002)

Table 2.7. Concentration of drug in 100 µl of inoculum and media.

Drug	1	2	3	4	5	6
Furazolidone (µg/ml)	1.6	0.8	0.4	0.2	0.1	Control
NAC (mg/ml)	1.4	0.7	0.35	1.45	0.87	

2.10.3. Time killed curve mucoadhesive formulations (L5, F2) and mucopenetrative formulation MP1 against *H. pylori*.

The time killed plot was made to check the actual time needed by the formulation MP1, free drug without formulation alone and in combination of 1% of NAC to kill the predetermined concentration of bacterial culture suspension. Two other formulations L5 (mucoadhesive liposomal formulation) and F2 (mucoadhesive spray dried microparticles formulation) were tested against *H. pylori*. For both of formulations MIC x 8 ±1% was considered as an optimum concentration of formulation bound drug. However, one additional concentration of MIC X 6 of furazolidone was also investigated for mucoadhesive liposomes to confirm the results. LC1 with the similar ratio of lipid and cholesterol to L5 was used as control for mucoadhesive liposomes to check the effect of chitosan on activity. Second control was blank microparticles without furazolidone which was similar to F2 without having furazolidone in it. This was used to check the effect of glutaraldehyde and chitosan on time killing curve of *H. pylori*. The test was conducted up to three hours for MP1 and six hours for L5 and F1 because the retention time of mucoadhesive formulation in this study is 6 hours in stomach.

2.10.3.1. Dilution of liposomal bound furazolidone and NAC

Encapsulation efficacy of mucopenetrative liposomal formulation MP1 is 58% for furazolidone and 51% for NAC in 5ml buffer and the initial amount of furazolidone

and NAC was 6mg and 14mg respectively. Liposomal formulation was centrifuged at 9500 rpm for 10minutes at 4ºC resulted in three separate layer. Supernatant was discarded that contained NAC and the white liposomal layer was separated carefully from yellow layer of furazolidone as shown in figure 2.10. Liposomes were washed with water and suspended in fresh 5ml of buffer.

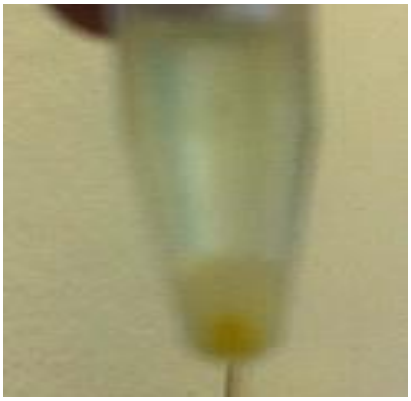


Figure 2.10: Three separate layer of liposomal formulation after centrifugation

After resuspending in 5ml fresh buffer each ml of suspension contained 0.696 mg of furazolidone and 1.42mg of NAC. 1ml of liposomal suspension was taken and diluted up to 10ml therefore each ml now contained 69.6 µg and 142 µg of furazolidone and NAC respectively. 50 µl of suspension was dispensed into microtiter plate that contained 3.48µg and 7.14µg of furazolidone and NAC respectively. After this 50µl of bacterial inoculum was added in the same well therefore the amount of drugs was in 100µl final volume. Hence the concentration used were 34.8 µg/µl of furazolidone that is approximately equals to 8 x MIC of furazolidone and concentration of NAC was 7.14µg/ 100µl that is 1% of minimum inhibitory concentration of NAC.

2.10.3.2. Procedure

Bacterial concentration of 5.5 x 10^4 cfu/ml was used in time killed study. Briefly, the culture of *H. pylori* containing 5.5 x 10^4 cfu/ml was incubated with different concentration of free and liposomal bound furazolidone. 1.25 % of MIC of NAC was added to all concentrations of furazolidone to study the modulation effect of NAC on

MIC of furazolidone and to check the synergistic effect of both antibiotic on killing time of *H. pylori*. Similar concentrations were used without 1.25% NAC as control to confirm the effect of NAC. The cultures were incubated at 37^0C for 1.5, 2, 3, 6 and 8 hours under microaeorophillic conditions. Concentration used for free furazolidone were 1/2, 4, and 8 x MIC with 1.25 % of NAC that was 87 µg.

After each time point tenfold serial dilutions were prepared with broth and 20µl were plated onto blood agar in triplicate. The CFU for each time point and each dilution were counted after 24 hours of incubation and the log of CFU were plotted against time for each concentration used. Single colonies from each plate of triplicate set were counted and average of the count was used to back calculate cfu/ml.

CHAPTER THREE

CHARACTERIZATION OF FURAZOLIDONE-CHITOSAN BASED MICROPARTICLES REGARDING THEIR DRUG RELEASE AND MUCIN ADSORPTIVE PROPERTIES

3.1. Introduction

This chapter is focused on the preparation and characterization of microparticles by using two different approaches in order to deliver the candidate drug to the stomach. The basic aim of the experiment behind this chapter was to keep the formulations in the stomach for an extended period of time that would be sufficient for completely killing the bacteria.

3.1.1. Spray Drying

Spray drying is a commonly used technique to transform liquid in the form of solutions, suspensions, emulsions, slurry pastes or melts (Rabbani *et al*.,2005; Gómez-Gaete *et al*., 2008; Li etal.,2010) to dry powder form. The whole process of spray drying is completed in four successive steps starting from atomization that is the liquid feed step followed by spray drying in hot air and then the final two steps include the formation of particles and collection of powder.

Figure 3.1. Laboratory scale bench top Spray dryer Buchi B-290 (commercial Boucher Masontechnology).

First of all, moisture is evaporated from the feed mixture by drying medium that is generally hot air. This process continues till the desired moisture content is achieved then the spray medium and the drying medium (air) are separated. The fluid is fed into the drying chamber by a peristaltic pump through an atomizer (Lee *et al*.,2011). This generates small droplets which are dried by drying medium through solvent evaporation and form dry particles as shown in figure 3.2. (Elversson *et al*.,2003; Fatnassi *et al*., 2016). Different energies are involved in conversion of feed mix into small droplet. if higher energy is used smaller droplet could be produced. In the classic Buchi B-290 lab scale appartus 100ml of feed mix can be converted into 8 x 10^8 particles of 25 microns. The separation of medium is carried out by a cyclone separator that dispense the dry powder into the glass collector separated from the hot air.

The second most important parameter after atomization is contact of air. There are basically two ways of drying air. The first is co current and the other is counter current. in co current both the droplets after atomization and air flows in same direction and it is used for heat labile products. However, in counter current droplets are vaporized by air flowing in the opposite direction (Mujumdar,2006; Raffin *et al*.,2006). In Buchi B-290 a wheel atomizer is used that converts the feed mix into a fine mist using the co current contact approach as shown in figure 3.1.

In third phase after contact with hot air the saturated vapors form a film on the surface of droplet are immediately dried. The factors that are responsible for efficient drying are high temperature, intense heat and moisture gradient. The rate of hot air flow is regulated by aspirator, increase aspirator rate introduces fast air flow in the system and vice versa.

Finally, for separation the chamber is designed in such a way that the air flow keeps the particles for enough resident time in chamber till the sufficient drying is achieved,

the particles are cooled down by sudden evaporation of solvent and once they equilibrate to the outlet temperature they are separated into collection chamber.

3.1.1.1. Lab scale mini Buchi B-290 spray dryer.

Most commonly used lab scale spray dryer is B-290 that could perform spray drying from 50ml to 1 litre in one hour as shown in figure 3.1.

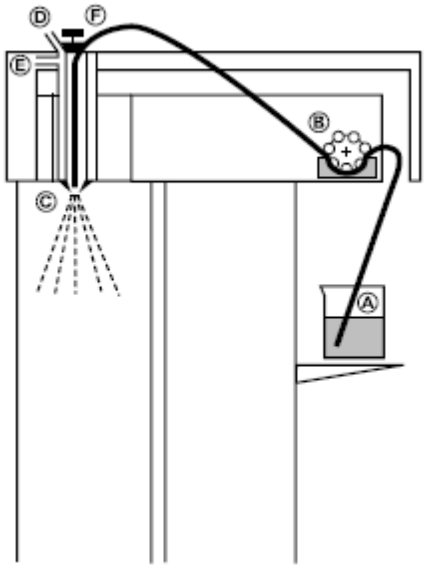


Figure 3.2. Basic layout of mini spray dryer B-290 with the description. a). Sample feed, b). peristaltic pump, c). Two fluid nozzle, d). Compressed air supply, e). Cooling water connection, f). Nozzle cleaning device. (BÜCHI Labortechnik AG)

Advantages of spray drying

1. Products obtained by spray drying are chemically and physically more stable than liquid formulations.
2. Final step is drying in most of the common approaches used for microparticles formation which is performed separately as an additional step but in spray drying it is done along with mainstream process. (Baras *et al*.,2006; Ren *et al*.,2013; Bowey *et al*.,2013)
3. By using spray drying approach polymeric nanoparticles and microparticles (MPs) can be produced efficiently with remarkably better flow properties and

final characteristics, high encapsulation efficiency (%EE) and loading capacity (%LC) provided that initial parameters are optimized properly (Mu *et al*.,2005; Stulzer *et al*.,2009)

4. The co-current mechanism of dry air makes the product at 15°C below the outlet temperature and therefore it could be used for encapsulation of antibiotic, peptide and proteins (Wan *et al*., 1992).

5. Spray drying approach produces shell-like structure around the polymer that helps in manufacturing of controlled-release products (Patel, 2009).

Disadvantages

Major drawback of using this technique is low yield in lab environment as compared to industrial scale where it can reach even up to 100% (Alejandro and Katia 2015). Lab scale yield usually ranges from 20 -70 %. This low process yield is attributed to different factors. Improper selection of initial parameters, including use of solvent that mainly increase the moisture content and the product stick to the wall of glass chamber that leads to low yield of product. Secondly, the small size particles flow out by the cyclone separator with air and presents low process yield. The rate of aspirator for separation and the combination of filter system can prevent this problem

3.1.2. Freeze Drying

Freeze drying is defined as removal of ice or other frozen solvents by sublimation and the removal of bound water molecules through the process of desorption. the process is also referred as lyophilization.

The theory of freezer drying is based on the sublimation and triple point where the ice is directly converted into vapors without intermediate liquid phase. This could be achieved if the samples are frozen followed by reducing of pressure below triple

point and energy is provided by heating (below the critical point) the sample to transform the sample into vapor phase as shown in figure 3.3.

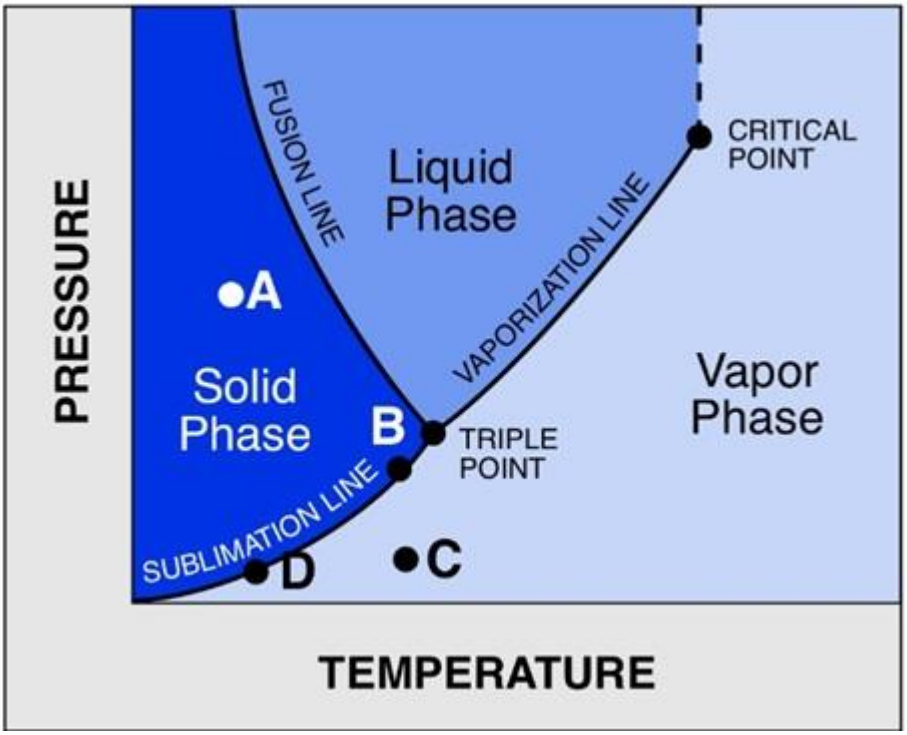


Figure 3.3. Typical triple phase sublimation diagram for freeze drying. (Nireesha *et al.,* 2013)

Freeze drying process is not as simple as it seems. Number of factors limits the versatility of freeze drying process. It is time taking process and needs series of optimization for the factors involved to get the final product with desired characteristics. Freeze drying is actually extended process that takes sublimation into account in addition to other steps. Basically freeze drying is consisting of three steps.

- Freezing
- Primary drying
- Secondary drying.

Freezing is the primary step and it is of paramount importance because the crystal structure of water and solute in frozen product greatly influence the final product. freezing make the component static in the sample and prevent formation of foam

during vacuum (Adam, 2007) secondly it forms the crystal structure of solution and suspension Number of factors must be taken into account while freezing the product. The freezing should be done at atmospheric pressure and the sample must be frozen below its eutectic point usually -20ºC, the rate of freezing is another parameter (Labconco, 2010)

During freezing when the temperature reaches to zero some of the particles changes into ice and this phenomenon is called nucleating. once started the ice crystals grows and spread into solutes. This intention of nucleating process should ideally make the spatial arrangement of all the components like initial suspension. Size of the crystals formed while freezing also effect the process of freeze drying because large crystals are difficult to dry during freeze drying because of the thick crust form at the surface presents more difficulties with regard to freeze drying, in that a thick crust forms at the surface (Nireesha *et al.,* 2013). One the other hand the small crystals are more homogeneous and easy to dry. The growth and size of crystals depends upon the nature of initial sample and rate of freezing (Labconco, 2010).

End of freezing phase is consolidation stage which refers to the holding time of the sample in the frozen state to make sure that it is adequately frozen. usually, this consolidation phase takes a long holding time. the quicker and efficient alternate is annealing that is not commonly practiced. Annealing could be achieved by increasing the temperature during freezing that transform the residual solute to recrystallize that was not crystalize in the first place. Annealing during freezing phase can form the more porous cake and the final product with very low moisture content.

There are two different ways of freezing the samples either in the freezer and transfer of frozen sample to freeze dryer but the disadvantage of this method is the

chances of melting of sample during transfer. However, in the second method the freezing is done in the freezing chamber of the freeze dryer but in this approach freeze dryer is used for extended time that decrease the life of bench type freeze dryer. Primary drying occurs through sublimation process. At triple point, all three phases coexist and while maintaining the freezing temperature the vapor pressure of water below sub atmospheric pressure convert ice into vapor state and leave the product dry and porous. vacuum is applied during this stage that reduce the air pressure above the product and encourages sublimation. In order to achieve the effectual primary drying the partial pressure of vapors in surrounding chamber must be lower than the partial pressure of ice at same temperature. Secondly, the energy in the form of heat that is introduced into sample must be lower than eutactic temperature. During freeze drying process pressure and temperature gradient must be maintained from sample to vacuum pump via condenser. The temperature of the sample must be higher than condenser to drive the water out of the sample. Secondary drying occurs mainly through desorption of water from the sample. After primary drying no more ice exists in sample and the temperature of the intermediate product started to rise and equilibrates with shelf temperature. At this stage the intermediate product still contains moisture content that varies from 5-7%, The aim of secondary drying is to bring the moisture content to acceptable limits 1-3% in the final product (Greiff, 1997). This process takes 30-40% of the time required to complete the complete freeze drying process. Relative humidity and the shelf temperature determines the effectiveness of secondary drying. Increase in the shelf temperature and high vacuum reduce the vapor pressure and relative humidity in the system that speed up the secondary drying. However, on the other hand if the temperature of product in the chamber is reduced below the temperature of condenser vapor pressure in the system is increased that makes the moisture reabsorbed into the sample.

Bench top freeze dryer majorly consists of the following components

- Refrigeration System
- Vacuum System
- Control System
- Product Chamber or Manifold
- Condenser

Refrigerated system works for freezing the sample to ice and it also maintains the low temperature of the chamber. In vacuum system the vacuum pump is the primary component that is attached to airtight condenser and the chamber. Control system consist of very sophisticated sensors that control the temperature and the pressure throughout the process. In modern systems they are designed to carry out the effective freeze drying process by changing at different time scale during the process. However, they can be adjusted manually to control the conditions depends up on the sample used and desired product. the product chamber consists of the shelf where the whole process takes place. Condenser system at the end is maintained at the low energy level and the vapor produced during sublimation are condensed back into ice on the condenser that is removed manually from the system at the end of the cycle.

Researchers sometimes suggest spray-drying technique as an effective alternative method to freeze-drying (Takashima *et al*., 2007). Because of its short duration that lasts in minutes instead of freeze drying that take days. Secondly, it can lead to complete dry product with no moisture content instead of freeze drying. however, from both spray-drying and freeze drying techniques the dehydration could be achieved that increased the stability and life span of dry product as compared to liquid form (Tshweu *et al*., 2013).

3.2. Results and discussion

3.2.1. Characteristics of spray dried microparticles

Characteristics of micro-particles prepared by spray dried method are listed in table 3.1 and 3.2. Particle size ranges from 1.2 µm to 2.5 µm (table 3.1). By adding the cross linking agent particle size was decreased which is in agreement with study conducted by (Genta *et al*., 1998 and P. He *et al*., 1999). Formulation F3 with highest amount of glutaraldehyde having particle size of 805/1424 nm. However, particles size was decreased by reducing the amount of glutaraldehyde. There were no significant number of particles observed by using 300 and 400nm nanopores.

Table 3.1. Particle size of formulation by using different nanopores. For formulation composition refer to table 2.2 in section 2.3.1, chapter 2

Formulation	Nanopore (nm)	Particle size (nm)			
		Maximum	Minimum	Mode	Mean
F1	1000	3640	987	**1137**	1616
	2000	4248	1866	**2597**	2377
F2	1000	2195	723	**939**	1163
	2000	4240	1688	**1818**	1860
F3	1000	2407	670	**805**	990
	2000	3386	1274	**1424**	1729

In spray drying there are initial variables that determine the characteristics of final products in terms of particles size, morphology and process yield. These parameters are inlet temperature, feed rate flow rate, pressure and aspirator rate.

Outlet temperature could not be controlled directly. However, it is very important parameter to be considered for characteristics of final product. Therefore, some initial parameters could be adjusted to control the outlet temperature. Increase in aspirator rate, air humidity and inlet temperature increase the outlet temperature because more heat and energy enters into system and increase the outlet temperature. On the other hand, increase in feed rate reduces outlet temperature due to evaporation of excessive solvent in short time (Schoubben *et al*., 2010; Park *et al*., 2013)

Similarly, the humidity in final product can be regulated e.g. increase in feed rate and aspirator increase the moisture content. However, increase inlet temperature, use of solvent instead of water reduce humidity. The particle size that is important parameter in any research could be controlled if the initial parameters are selected with care. Inlet temperature, and aspirator has minor or no effect on particles size. But the use of organic solvent instead of water decrease the particles size because of less surface tension. However, increase in the feed rate increase the particles size due to dispersion of more liquid in less time. Efficient fluid dispersion and fast drying is achieved by increase in flow of air which intern decrease the particles size (BÜCHI Labortechnik AG, 2002).

Regarding the particles morphology, quick solvent evaporation with organic solvent can produce the particles that are more porous due to shorter time for the droplets shrinkage (Littringer *et al*., 2013; Wan *et al*., 2013)

Finally, the process yield that untimely plays the role in cost and research parameters like encapsulation activity and in vitro release study is also worth mentioning. High inlet temperature increase produces the final dry product and prevents the sticking on cyclone chamber. Increase aspirator gives better separation in cyclone leads to high process yield. Use of organic solvent instead of

water gives good yield because of no hygroscopic solvent residue. Therefore, all the parameters were optimized in this study to get the final product and organic solvent was used instead of water to improve the characteristics of the final product and increase the yield.

According to study conducted by (Bilancetti *et al*., 2010) increase in viscosity of the solution, resulted in low recovery. Similarly, in the current study by increasing the amount of chitosan in formulation the yield decreased. F1 having chitosan to drug ratio 1.5: 1 showed process yield of 49% in contrast to F2 having 1:1 ratio with process yield 57.3% indicated in table 3.2.

The selection of solvent also affects the process yield. Furazolidone being poorly water soluble drug was not completely dissolved when only water was used as solvent before spray drying in case of formulation F3 that gave 44.3% process yield. However, the yield was increased in F1 and F2 (shown in table 3.2), when drug was first dissolved in acetonitrile to make it completely soluble. The use of co-solvent described by another study bring forth two possibilities, solvent facilitates evaporation process that in turn decreases the time and energy required and shows positive effect on the percentage recovery. It was reported in another study that selection of solvent type influences the structure of resultant microparticles which influences different characteristics (Marjana Du ¨rrig *et al*., 2011)

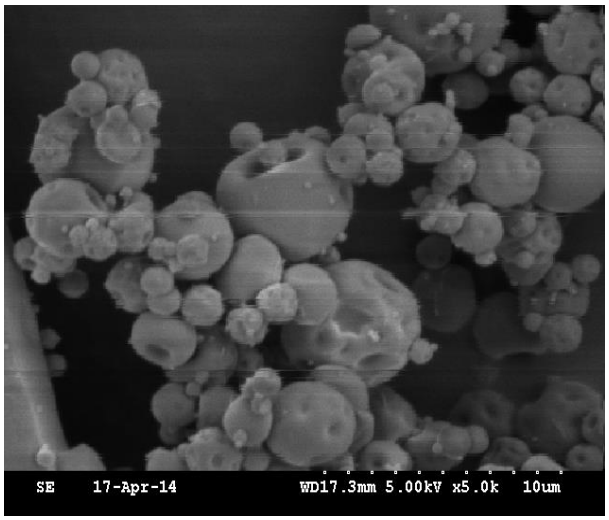
Similarly, drug content of micro-particles dependent on selection of solvent i.e. in F1 and F2 when acetonitrile was used as co-solvent with water the drug content was 60 and 66.6% respectively. But in case of F3 when water was only used as solvent drug content was only 51.5% as shown in table 3.2. In the current study, process yield did not vary much with different amount of glutaraldehyde however it was mainly influenced by selection of solvent.

Table 3.2: Percent drug content and process yield of formulations. For formulation composition refer to table no 2.2 in section 2.3.1, chapter 2

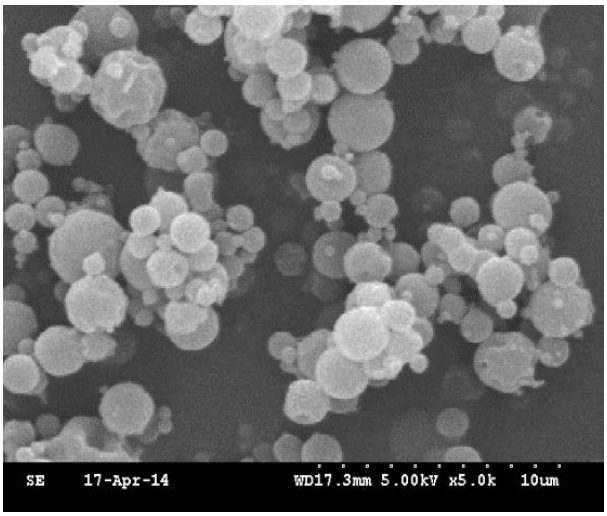
Formulation	Drug content (%)	Process yield (%)	Zeta potential (mV)
F1	60 ± 0.34	49.2 ± 3.1	+36
F2	66.6 ± 0.65	57.3 ± 3.9	+25
F3	51.5 ± 0.76	44.3 ± 2.8	+14

3.2.1.1. Particle morphology

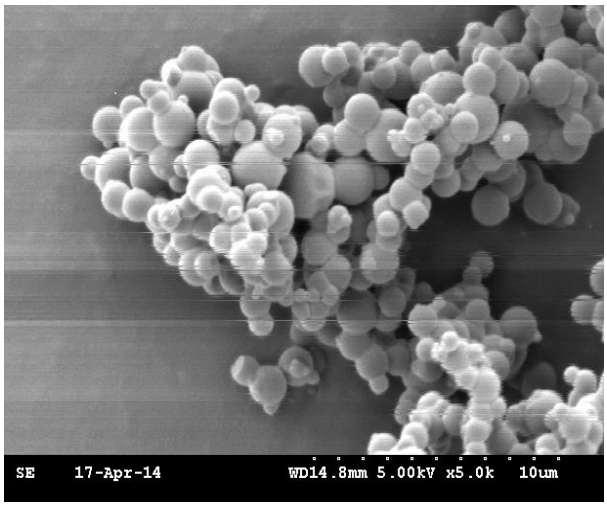
The microparticles were spherical and smooth as shown in figure 3.4 but the morphology of microsphere with low concentration of glutaraldehyde showed slightly wrinkled and somewhat distorted surface**.**



a). F1



b). F2

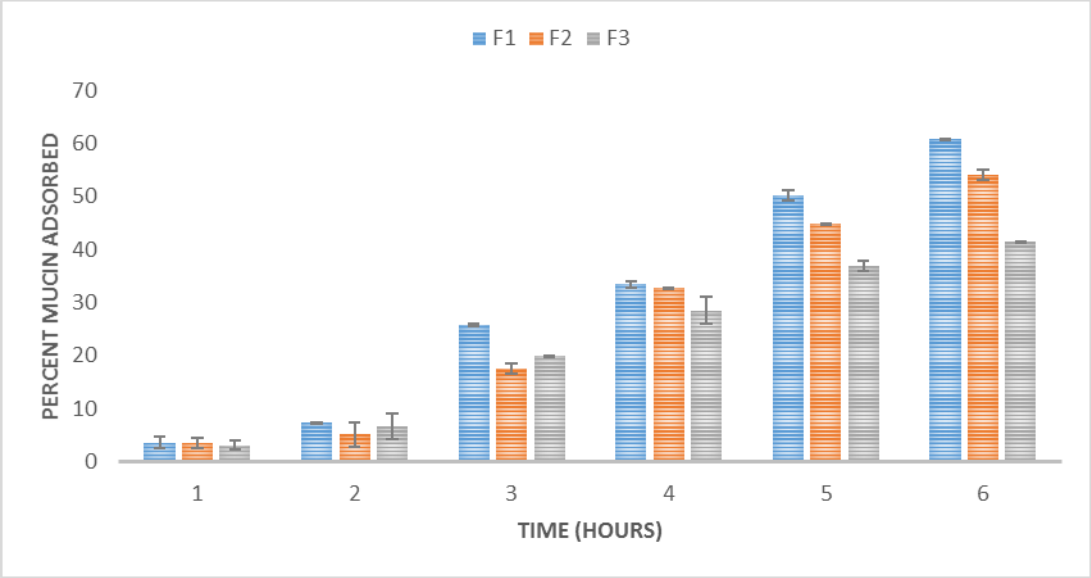


c). F3

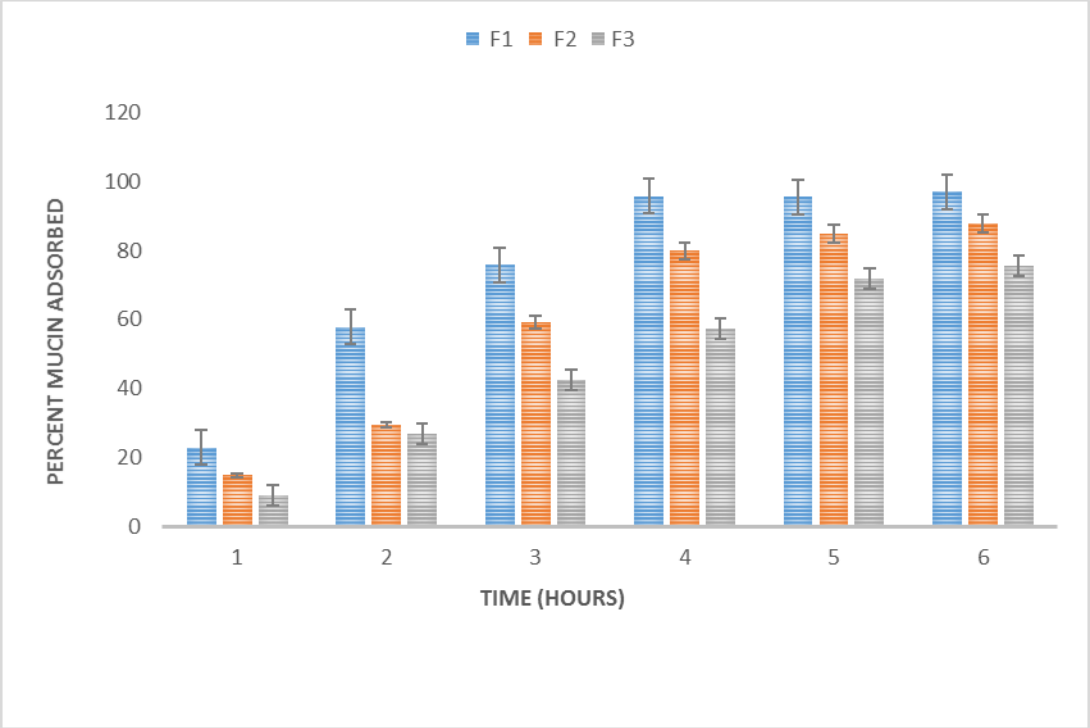
Figure 3.4: SEM micrograph of mucoadhesive spray dried micro-particles. For formulation composition refer to table no 2.2 in section 2.3.1, chapter 2

3.2.1.2. Mucin adsorption

The amount of chitosan micro-particles adsorbed on mucin was determined indirectly by measuring the concentration of free mucin after reaction between chitosan and mucin at different time intervals. The crosslinking agent showed the negative effect on mucin adsorption as depicted by the Figure 3.5 because high glutaraldehyde concentration makes micro-particles harder which consequently decrease the percentage of mucin adhesion. (Kotadiya *et al*., 2009). In current study, at pH 4.5 the formulation F1 having lowest amount of glutaraldehyde attained the maximum adsorption after four hours, followed by steady phase. In contrast, the formulation F2 achieved 50% after three hours and reached to 87% in six hours. Formulation F3 with highest amount of glutaraldehyde attained 50% at fourth hour and showed maximum adsorption of 75% after six hours as shown in figure 3.5. However, at pH 1.3 similar formulations demonstrated different adhesion behaviour. In the current study mount of mucin adsorbed was decreased by low pH because generally acidic pH has negative impact on mucin adsorption as shown in figure 3.5 (P. He *et al.,* 1998). At pH 1.3 F1 gave only 7% adhesion in two hours and maximally reached to 60%. However, F2 and F3 the adhesion was very low after two hours and hardly achieved 50% after six hours as shown in figure 3.5. The results at two different pH levels were significant with significant difference less than 0.05. Similarly, variable amount of crosslinking agent also has significant impact on mucin adsorption. But the combined effect of both crosslinking agent and pH has showed non-significant results with p value of 0.695.



a).



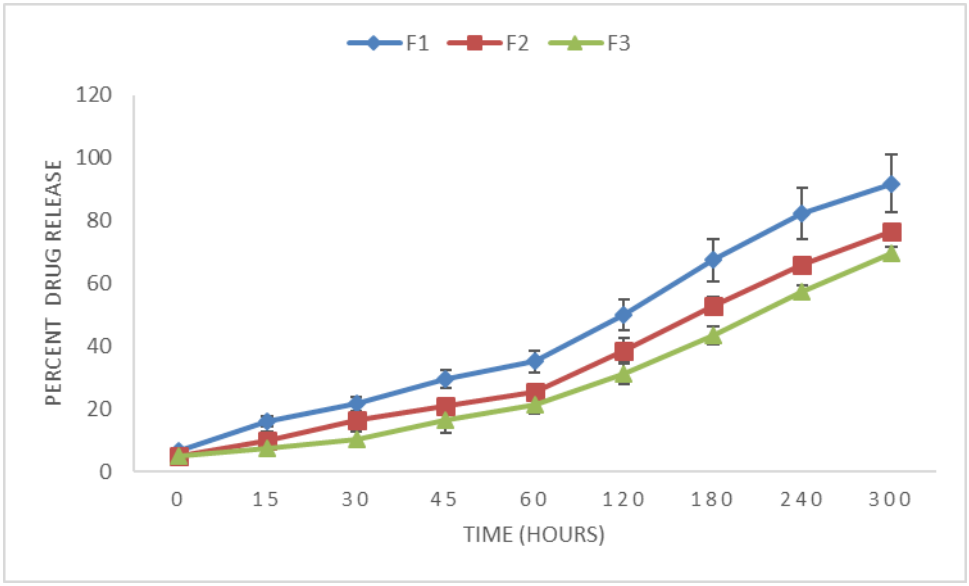
b).

Figure 3.5. Mucin adsorption of chitosan micro-particle formulations at a) pH 1.3 and b) pH 4.5. For formulation composition refer to table no 2.2 in section 2.3.1, chapter 2

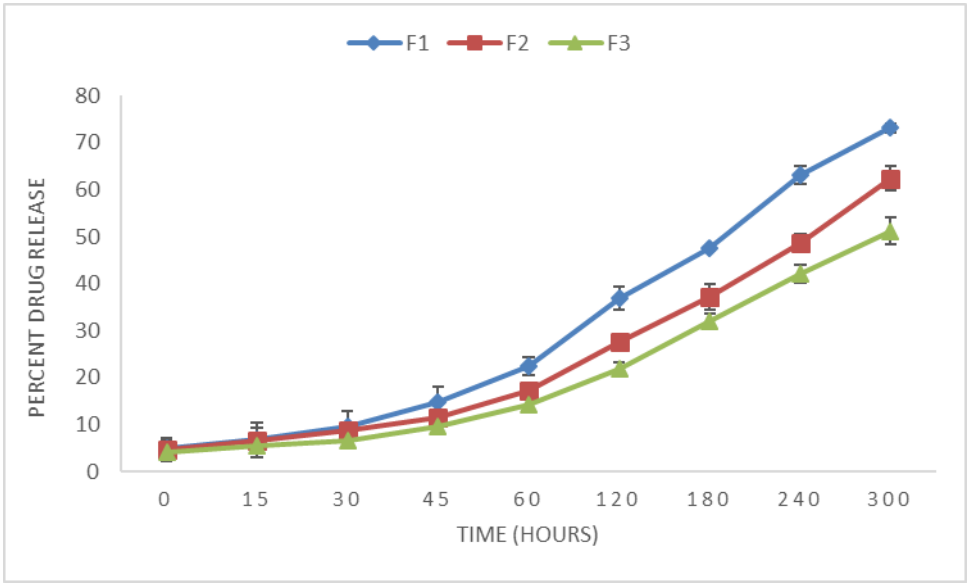
3.2.1.3. *In vitro* Drug release

In vitro drug release from micro particles are shown in figure 3.6. Results showed that release was increased by decreasing pH F1 released 96% of drug after 5 hours at pH 1.3, however the same formulation released only 73% at pH 4.5. Similarly, F2 and F3 released 76% and 79% of drug respectively at pH 1.3 while the release was 62% and 51% at pH 4.5 as shown in figure 3.6. Chitosan used as a polymer for micro-particles becomes soluble under low pH condition due to protonation of amino group that leads to increase in the release of drug at lower pH (Du *et al.,* 2015). *In another* study microsphere almost released all entrapped drug at pH 1.2, however, nearly 70% of the drug was released over 3 hours at pH 3.0 (Li *et al.,* 2011). These results are in agreement with the current study showing that increase in pH has negative impact on release of drug. The other factor that influenced the release rate is the concentration crosslinking agent (GTA). The results showed that formulation containing higher concentration of glutaraldehyde (table 2.2. section 2.3.1 chapter 2), showed lower drug release as shown in figure 3.6. Similar results were obtained from another study conducted by (He *et al.,* 1999) where increasing glutaraldehyde concentration reduced the release. The effect of both pH and crosslinking agent have significant impact on release from zero time till five hours' time. But the combined effect of crosslinking agent and pH was significant at zero time and became non-significant after five hours

All the formulation at both pH levels demonstrated zero order kinetics as the correlation coefficient values of zero order kinetics are higher than first order kinetic as shown in table 3.3.



a).



b).

Figure 3.6. In vitro drug release of micro-particle formulations at a) pH 1.3 and b) pH 4.5. For formulation composition refer to table 2.2 in section 2.3, chapter 2

Table 3.3. Correlation coefficient of formulations at different pH levels. For formulation composition refer to table 2.2 in section 2.3, chapter 2

Formulation	pH 1.3		pH 4.5	
	Zero order r^2	First order r^2	Zero order r^2	First order r^2
F1	0.9801	0.9706	0.9919	0.988
F2	0.9917	0.9891	0.997	0.977
F3	0.9958	0.98	0.997	0.990

3.2.1.4. Effect of zeta potential on the characteristics of microspheres

Zeta potential is the representation of the surface charge on any particulate system in specific medium. In current study the amount of crosslinking agent and the amount of chitosan were considered in relation to zeta potential to achieve the final aim of the delivery system. As shown in tables 2.2 and 3.2 the amount of the glutaraldehyde directly affects the zeta potential. The zeta potential of F1 was highest with value of +36mV, however by increasing the amount of glutaraldehyde from 2mg to 6mg in formulation F2 and F3 respectively, the zeta potential dropped down to +25mV and +14mV. These results are in agreement with the study that states that increase in the amount of crosslinking agent (GTA) decreases the zeta potential (Oliveira *et al*.,1996). In current study the increase in cationic charge of chitosan impart more positive charge and mask the negative charge imparting property of glutaraldehyde.

Chitosan was used as a polymer for preparation of microparticles and according to literature the addition of chitosan increases the zeta potential. This could be explained by the fact that chitosan possesses high positive charge therefore its addition in the formulation could possibly increase the overall charge of the

particulate system. It is supported by literature that chitosan microspheres have the ability to adsorb mucus glycoprotein and the degree of adsorption is dependent upon zeta potential of chitosan microspheres (Mady *et al.,* 2010).

According to one study the zeta potential is more stable in acidic environment (Duffy and Hill, 2011) and the zeta potential could drop down if pH of the medium increase. Therefore, the experiment was conducted at the pH value above 4 to check the effect of zeta potential in alkaline pH on electrostatic interaction between positively charge chitosan microparticles and negatively charged mucus. The results in this study showed that muco-adsorption increased by increasing the pH. However, in contrast the increase in pH decreases the zeta potential that may weaken the electrostatic interaction between mucus and microparticles. But the strong mucus adhesion at pH 4 in this study is justified because, lower negative charge of mucin decreases the strength of ionic interaction of mucin and chitosan which in turn reduce the mucin adsorption on chitosan at low pH. Zeta potential with stand the effect of pH up to certain level as presented by Sunee *et al* in 2010 that if the pH values decreases from 8 to 4 there is gradual increase in the zeta potential but below 4 pH the zeta potential drastically overshoot towards positive values (Sunee *et al*.,2010)

3.2.2. Characterization of freeze dried microparticles

Characterization of freeze dried microparticles are summarized in table 3.4 and 3.5. The use of crosslinking agent affects the drug content. However, process yield remained unaffected by use of crosslinking agent. As shown in table 3.4 addition of crosslinking agent in formulation F*f*2 process yield remains almost same (81%) as in formulation F*f*2 with 4mg of crosslinking agent (79.5%). However, by increasing the crosslinking agent to 6mg in formulation F*f*3 the process yield was 90%. This increase of process yield in F*f*3 was due to presence of chitosan because increase

of glutaraldehyde in the formulations has no effect on process yield as shown in formulation F*f*1 and F*f*2 where process yield remained unaffected by increase of glutaraldehyde.

The loss in overall weight of powder in final product during freeze drying as in current study was also observed in number of other studies but the phenomena is still not well explained in literature. One study reported process yield even up to 73% of the final product (Cho *et al.,* 2010) however, the process yield after freeze drying ranges from 78% to 93% in the study conducted by Cho et al (2011).

Table 3.4. Characterization of freeze dried microparticles. For formulation composition refer to table 2.2 in section 2.3, chapter 2

Formulations	Glutaraldehyde (mg)	Chitosan (mg)	Drug (mg)	Drug Content	Process yield
F*f*1	-	10	10	56.3 % ± 1.2	79.5%
F*f*2	4	10	10	59.1% ± 0.9	81%
F*f*3	6	15	10	42.0% ± 1.4	90%

The amount of chitosan has shown obvious effect on the process yield and drug content. By increasing the amount of chitosan in formulation F*f*3 the process yield was increased from 80% to 90%. However, on the other hand the drug content was decreased to 42%. Most likely, increase in the chitosan increase the bulk viscosity which decreases the efficient encapsulation of drug in polymeric layer and most of the drug remained un-encapsulated. This result was further supported by evidence that only parameter that is changing is the amount of the chitosan however, the solvent was kept constant for all three formulations. However, in spray drying increase in the amount of chitosan decrease the process yield because increase

the amount of chitosan initially increase the bulk of feed mixture but during processing chitosan being the adhesive polymer may stick to the walls of the glass chamber due to residual moisture content and decrease overall process yield.

The particle size of all the freeze dried formulations are almost same ranges from 2.34µm to 2.55 µm. Which means particle size remained unaffected in freeze drying by adding crosslinking agent in contrary to spray drying where increase the amount of glutaraldehyde decreased the particle size. Most likely freeze drying process recover high amount of polymers in the final product and the amount of crosslinking agent is insufficient for the crosslinking when used in comparable concentration to spray drying process and the resultant particles are bigger than spray dried particles as shown in table 3.5.

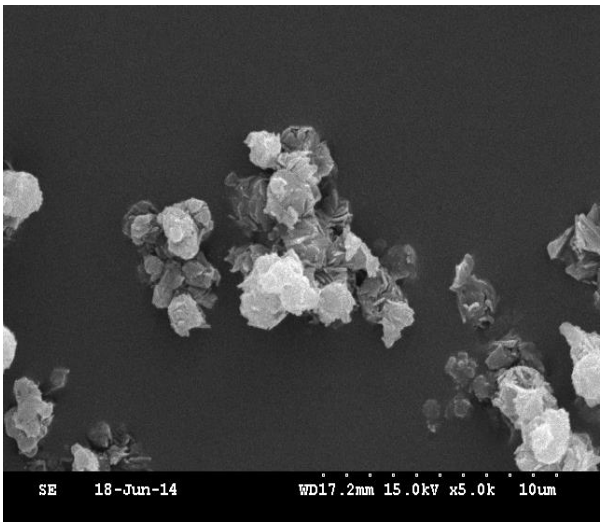
Table.3.5. Particle size of freeze dried formulations by using different nanopores. For formulation composition refer to table 2.2 in section 2.3, chapter 2

Formula	Nanopore (nm)	Particle size (nm)			
		Maximum	Minimum	Mode	Mean
F*f*1					
	2000	4289	1954	**2550**	2475
F*f*2					
	2000	3968	1722	**2341**	2110
F*f*3					
	2000	3744	1782	**2487**	2374

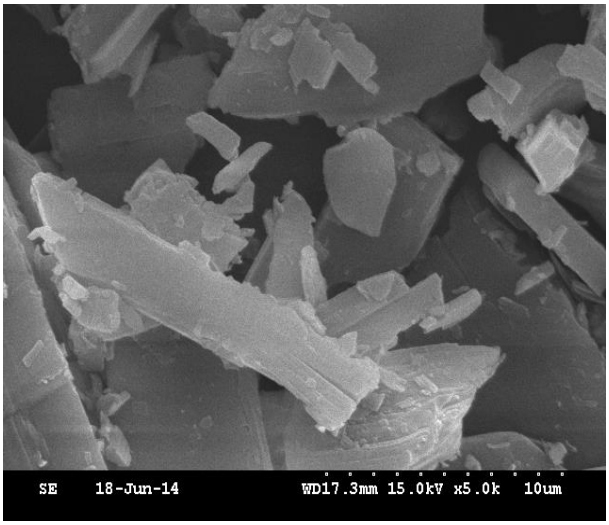
SEM micrographs of freeze dried microparticles are shown in figure 3.7 show the dispersed, irregular and fluffy powder in comparison with spray dried particles that are more spherical and possessed defined structure. Study conducted by Hyun-

Jong *et al*, in 2010 revealed that the microparticles prepared by using freeze drying approach are irregular in shape. There is another study conducted by Hyun-Jong *et al* in 2011 also reported irregular shaped freeze dried microparticles (Hyun-Jong *et al*., 2010; Hyun-Jong *et al*., 2011)

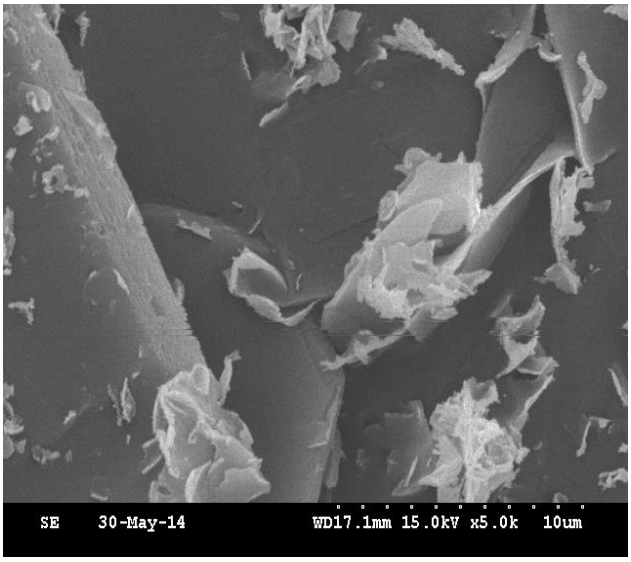
Figure 3.7. A and B shows the morphology of microparticles that depicts the insufficient crosslinking and the fluffy powder represents the dispersed polymer along with drug which is not fully incorporated inside the polymer.



a). F*f*1



b). F*f*2



c). F*f*3

Figure 3.7. (A). SEM photo micrographs of freeze dried furazolidone microparticles with chitosan at 5X magnification a). with 2mg of glutaraldehyde b). without glutaraldehyde and c). with 6mg of glutaraldehyde. For formulation composition refer to table 2.2 in section 2.3, chapter 2

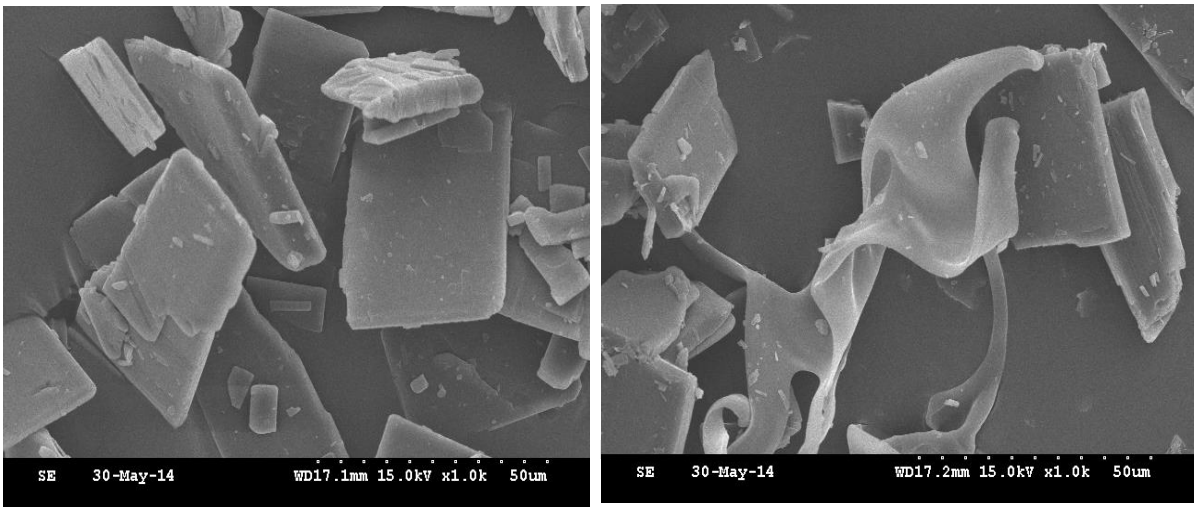


Figure 3.7. (B). SEM photomicrographs of freeze dried furazolidone microparticles with chitosan at 1X magnification a). without glutaraldehyde and b). with 6mg of glutaraldehyde. For formulation composition refer to table 2.2 in section 2.3, chapter 2

Figure 3.7 represents F*f*1 with chitosan to drug ratio 1:1 and 4mg glutaraldehyde showed more geometrical shape because less amount of chitosan was available in final product crosslinked by glutaraldehyde. However, in F*f*2 where there was no glutaraldehyde there were solid drug particles. In the figure for F*f*3 at both magnifications, 3.7 A and B microparticles started to mold with the presence of crosslinking agent that was 6mg but due to excessive amount of chitosan in F*f*3 crosslinking agent was not enough to mold microparticles into defined shape.

In vitro drug release profile is shown in figure 3.8. The drug release profile shows that all of the formulations released the drug up to 3 hours maximally followed by steady state. Drug release from chitosan-based particulate systems depends upon the extent of cross–linking, morphology and the size of the particles (Analava and Baishakhi 2011). Formulation F*f*1 showed release of 86% of drug in two and half hours. Similarly, formulation F*f*2 without crosslinking agent released 95% drug in three hours and F*f*3 showed 97% of drug in three hours. Similar kind of results were reported in one study conducted by Ruth *et al*, in 2010 who showed that the non-crosslinked clarithromycin released 100% of its drug in three hours. In the same

study, the un-crosslinked tramadol microparticles revealed burst release and a total of 80% drug release in first two hours (Ruth *et al*.,2010).

The results in this study showed that addition of crosslinking agent and the absence of crosslinking agent produce similar release profile which explains the possibility of interaction of crosslinking agent, drug and chitosan differently in each formulation during freeze drying. Freeze drying did not efficiently encapsulate the drug into polymer in the absence of crosslinking agent in formulation F*f*2. In case of F*f*3 the excessive chitosan recovered limits the availability of crosslinking agent and most of chitosan renders un-crosslinked in formulation F*f*3. However, F*f*1 indicated the limitation of freeze drying process itself as the amount of the chitosan and crosslinking were comparable to the proportion used in the spray drying and the results of spray dried sample showed delayed release as shown in figure 3.6 in contrast to burst release in the case of freeze dried particles. These results are supported by the evidence from one other study that stated that the cimetidine loaded microparticles with chitosan showed the burst release due to absence of crosslinking agent (He *et al*., 1999).

If the formulation lacks the appropriate proportion of crosslinking agent the drug adsorbed onto the surface of polymer and instantly released with dissolution media in first few hours (burst release). However, crosslinking agent produces a firm contact between the polymer and the drug that helps in maintaining the sustained release pattern.

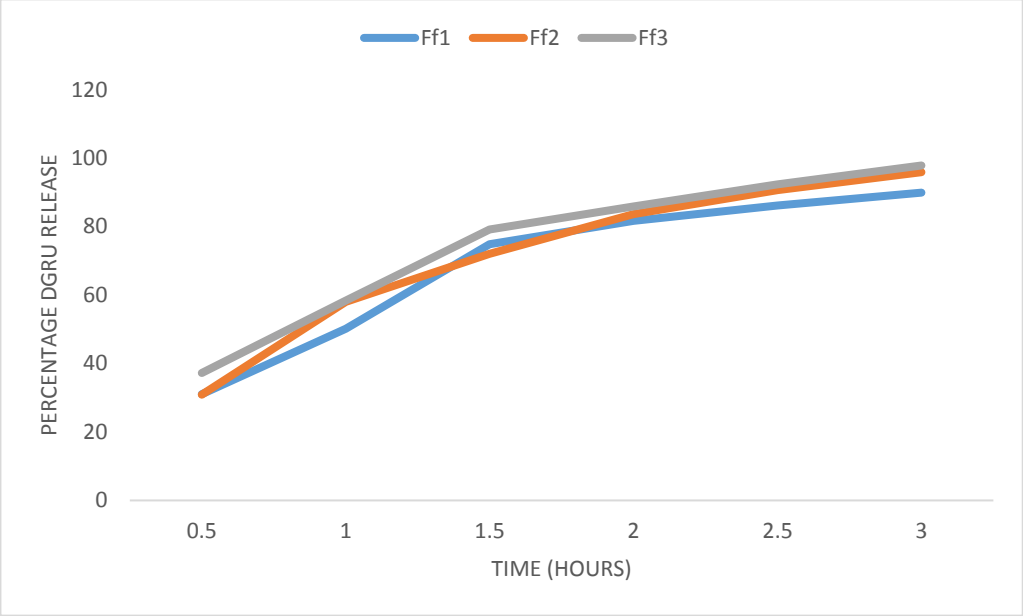


Figure 3.8. Drug release profile of freeze dried microparticles in 5ml dissolution media by dispersion method presented by shazly *et al* in 2013. For formulation composition refer to table 2.2 in section 2.3, chapter 2

3.3. Conclusion

This study demonstrated that mucoadhesive furazolidone microparticles were successfully prepared by spray drying process. Those particles showed better adherence to mucin at low acidic condition (pH 4.5), this favours the local gastric delivery of furazolidone against for example *H. pylori* which increases gastric pH. At pH 4.5, formulations F1, F2 and F3 accomplished 50% mucin adsorption after 2 h, 3 h and 4 h respectively.

CHAPTER FOUR

Formulation and advantages of furazolidone in liposomal drug delivery systems

4.1. Introduction

The major aim of this chapter is to produce mucoadhesive liposomes that deliver furazolidone for prolonged period of time at pH 4.5, to optimize the cholesterol concentration, to use optimum amount of drug that can give maximum encapsulation activity and to compare the results of sephadex gel filtration and refrigerated centrifugation method for determination of encapsulation activity.

4.2. Results and discussion

4.2.1. Effect of cholesterol and drug on encapsulation efficacy

Encapsulation efficiency of liposomes was determined in relation to varying amount of cholesterol and drug; however, the type and the amount of lipid were kept constant. Decreasing the cholesterol content in all formulations increased the encapsulation efficiency correspondingly as shown in (table 4.1, figure 4.1). These results are in agreement with fact that increasing the cholesterol content beyond saturation point may cause disruption of linear structure of membrane (Nounou et al., 2005) that leads to decrease entrapment efficacy; this also was shown in this study which was revealed by transmission electron microscopic images (refer to Section 4.2.7 and figure 4.7). The fact explained in a study conducted by Begum et al (2012) that increase the cholesterol content occupies the space between the alkyl chain of phospholipids and decrease the entrapment efficacy of lipophilic drug that accommodate in lipid bilayer (Begum et al., 2012).

However, it has been shown in some studies (Nounou et al., 2005; Rojanapanthu et al., 2013) that cholesterol increase rigidity of membrane and decreased permeability and leakage of liposomes ultimately enhancing encapsulation activity. Cholesterol changes micro viscosity of lipid membrane that effects fluidity therefore improves the integrity, stability of vesicle membrane (Sankaram and Thompson,

1990; Haeria et al., 2014). It is presumed that cholesterol depicts variable effects on encapsulation activity for hydrophobic drug molecules possibly due to molecular interaction between lipid, candidate drug and cholesterol. Nevertheless, by increasing the amount of the drug, encapsulation efficiency increases up to certain limit and then decreases. Maximum encapsulation activity by using 2:1 lipid and cholesterol was 44.7% for L2 when 5mg of drug was used initially but increasing or decreasing the initial amount of drug by keeping lipid to cholesterol ratio same decreased encapsulation activity to 29.9% for L3 and 43.3% for L1 (table 4.1, figure 4.1). In one another study i.e. percent entrapment efficacy increased initially by increasing the concentration of drugs up to certain limit however, it start decreasing by further addition of the drug beyond saturation point of the lipid bilayer(Karen et al.,2011). Similarly, by using low cholesterol content in formulations L4, L5 and L6 the relationship between initial amount of drug and encapsulation efficacy demonstrated the same trend as revealed by using high cholesterol content (table 4.1, figure 4.1). It was shown revealed from statistical analysis using two-way ANOVA Tukey test that variable amount of drug and cholesterol has significant effect on encapsulation efficiency individually as well as interaction of drug with cholesterol also influenced encapsulation efficiency as significance factor was less than 0.005 for all three cases. Impact of effect of changing amount of drug was more influential than variable amount of cholesterol i.e. the amount of drug (8 mg in L3, L6) responsible for lowest encapsulation efficiency got lowest two EE values (table 4.1) irrespective to the amount of cholesterol.

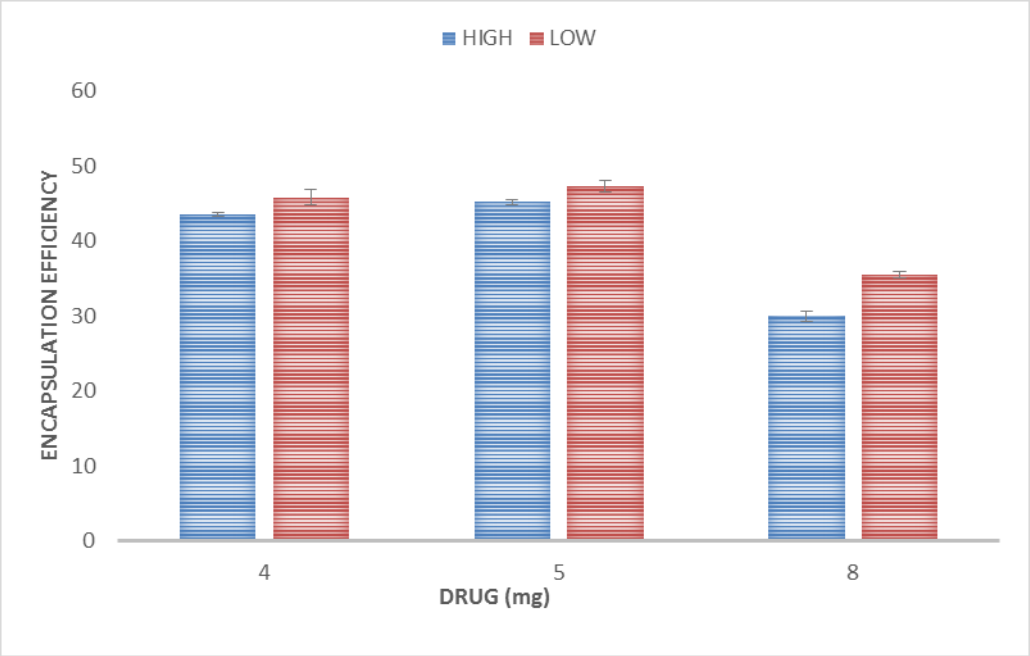


Figure 4.1. Effect of the drug (4,5 and 8mg) and cholesterol (High and Low) contents on encapsulation efficiency of liposomes. composition of mucoadhesive formulations are given in table 4.1.

Table 4.1. Z-potential of formulations and effect of cholesterol content and drug on encapsulation efficiency (EE) and drug loading

Formulation	Drug	Lipid: Cholesterol	% EE	Entrapment	Z-potential
L1	4mg	106:53 mg	43.3 ± 0.22	1.73 mg	-17 mV
L2	5 mg	106:53 mg	44.7 ± 0.36	2.23 mg	-19 mV
L3	8 mg	106:53 mg	29.9 ± 0.70	2.39 mg	-12 mV
L4	4 mg	106:10.6 mg	45.7 ± 1.07	1.82 mg	-29 mV
L5	5 mg	106:10.6 mg	47.2 ± 0.73	2.36 mg	-32 mV
L6	8 mg	106:10.6 mg	35.4 ± 2.5	2.83 mg	-26 mV

4.2.2. Encapsulation efficiency determination by sephadex method

As shown in figure 4.2. liposomal encapsulation activity of all the formulations are almost similar when determined from both methods. i.e. refrigerated centrifugation and sephadex filtration

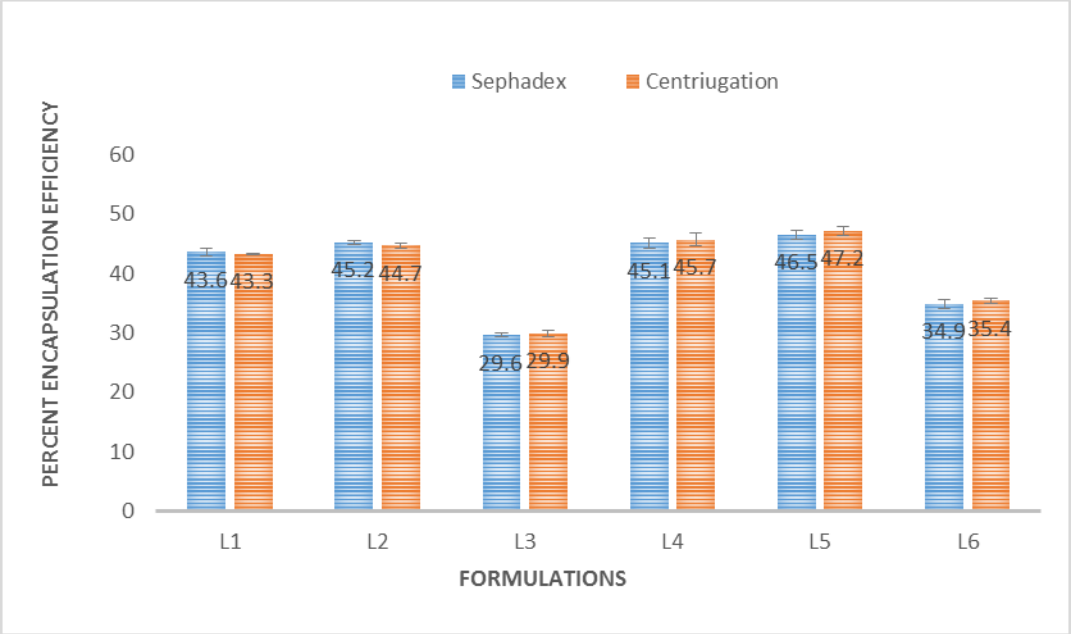


Fig 4.2. Comparison of encapsulation efficiency by using sephadex chromatography and refrigerated centrifugation

4.2.3. Effect of pH on mucoadhesion of liposomes:

In mucoadhesion analysis LC_1 formulation (table 4.2) containing chitosan as a mucoadhesive polymer showed 32% mucoadhesion at pH 1.3 up to 6 hours' however, the same formulation showed 59% mucoadhesion at pH 4.5 (figure 4.3). Liposomal formulation NLC_1 containing no chitosan showed almost no mucoadhesion over four-hour time duration at both pH as shown in (figure 4.3). Fifty-five percent of mucoadhesive liposomes, LC_1, dropped from stomach mucus within 3 hours' time period at pH 1.3 and only 35% at pH 4.5, while non mucoadhesive liposomes almost dropped off completely (80%) from stomach tissue between 1.5 to 3 hours as shown in (figure 4.3). The results were further confirmed by

fluorescence microscopy that demonstrated progressive decrease of liposomal adhesion on stomach mucus with the passage of time (figure 4.4). Chitosan being cationic polymer interacts with negatively charged sulfonic and sialic acid residues of mucus (Han et al., 2012). Chitosan consists of primary amino groups and therefore regarded as strong base having pKa 6.3. Therefore, lower pH makes it soluble as its amino groups get protonated below pH 4 that causes repulsion between them, subsequently hydrophobic interaction and hydrogen bonding becomes week and makes chitosan soluble (Jayakumar et al., 2010). But in this study, mucoadhesion was investigated with reference to *H. pylori* that can survive over wide range of pH extending from acidic to basic conditions. Ideal pH of its growth is between 6 to 8 however it can survive at pH 4. *H. pylori* evolved acid tolerant mechanism by urease enzyme activity that could increase the pH of microenvironment of mucus (Scott et al., 1998). Therefore, in this study, mucoadhesion of chitosan coated liposomes were investigated at two different pH i.e. natural pH of stomach 1.3 and pH 4.5 to mimic pH conditions produced due to *H. pylori.* As shown in results (figure 4.4 b,c), liposomes remain adherent to mucus for prolonged period at pH 4.5 as compare to pH 1.3. These results are in agreement with a study states that drug microspheres coated by chitosan release instantly at pH 1.2. However, 70% of drug released at pH 3 over three hours' time period (Du et al., in press). The possible explanation for non-instant release in this study (refer to Section 4.2.5) could be the drug encapsulation inside liposomes. Mucoadhesion at pH 4.5 is ascribed by the study which revealed that pH of gastric surface is significantly higher and close to neutral pH as compare to lumen where pH is 1.2. Proposed theory for high surface pH is secretion of bicarbonate from *H. pylori* that could only neutralize 10% acid secretion. But near HCl source or fundic glands pH is much lower (Scott et al., 1998). However, mucoadhesive properties of chitosan

could be tailored for acidic pH in stomach by using different strategies like chitosan could be decorated with number of other polymers like Eudragit (Zhu et al., 2012).

Regarding statistical analysis for mucoadhesion data, the adhesion of liposomal drug formulation LC_1 to stomach mucus was significantly different at pH 1.3 and pH 4.5 as revealed by two tail test with ($p < 0.003$). However, non-chitosan formulation NLC_1 showed no significant difference at different pH.

Table 4.2. Composition of fluorescence labelled liposomes. LC1: Liposomes containing coumarin-6 with chitosan; NLC1: Liposomes containing coumarin-6 without chitosan

Formulation	Composition	Mucoadhesive liposomes
	Coumarin6: Lipid: Cholesterol (weight)	Chitosan
LC_1	2.5 µg:26.5mg: 2.5mg	0.6% (w/v)
NLC_1	2.5 µg:26.5mg: 2.5mg	0

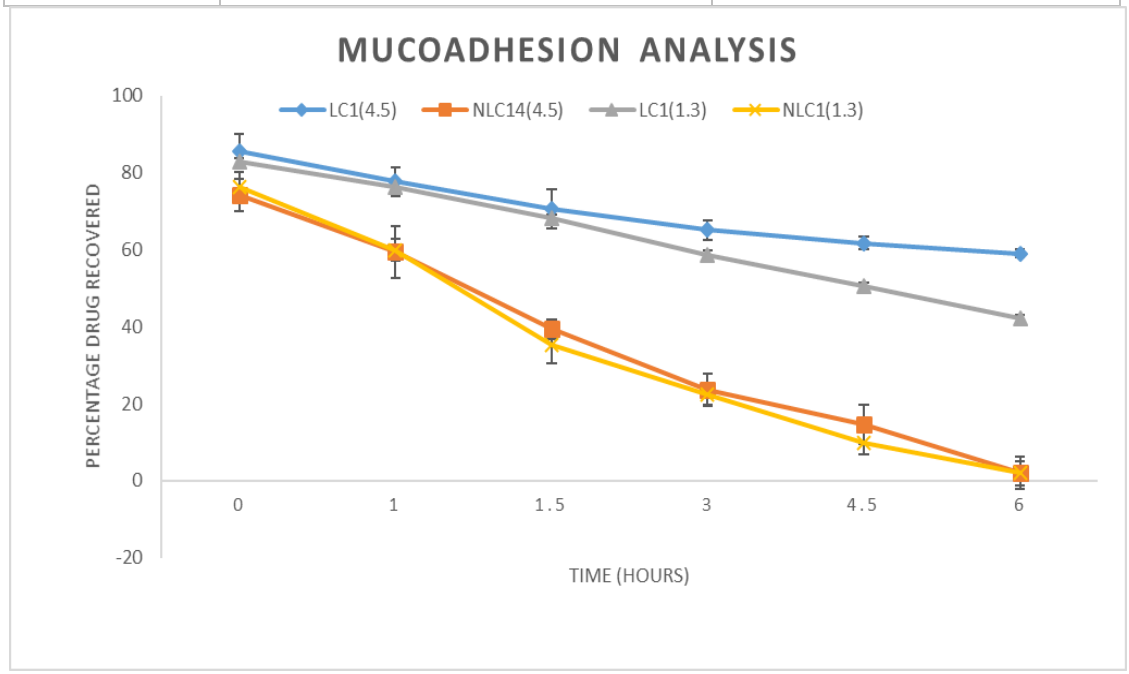
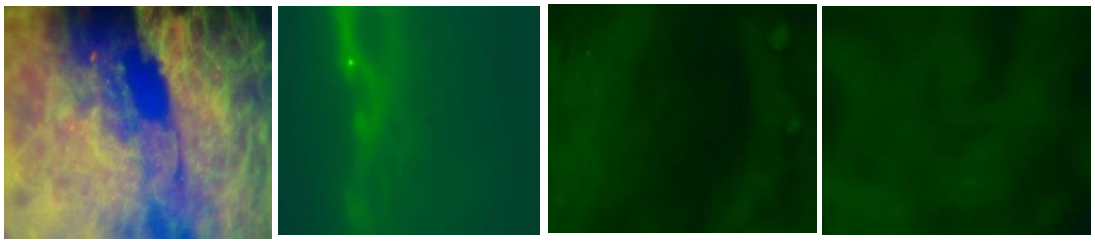
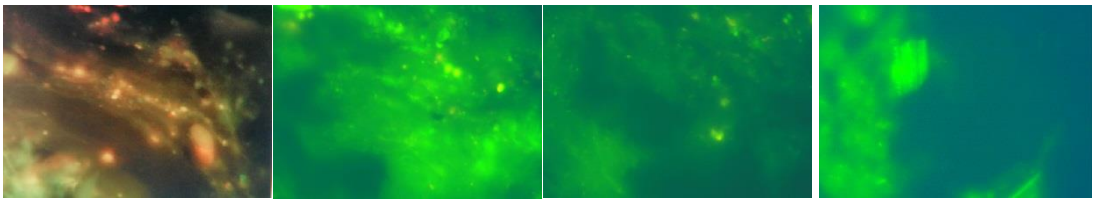


Figure 4.3. Mucoadhesion analysis: Percentage of fluorescent dye liposomes recovered on stomach tissue in SGF at pH 1.3 and 4.5. Refer to table 4.2 for formulation composition



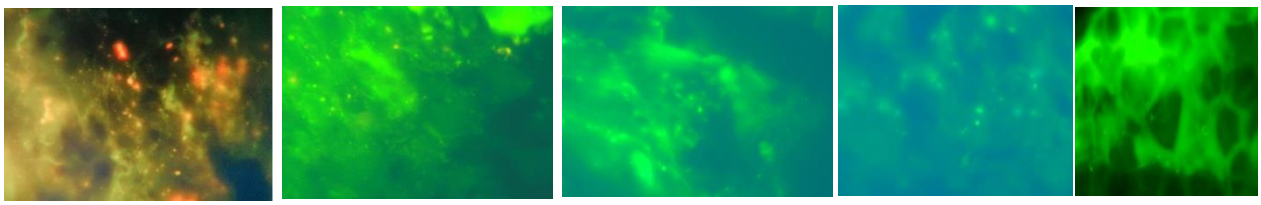
0.5h 3h 4.5h 6h

a).



0.5h 2h 4h 6h

b).



0.5h 1.5h 3h 4.5h 6h

c).

Figure 4.4. Fluorescence microscopic images of stomach tissue with mucoadhesive liposomal formulations: **a**. Non Chitosan liposomes, NLC_1, at pH 4.5 over 6 hours' time period in simulated gastric fluid (SGF) on stomach mucus; **b**. Chitosan liposomes, LC_1, at pH 1.3 over 6 hours' time period in simulated gastric fluid (SGF) on stomach mucus and **c**. Chitosan liposomes, LC_1, at pH 4.5 over 6 hours' time period in SGF on stomach mucus. Refer to table 4.2 for formulations

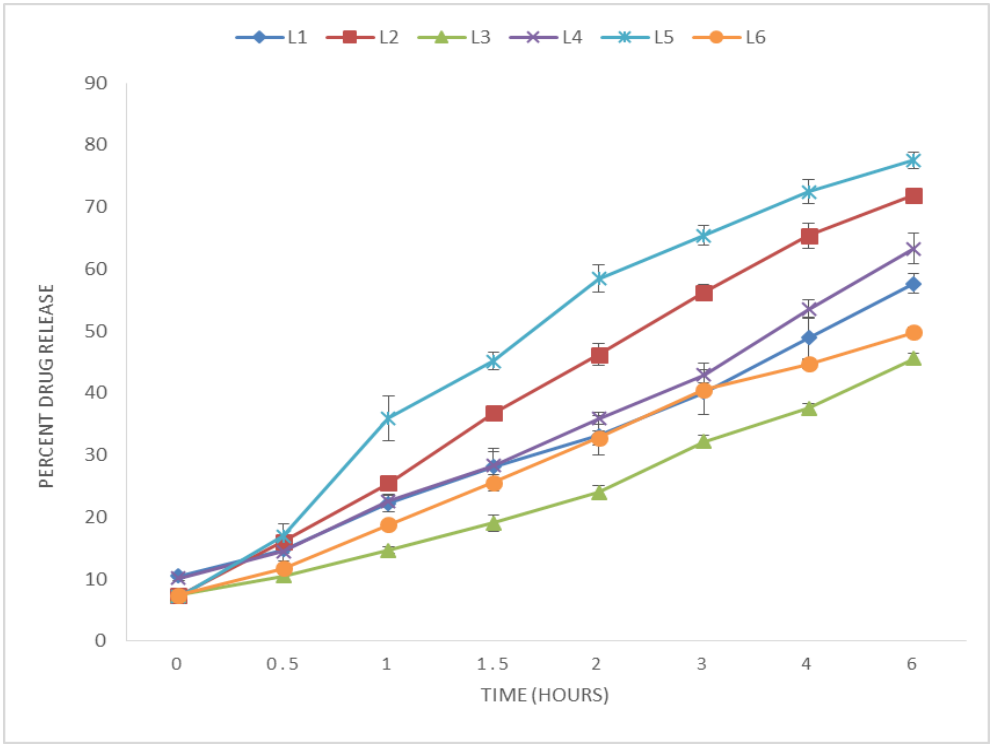
4.2.4. HPLC Analysis of Liposomes

Retention time of standard furazolidone injected into HPLC with gradient elution approach by using two mobile phases was 4.388 minutes' (figure 2.1.a)

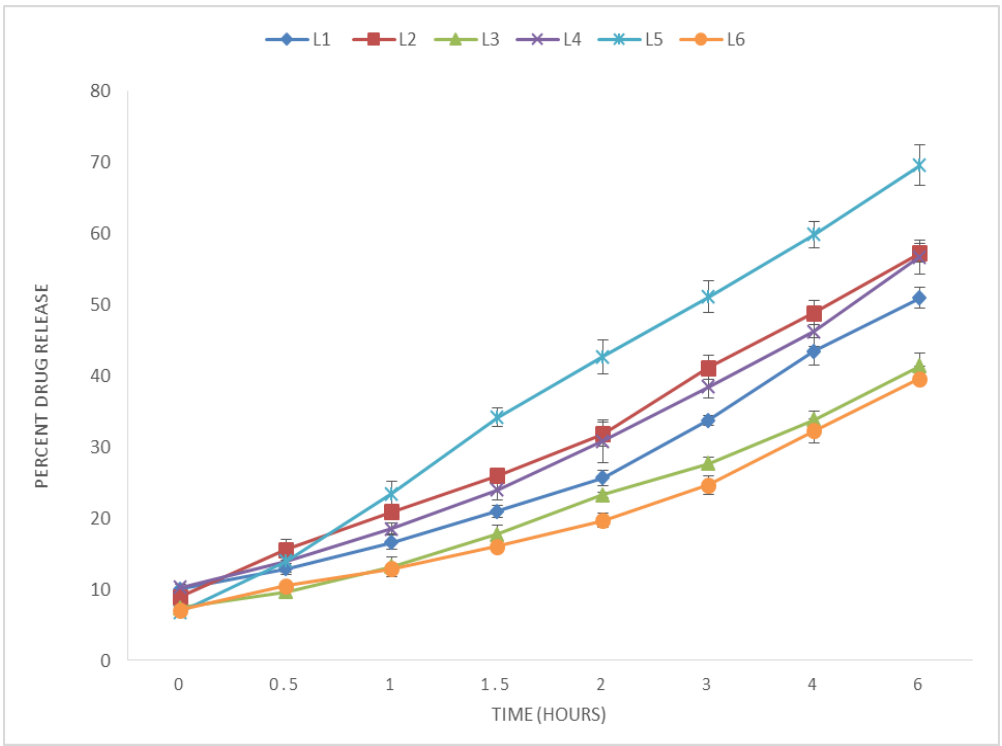
4.2.5. *In vitro* drug release

In vitro drug release of all six formulations showed inverse relationship with amount of cholesterol at both pH 4.5 and 1.3 (figure 4.5 a and b). L2 with more amount of cholesterol (table 4.1) demonstrated 71% percent drug release as compare to L5 that gave 77% release at pH 4.5 (figure 4.5 a). In addition to cholesterol content, pH also influenced the percentage of drug release. At pH 1.3 the overall release of both formulations decreased, L5 gave 70% in contrast to L2 that gave maximum release of 58%. L3 formation with highest amount of cholesterol gave 42% release at pH 1.3 (figure 4.5 b) and 47% at pH 4.5 (figure 4.5 a). In the current study, results revealed that by increasing the cholesterol content the overall release of furazolidone was decreased, one of the possibility could be accumulation of cholesterol and furazolidone being lipophilic drug in phospholipid layer which further restricts the drug deep into lipid layers and limit its release (Begum et al., 2012; Deniz et al., 2010). According to this study results of furazolidone release profile (figure 4.5), the drug release is considered as sustained release but as current study is focused on stomach mucoadhesion mechanism and physiological shedding time for mucus is 6 hours the point of interest was more focused on six hours instead of 12 or 24 hours recommended for sustained release (Lai *et al.,*2009)Three different time intervals i.e. half an hour, 3 hours and after 6 hours, were selected for drug release in order to perform statistical analysis to study the effect of change in pH, initial amount of drug and amount of cholesterol on drug release. According to analysis it is confirmed that cholesterol in formulation has no significant impact on drug release at 0.5 hour as the significance factor for cholesterol is 0.832. However, increasing pH has significant effect on drug release at 30 min as P is 0.002 and amount of drug also has significant effect on release ($p < 0.001$ at 30 minute). There is no significant impact of interaction between drug, pH condition and amount of

cholesterol as significant factor is $P < 0.83$. But the effect of all three factors individually and in combination was significant from three hours up to six hours' time period ($p < 0.05$).



a).



b).

Figure 4.5. Drug release from liposomal formulations: a. at pH 4.5 and b. at pH 1.3. For composition of formulation refer to table 4.1

4.2.6. Particle size analysis of L5:

Liposomal formulation L5 (table 4.1) gave maximum encapsulation efficacy and maximum release during *in vitro* release test and by keeping same lipid to cholesterol ratio as L5 the mucoadhesive formulation LC_1 gave maximum mucoadhesion therefore L5 was considered for particle size analysis and other formulations were overlooked as the main aim of study was not focused on particle size. Izon qnano was new approach used for particle size analysis in this study. Particles with maximum, minimum size, average size of population is given in (table 4.3). Mode by using nanopore with greater particle count was 482nm L5 (table 4.3) that represents overall size of maximum number of particles in population. According to (figure 4.6) the particle size distribution of L5 gave cut off data shown by nanopore 200 which revealed there is only small proportion of smaller size particles less than 266nm in overall population. This technology works by tunable resistive pulse sensing with nanopores that makes particle-by-particle characterization on the nanoscale. Secondly it performs real-time analysis, therefore it gets insight into dynamic properties. Working principle or algorithm of this technology is Coulter principle that is when voltage is applied through fluid cell by silver-silver chloride (Ag/AgCl) electrodes; the movement of ions in sample electrolyte generates the current across nanopore. A voltage detector recognizes changes in current during the movement of particle through nanopore which is interpreted as blockage magnitude in current flow that is proportional to particle size which gives size and count of particles in an electrolyte solution.

As shown in (figure 4.6) two main populations at two different nanopores for L5 formulation (table 4.3) generate a data of discrete particles over range of sizes including mode, maximum, minimum and mean therefore working by particle-by-

particle analsis, results are neither averaged nor influenced by large particles (Izon qnano training modules, 2014) (Nounou et al.,2005)

Table 4.3. Particles size measurement of liposomes with highest encapsulation efficiency, L5

Formulation	Particles size (nm)					
	Nanopore	Mean	Mode	Max	Min	Particle count
L5	200	535	374	2082	266	435
	400	692	**482**	2236	328	1070

L5: Liposomes contain 5mg drug: 106mg lipid: 10.6mg cholesterol

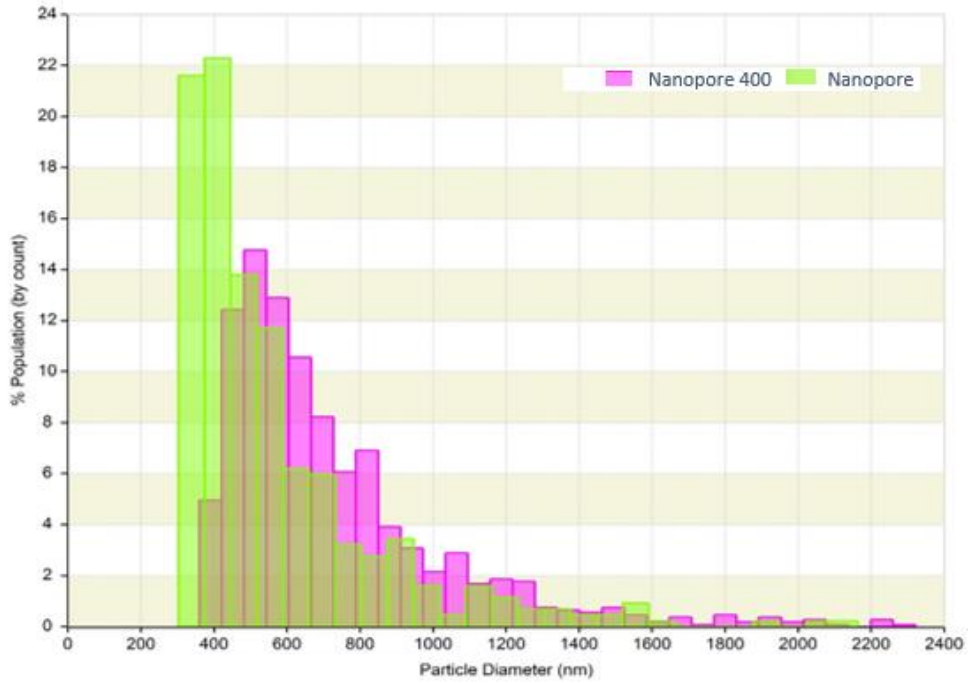
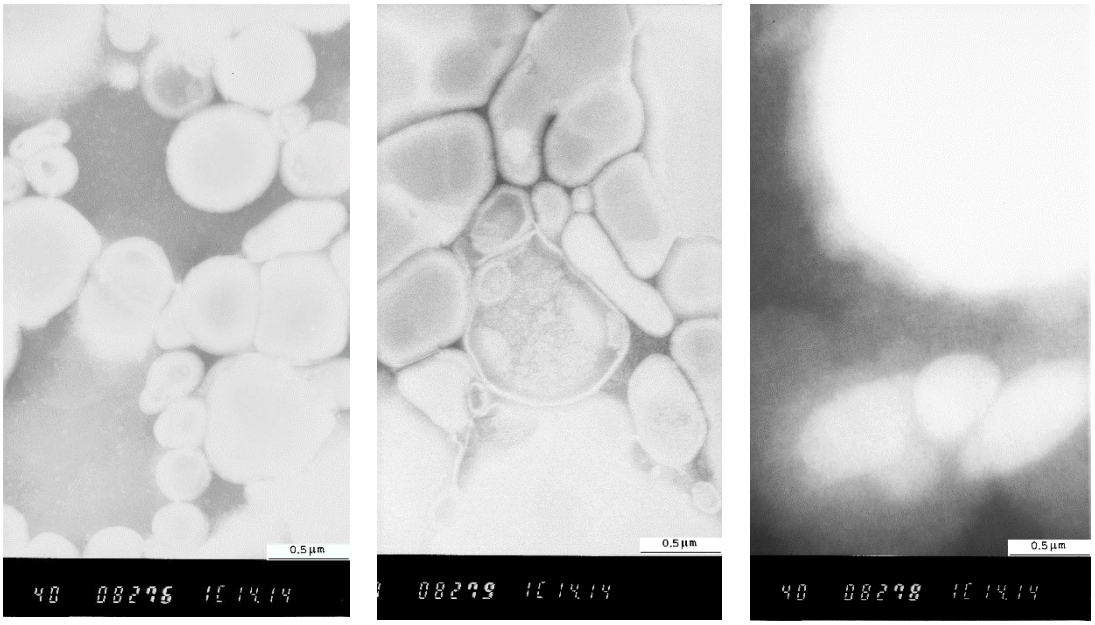


Figure 4.6. Particle size and distribution curve of L5 (Liposomes contain 5mg drug: 106mg lipid: 10.6mg cholesterol) by using Nanopore 200 nm (green bars) and 400 nm (pink bars).

4.2.7. Particle Morphology

Two types of formulations having similar amount of drug and lipids with variable amount of cholesterol were selected for particle morphology based on the amount of encapsulated drug. L5 appeared as smooth round shaped liposomes as shown

in TEM micrograph in (figure 4.7 a). When the amount of cholesterol was increased by keeping similar amount of drug, lipid and surfactant, surface morphology of L2 starts deforming as shown in (figure 4.7b) Micrograph of LC_1 as taken from TEM showed round regular shaped liposomes embedded in chitosan polymer as shown in (figure 4.7c).



a. b. c.

Figure 4.7. Transmission electron microscopy (TEM) of liposomes: a. Liposomes with low cholesterol content (L5); b. Liposomes with high cholesterol content (L2) and c. Liposomes with chitosan (LC_1). For composition of formulation refer to table 4.1 and 4.2

4.2.8. Effects of Zeta-potential

Zeta-potential was determined by q-nano to determine the surface charge of the liposomes. As shown in table 4.1. ζ -potential of the liposomes increased by increasing the amount of cholesterol in the formulation. Maximum ζ-potential reaches to -19 mV when high amount of cholesterol was used. This value of ζ-potential is lower than the lowest value formulation L6 that contains increased amount of cholesterol. This effect of increase in negativity of liposomal surface charge by adding cholesterol is explained in the study that cholesterol settles in the

lipid bilayer which in turns reduces binding affinity of lipid layer to cations in the buffer system (Yandrapati.,2012). Negative charge on all liposomal formulations may also be attributed to lipid (egg-PC) used in the formulation because use of egg-PC has slightly negative tendency due to the presence of impurities that could be oxidation of fatty acid in PC as fatty acid has known effect on ζ-potential (Guo et al.,2003).

The aim of the current study is to formulate the mucoadhesive liposomes and chitosan was selected as mucoadhesive polymer. Chitosan being cationic polymer needs the slightly negative surface of liposomes for better coating. According to one study negative charge on liposomes increases the ionic interaction between chitosan and liposomes (Yandrapati.,2012). The negative surface charge could be imparted by adding cholesterol but as discussed in previous section that increase in cholesterol decreases the encapsulation efficiency of liposomes. Therefore, egg-PC was selected in the formulations to create the electrostatic attraction and to achieve better coating of chitosan on formulations. The second important factor that was considered in this chapter is the selection of pH i.e. 1.3 and 4.5. It has been postulated in one study that decrease in the pH of the medium has effect of ζ-potential (Maria *et al.,* 2011). Decreasing the pH of the medium increase the ζ-potential depending upon the lipid used. This increase in ζ-potential could be possibly due to acid neutralization of polar head in phosphate group. The H^+ from the acidic medium adsorbed onto the surface and increase the ζ-potential.

According to Channarong (2010) the pH of the medium has slight effect on ζ-potential from pH 8 to 4 but if the pH dropped below 4 there is sudden increase in the ζ-potential. The aim of current study is to promote the mucoadhesion therefore the pH selected was 4.5 to check the effect of lowest pH that could bears negative charge on the mucoadhesion behavior of chitosan liposomes with negatively

charged mucus. Second pH selected was highly acidic 1.3 that keeps the overall ζ-potential positive. The results of ζ-potential in this chapter proves two major findings. Assuming that liposomes being negatively charged and reduce the mucoadhesion at pH above 4.5 as mucus is negatively charged and repulsive forces could come into action. But in this study pH above 4.5 demonstrated good mucoadhesion and this effect is possibly due to coating of liposomes with chitosan. However, at pH 1.3 liposomes also demonstrated certain degree of mucoadhesion although at this low pH the chitosan tends to solubilize. However, it is possible that at acidic pH 1.3 liposomes absorb H^+ from the surrounding medium that leads to increase of ζ-potential which could contribute towards mucoadhesion of liposomal particles.

4.3. Conclusion:

It can concluded from current study that mucoadhesive liposomes can be used in low acidic condition for local delivery of furazolidone against *H. pylori* which increases gastric pH that can add-on additional advantage of enhanced release of the drug from liposomes. Secondly optimum cholesterol and drug content can increase encapsulation efficiency that shows subsequent effect on the drug release. L5 liposomal formulation with 5mg drug: 106mg lipid: 10.6mg cholesterol after coating with chitosan (LC_1) shows promise for furazolidone delivery to stomach to target/treat *H. pylori* (with the drug being tolerate bacterial resistant) and the drug release study was in accordance with (and supported) muchoadhesion results.

CHAPTER FIVE

PREPARATION AND CHARACTERIZATION OF MUCOPENETRATIVE LIPOSOMES FOR CO-ENCAPSULATION OF FURAZOLIDONE AND N-ACETYL CYSTEINE

5.1. Introduction

The use of liposomes for oral drug delivery is widely selected approach by different researchers. The major challenge of oral drug delivery based liposomal vesicles to treat *H. pylori* is gastro intestinal mucosa (Zhao *et al.,*2014). Mucus is a protective covering present in different parts of the body including lungs, nose, female reproductive tract and gastrointestinal tract. It protects the epithelial cell layer from invading foreign materials including bacterial and viral pathogens (Knowles and Boucher,2002). Like all other epithelial surfaces gastric epithelium is also protected by mucus layer and *H. pylori* resides in deep mucus layer towards epithelium surface and in order to kill the bacteria drug must reach to local site (Conway, 2005). The concept of using liposomes for antibiotic deliver locally to H. pylori in mucus has gained more interest in this decade.

The effectiveness of liposomal drug delivery systems depends upon two major factors: The effective loading of the drug and the site directed delivery of liposomal vesicles.

AIMS:

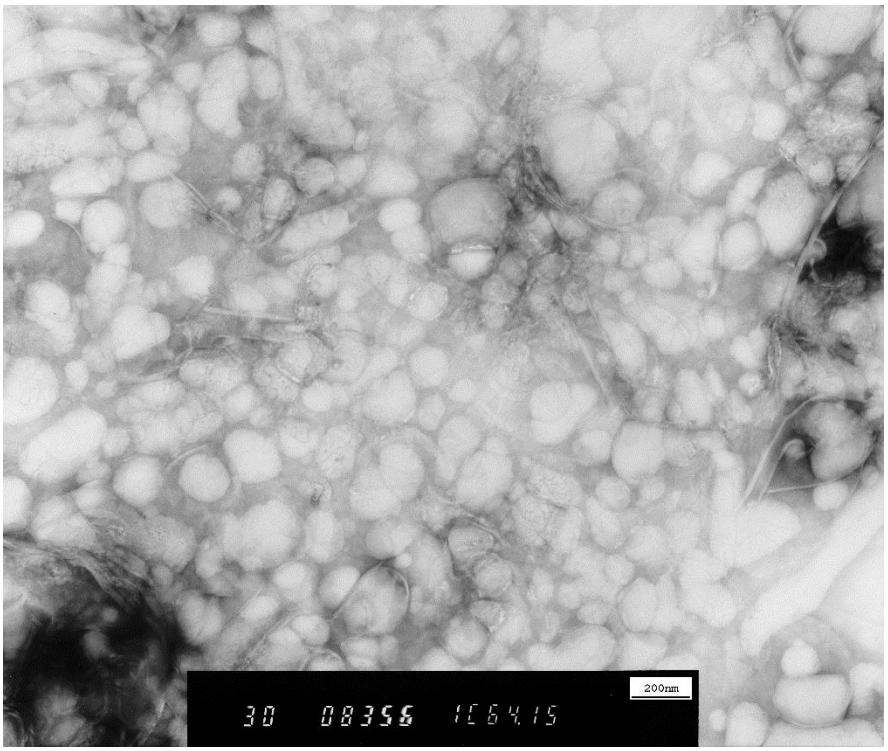
1. The aim of the current chapter is to formulate liposomal systems that can co-encapsulate hydrophilic and hydrophobic drugs.
2. To prepare mucopenetrative liposomal drug delivery systems and to check the effect of liposomal charge, pluronic F-127 and NAC on the mucus pene-tration.

5.2. Results and discussion

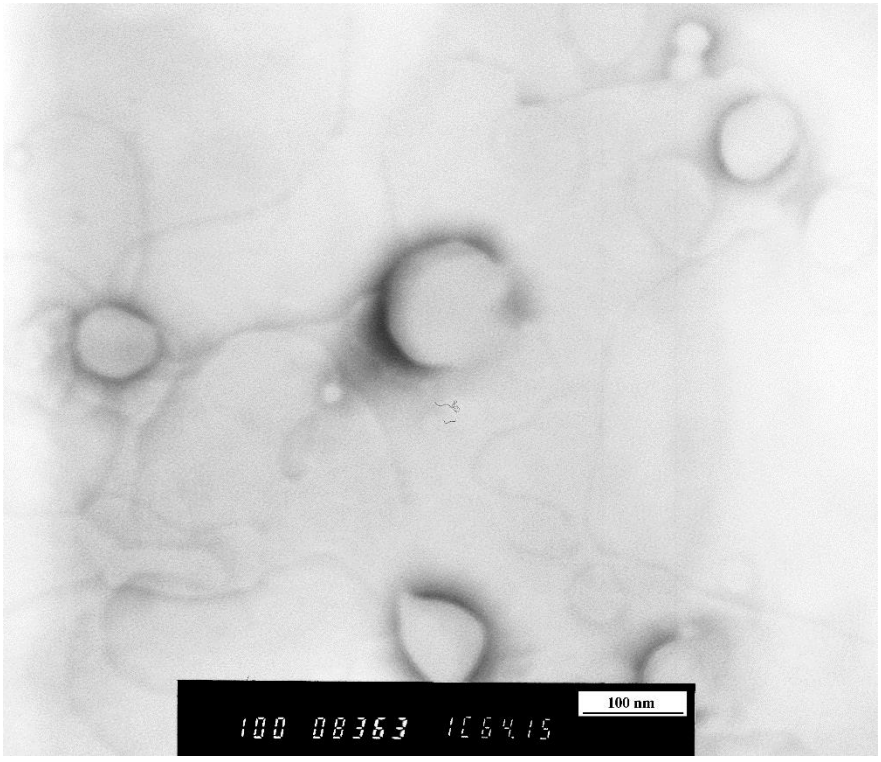
5.2.1. Morphology of mucopenetrative liposomes.

Morphology and composition of mucopenetrative liposomal formulations is presented in figure 5.1, table 5.1. The figure shows that when pluronic F-127 was added to the formulations in figure 5.1. b and c. liposomes produced were more regular in shape than MP4 without pluronic F-127 as shown in figure 5.1.a. Second finding revealed from the TEM micrographs showed that irrespective of the charge both formulations MP3 and MP1 in figure 5.1.b and 5.1.c respectively showed good regular shape. However, neutral liposomes demonstrated more aggregation as shown in figure 5.1.c as compared to cationic liposomes that were more evenly distributed in the field shown in figure 5.1.b.

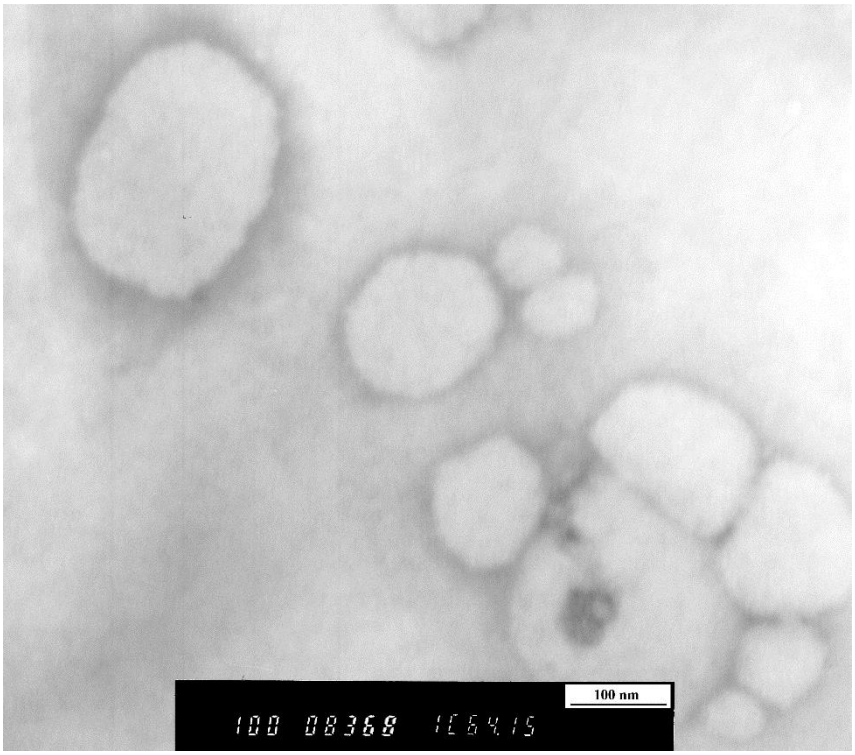
Liposomes		**Lipid**	**Cholesterol**	**DDAB**	**DCP**	**Furazolidone**	**NAC**	**Pluronic**
Positive	MP3	65	6.5	1	-	6	14	+
	MP6	65	6.5	1	-	6	14	-
Negative	MP2	65	6.5	-	1	6	14	+
	MP5	65	6.5	-	1	6	14	-
Neutral	MP1	65	6.5	-	-	6	14	+
	MP4	65	6.5	-	-	6	144	-



a).



b).



c).

Figure 5.1. Transmission electron microscopic images of mucopenetrative liposomes a). MP4, neutral liposomes without pluronic F-127, b). MP3, cationic liposomes with pluronic F-127 c). MP1, neutral liposomes with pluronic F-127.

5.2.2. Encapsulation efficiency of mucopenetrative liposomal formulations

Encapsulation efficiency of all six formulations for both drugs are listed in table 5.1. and determined by HPLC methods stated in chapter 2, sections 2.6.3.3 and 2.8.3.1 for furazolidone and NAC. Maximum encapsulation efficiency was attained by (MP6) including 62% for NAC and 65 % for furazolidone. However, the formulation MP3 contains pluronic F-127 NAC shows encapsulation of 54% and Furazolidone entrapment of 67.9%. The lowest percentage of drugs was encapsulated in MP4 and MP1 that are similar in composition apart from pluronic F-127 in MP4 refer to table 2.5. and 5.1. The overall encapsulation efficiency in all the formulations was considerably high. This high encapsulation efficiency is attributed to use of long

carbon chain lipids and reverse phase evaporation process for the preparation of those mucopenetration liposomes.

Table. 5.1. Encapsulation efficacy and zeta potential of liposomal formulation containing furazolidone and NAC along with zeta potential. For formulation composition refer to table 2.5 in section 2.7.

Formulation	**Encapsulation efficiency (%)**		**Zeta potential (mV)**	**Particle size (nm)**
	Furazolidone	NAC		
MP1	58.9 ± 3.4	51.41 ± 1.2	+3.5	490
MP2	62.2 ± 3.2	52.35 ± 0.9	-19.1	520
MP3	67.9 ± 3.8	54.25 ± 1.5	+10.3	600
MP4	56.0 ± 4.2	50.53 ± 2.1	-1.4	530
MP5	63.9 ± 6.5	59.52 ± 1.2	-36.1	570
MP6	65.6 ± 7.5	62.45 ± 2.3	+7.2	740

5.2.2.1. Reverse phase evaporation and encapsulation activity

In reverse phase evaporation (REV) organic phase is added to aqueous phase as a result phospholipids are arranged at interphase of two different phases (Cortesi et al.,1999). After evaporation of organic phase, they rearrange themselves into vesicles in aqueous system. The results in table 5.1 shows the successful encapsulation of lipophilic and hydrophilic drug into one vesicle by using this reverse phase evaporation method. This approach creates high aqueous space to lipid ratio which allows encapsulation of high amount of aqueous soluble drug. Previous studies reported four fold increase in aqueous volume to lipid ratio by using REV as compared to hand shaking method (Himanshu et al.,2011; Kataria et al., 2011). The maximum encapsulation efficiency using REV in this current chapter was 62% for

MP5. More or less similar values were reported in other study by using REV (Akbarzadeh et al.,2013). One other study suggested that encapsulation activity can be increased up to 85% by using REV approach (Handa et al.,2006). In order to study the effect of charge on encapsulation of drugs, charged liposomal formulations were prepared by REV shown in table 5.1. NAC being acidic drug interact electrostatically with cationic Didodecyldimethylammonium bromide (DDAB) that leads to higher entrapment of the hydrophilic drug (NAC).

5.2.2.2. Effect of charge on encapsulation efficiency

Encapsulation efficiency of cationic liposomes was highest followed by anionic liposomes as shown in table 2.5 and 5.1. However, the neutral liposomes showed the least encapsulation efficiency when compared to charged liposomes. Data suggested that encapsulation depends upon the electrostatic interaction between the charged lipids layer. This high encapsulation efficiency of NAC in charged liposomes could be possibly due to the electrostatic repulsion between the lipid layers of multi-layered liposomes that in turn increase the aqueous cavity of liposomes to accommodates high amount of water soluble NAC (Poyner et al., 1993). Another study suggested that the negatively charged lipids push the bilayer apart and increase the encapsulation efficiency of liposomes (Çağdaş et al., 2014). The encapsulation of NAC was influenced by the addition of pluronic F-127 in formulations. Both charged and neutral liposomes entrapped less amount of NAC in aqueous space in the presence of pluronic F-127. However, when pluronic F-127 was not present in the formulations all three formulations showed increased entrapment efficacy with maximum entrapment of 62%. However, entrapment of furazolidone that accumulates in the lipid layers was not influenced by pluronic F-127.

5.2.3. Mucopenetration

Figure 5.2 shows percentage of mucopenetration of six different formulations in the reconstituted mucin type III at concentration of 60mg/ml in 1mm depth (Dowsan et al, 2004). 1 mm of depth was selected as the upper shallow mucus layer of the stomach is more or less equal to 1mm (Gartner and Hiatt, 2001). MP1 demonstrated the highest percentage of mucopenetration that of 52% followed by MP4 that showed 22% of mucopenetration.

Liposomal formulation (MP1) achieved 24% in the first hour and 36% in the second hour but the maximum penetration achieved was 52% after three hours. The experiment was conducted for three hours because the normal resident time in the stomach is about three hours. Liposomal formulation (MP4) showed maximum of 25% in three hours however in first and second hours it was 6% and 18% respectively.

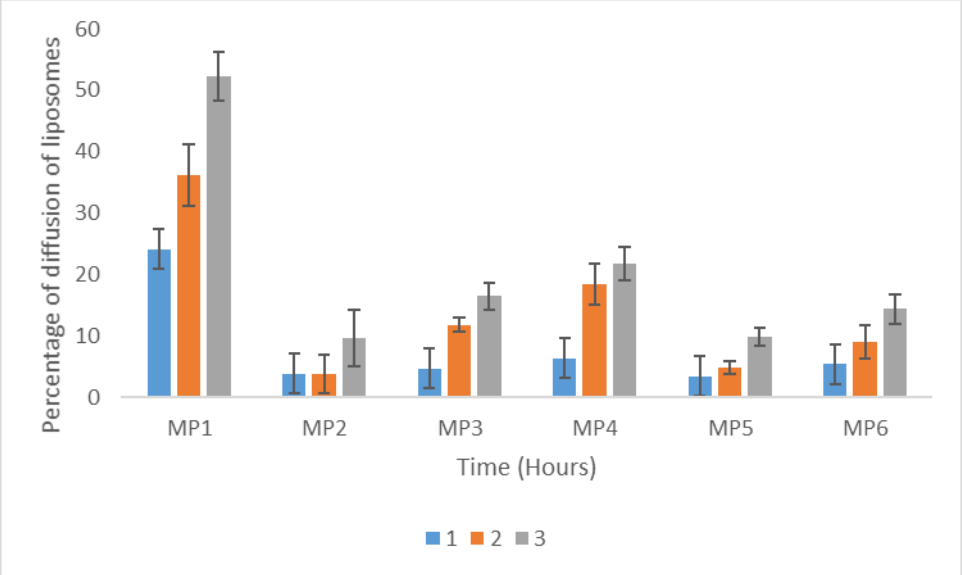


Figure 5.2. Diffusion of mucopenetrative liposomal particles from MP1 to MP6 through 1 mm thick sigma mucin type III in silicon tube maintained at pH 6.0 at 37ºC at 1, 2 and 3 hours' time. For composition of mucopenetrative formulation refer table 2.5 in chapter 2.

5.2.3.1. Effect of charge on mucopenetration

According to the results in the current study, the formulation with no net charge penetrated up to 52% in mucus. However negatively and positively charged liposomes have not shown any promising results in terms of mucopenetration. Similarly, it was concluded in a study that cationic liposomes adhere to negatively charged mucus 3 folds more than electrically neutral liposomes (Samuel et al., 2009). According to the study electrically neutral particles diffuse more efficiently in cystic fibrosis sputum as compared to anionic particles (Min et al., 2015). It was hypothesized in one study that positively charged particles can bind to anionic glycosylated region due to polyvalent electrostatic interactions (Aljayyoussi et al., 2012). However electrically neutral nanoparticles could easily diffuse mucus layer (Jyssum., 2012). According to one study both negatively and positively charged nanoparticles have shown limited diffusion as compare to nanoparticle with net neutral charge that shows surface charge has high impact on diffusion of nanoparticles through mucus (Flavia and Andreas 2012).

5.2.3.2. Effect of Pluronic F-127 on mucopenetration

Triblock copolymer of poly (ethylene glycol)-poly (propylene oxide)-poly (ethylene glycol) (PEG-PPO-PEG; known as Pluronic F-127) has proven effect on mucopenetration in different studies (Min et al.,2015). In one study pre-treatment of cervical mucus with pluronic F-127 increase the penetration of nanoparticle through mucus (Samuel et al., 2009). Figure 5.2 shows highest degree of mucopenetration which was achieved by MP1 that contains pluronic F-127 and bearing a neutral charge. This high percentage of mucopenetration could be the effect of neutral charge or due to the presence of pluronic F-127. Research has shown that electrically neutral nanoparticles are retarded in mucus due to hydrophobic nature and as such hydrophilicity of liposomal formulation also contributes to the degree of

mucus penetration (Mantle et al.,1989). However, increase in hydrophilicity can be used to enhance the mucus penetration (Olmsted et al., 2001).

Therefore, the addition of pluronic F-127 in the current study imparted the hydrophilicity to the liposomal formulation that in turn increase mucus penetration. Secondly the effect of pluronic F-127 on mucopenetration was only obvious in MP1 whereas its effect was not clear in formulation (MP2) and (MP3) having the same concentration of pluronic F-127. The apparent reason seems to be the presence of charged moieties that masked the mucopenetrative effect of pluronic F-127 in formulations. The effect of pluronic F-127 on penetration through mucus is explained in another study conducted in study in 2011 suggested that treatment of nanoparticles with pluronic F-127 can improve the diffusion rate (Yang et al., 2011). It is also reported in a study that nanoparticles with hydrophilic long chain polymers like pluronic F-127 increased the mucopenetration. (lie et al., 2007).

5.2.3.3. Synergistic effect of pluronic F-127 and NAC on mucopenetration.

NAC is a mucolytic agent that has shown improved mucus penetration of nano scale particles and liposomes (Samuel et al., 2009). The results in this chapter indicate the substantial increase in mucus penetration of liposomal formulations containing NAC with no net surface charge (MP1). These findings are in good agreement with study conducted by Ferrari and Alton that mentioned the partial improvement of non-viral gene vector could be improved by use of NAC in sheep tracheal model (S. Ferrari et al., 2015) The mucolytic properties of NAC could be due to multiple mechanism. It can reduce the bulk rheology of mucus (Sheffner et al.,1964; Henke and Ratjen,2007) and replace disulphide bond of mucin polymer connecting the mucin protein by thiol group which in turn reduce the viscosity of mucin (Henke and Ratjen,2007). All six formulations contained equal amount of NAC bearing different surface charges. MP1 and MP4 having no net charge have shown the good diffusion

through mucin. However, there was no considerable diffusion through mucus for the of the formulations. Similarly, the use of NAC on its own in formulation MP4 has showed lower degree of penetration as compared to MP1 that contained both NAC and pluronic F-127. These finding indicates that both pluronic F-127 and NAC in combination demonstrate the synergistic effect for mucus penetration

5.2.3.4. Effect of particle/pore size and pH on mucopenetration.

Size of the liposomes is an important factor that must be kept in view for the particles diffuse very smoothly through mucus. The mucus exhibits different mesh size depending on its location and ranges from 340nm to 550nm in cervical vaginal mucus (CVM) (Min et al.,2015) and may be reach up to 1 µm (Yen and W. Mark 2009). However, mesh size of gastric mucin ranges from 200-650 (Celli *et al.,* 2005) Particle size of liposomes in this chapter range from 350nm-800nm is listed in table 5.1. In addition to mesh size and particles size other contributing factors that support the diffusion of liposomal particles through reconstituted gastric mucus were pre-determined. These factors were selected on the basis of their potential to achieve the overall aim of the study.

Experiment of mucopenetration was carried out on pH 6.0 *H. pylori* survive in high pH conditions and the presence of *H. pylori* and the use of PPI that increase the pH of stomach between 6-7. This alkaline pH adds the benefit of enhancement in the penetration as described in one study that acidic pH below 4 can increase the viscosity of mucus (Hong et al.,2005). Another study explained the relation of pH with bulk viscosity of mucus where a drop in pH from 6 to 4 increases the viscosity and bulk moduli up to 1000 fold magnitude. Further decrease in pH from 4 to 2 increased the viscosity by 10 fold. This decrease in bulk viscosity decrease the mesh size even up to 100 folds (Ying-Ying et al.,2013). Therefore, keeping the pH

alkaline by the use of proton pump inhibitor increases the mesh size and facilitates the diffusion of neutral particles through mucus.

NAC in formulations also increases the mesh size of the mucus ascribed by another study conducted by (Jung et al.,2011) and claimed that treatment of mucus with NAC increase the mesh size of native mucin from 300 to 1300 nm.

5.2.3.5. Statistical analysis of mucopenetration.

The combined effect of charge and pluronic F-127 was analysed statistically and the finding proved that the effect of charge as well as pluronic F-127 was significant at all time point. According to post hoc Tukey test the effect of charge was less influential in mucopenetration. Neutral formulation in the absence of pluronic F-127 showed high efficiency (21% after 3 hours) as compared to positively (14% after 3 hours) and negatively charged (9% after 3 hours) liposomes. However negatively charged liposomes showed lesser degree of penetration as compared to positive.

Similarly, in the case of formulation with pluronic F-127 the effect of charged particles on mucopenetration was significantly reduced ($P<0.05$) as compared to neutral particles. Neutral particles demonstrated 52% of diffusion after three hours whereas positive and negative formulations showed only 16% and 9% respectively. Therefore, presence or absence of pluronic F-127 has no effect on negatively charged formulations after three hours.

The time taken by particles for mucopenetration also has effect on the extent of mucopenetration. In the first hour, the effect of pluronic F-127 on charged particles is less as almost all the charged formulations diffuse equally. The possible explanation is short time span at which the samples were analyzed from the beginning of experiment but with the passage of time in the presence of pluronic F-127 there was significant ($P<0.05$) diffusion of positive and negative formulations up

to two hours. In third hour the presence of pluronic F-127 cationic liposomes showed more penetration than cationic non pluronic liposomes. Anionic liposomes have not shown any significant difference in diffusion with the presence of pluronic. This could be due to high release of pluronic F-127 in positively charged liposomes as compared to negatively charged liposomes. However, the release profile demonstrated the high release of NAC in negatively charged liposomes therefore, in theory, negatively charged liposomes should show high diffusion rates. But in actually experiment positively charge liposomes that release lesser degree of NAC showed more penetration. This paradox is explained by the fact that the repulsive forces between negatively charge mucus and anionic liposomes is more than binding forces of cationic liposomes and mucus membrane.

5.2.4. *In vitro* drug release of mucopenetrative liposomes

5.2.4.1. *In vitro* drug release of furazolidone

The release of furazolidone and NAC from the formulations were analysed at three different time points to check the effect of charge and pluronic F-127 on the release behaviour of liposomes. After 30 min of *in vitro* drug release test there was no significant difference in release profile of different formulations ($p = 0.138$) containing of pluronic F-127. As represented in figure 5.3 (a) the release rate for first three formulations was almost similar to their counterparts without pluronic F-127. However, the effect of charge was significant. Both formulations bearing the positive surface charge i.e. MP6 and MP3 gave the maximum release of 12%. However, the neutral formulations MP1 and MP4 released 12% and 11% drug respectively which was lower than positive formulations followed by negatively charged formulations MP2 and MP5 that released least amount of drug in 30minutes. After 60 minutes of drug release the effect of pluronic F-127 became significant. At 60 minute the

positively charged formulations MP6 and MP3 release the maximum amount of drug 25% and 20% respectively.

However, 23% of the drug was released by MP5 and 19% of the drug released from MP2 which shows less release as compared to positively charged liposomes. In the case of neutral liposomes, the release of the drug remained unaffected by the presence of pluronic F-127 as shown in figure 5.3 (a) where both formulations MP1 and MP4 released almost 18-19% of drug. Similarly, at 240 minutes the formulations with pluronic F-127 released less amount of the drug in the presence of charged moieties and demonstrated unaffected drug release in neutral liposomes. However, at this time point the negative formulations release maximum amount of the drug as compared to positively charged and neutral formulation. It was observed in the current study that the drug release rates of positively charged liposomes were higher in the first two hours as compared to negative and neutral liposomal formulations. These findings are explained in a study that also claimed the high release from positively charged liposomes in first two hours (Mohamed et al.,2006).

5.2.4.2. *In vitro* drug release of NAC

NAC that is hydrophilic drug remained in aqueous cavity and showed non consistent release profile. On the other hand, the effect of pluronic F-127 on furazolidone (that is hydrophobic drug and stays in lipid layers) was observed on drug release from charged liposomes as compared to neutral liposomes. The effect of charge on release of NAC demonstrated that negatively charged liposomes gave maximum release for all of the selected time point when compared with positive and neutral as shown in figure 5.3 (b).

The results of *in vitro* drug release for both drugs mentioned above were in agreement with a study stated negatively charged liposomes release maximum amount of drug as compared to positive and neutral liposomes (EL-Nesr et., al

2010). In another study that imparting the negative charge in liposomes enhance the release rate over the neutral particles at high pH similar to current study conducted at pH 6.0. (Mohamed et al.,2006)

The release rate of NAC from neutral liposomes was less at final time point as compared to negative and positivity charged liposomes which is in agreement with the results, showed that release of doxorubicin was less from neutral particles as compared to anionic and cationic liposomes in another study (Yu et al.,2012). However, in contrast liposomes provided high drug release in first two hours. The possible explanation could be the short time span of the sample taken from the start of the experiment.

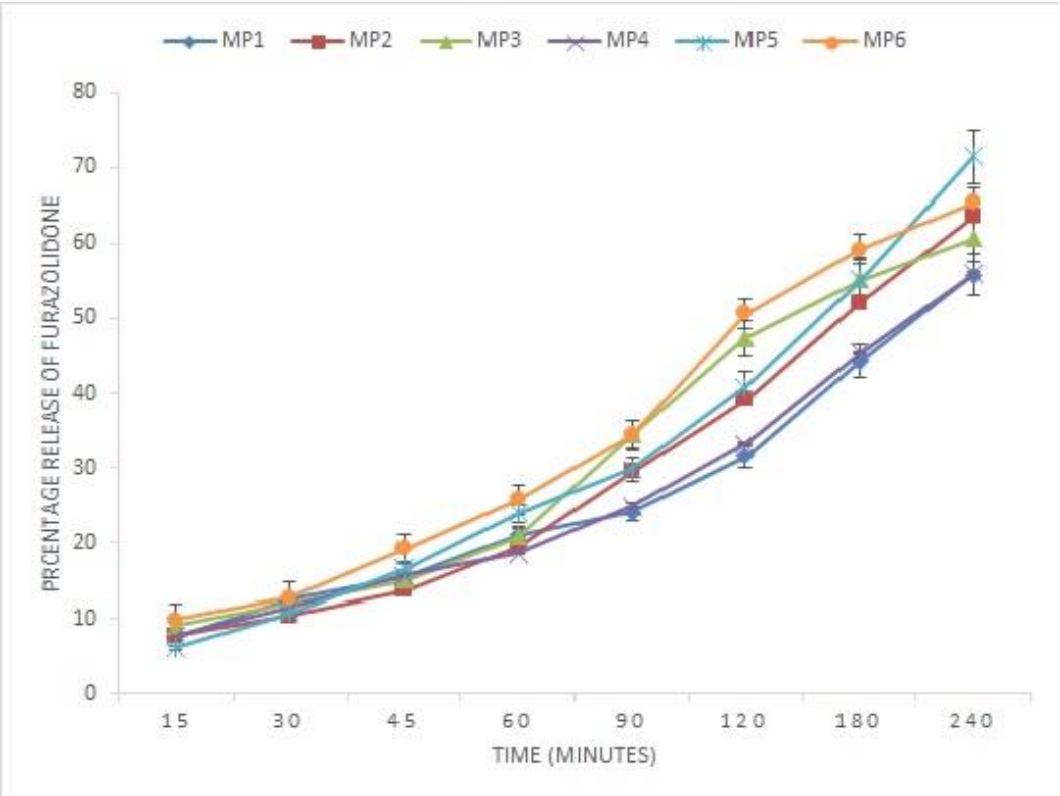


Figure. 5.3 (a). In vitro drug release of furazolidone from mucopenetrative formulation from MP1 to MP6 up to 4-hour time at pH 6.0. For formulation compositions refer to table 2.5.in chapter 2

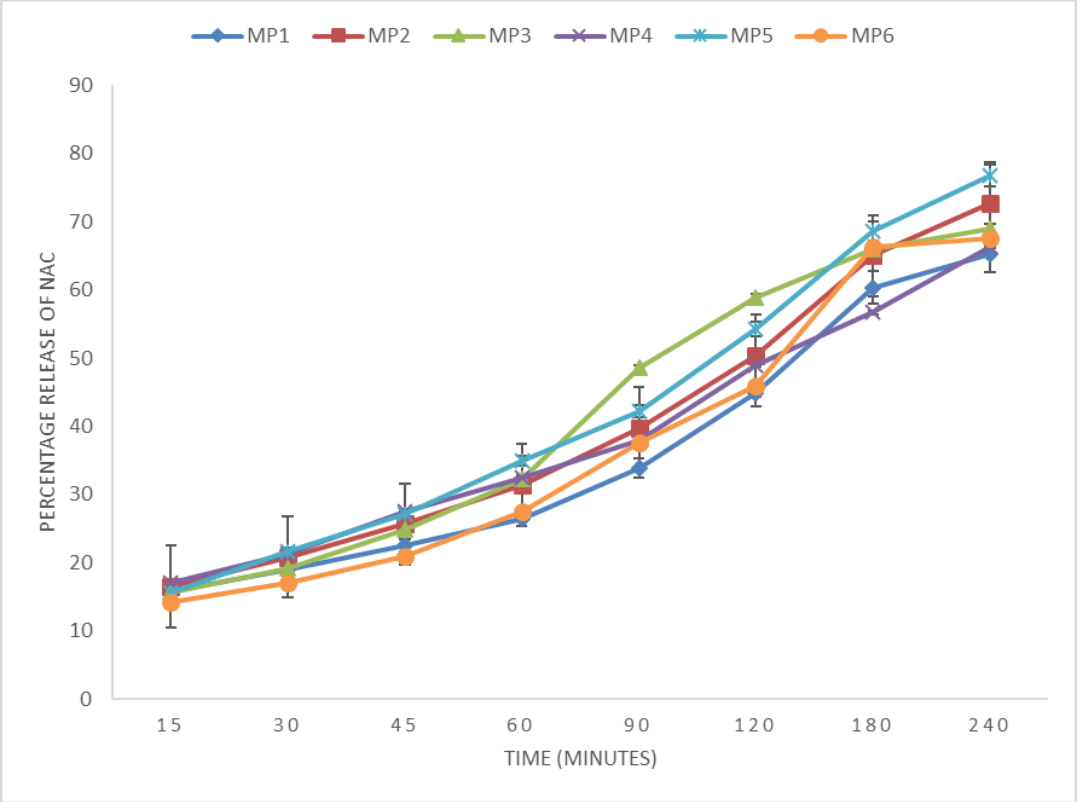


Figure. 5.3 (b). In vitro drug release of NAC from mucopenetrative formulation from MP1 to MP6 up to 4-hour time at pH 6.0. For formulation compositions refer to table 2.5.in chapter 2

The liposomal particles with neutral charge showed better mucopenetration in the presence of pluronic, that shows the positive effect of pluronic on mucopenetration when used in combination with NAC. However, the release and encapsulation efficiency of charged liposomes (cationic and anionic) have shown better results as compared to neutral liposomes. But the drug encapsulated in neutral liposomes can maintain enough concentration on site for required period of time to completely kill the bacteria.

CHAPTER NO 6

Antimicrobial activity of furazolidone based formulations against *Escherichia coli* and *Helicobacter pylori*

6.1. Introduction

The success of first line triple therapy for *Helicobacter pylori* is now becoming a global challenge. Multiple factors account for treatment failure but two most important factors are resistance and therapeutic approach. Introducing low resistance antibiotics in therapeutic regimens is not enough. However, finding appropriate therapeutic approach can achieve the overall eradication. *H. pylori* resides in gastric epithelial surface but it does not invade the epithelial cells therefore, local therapeutic approach with combination of two different drug was used to make augmented therapy. The experiment for antimicrobial assay was first performed on *E. coli* house strain to optimize the method for *H. pylori* because it is difficult to grow *H. Pylori* in short time scale to establish the protocol. The protocols for calibration curve, inoculum size determination, minimum inhibitor concentration and time killed curve of bacterial population with liposomal bound drug were optimized on *E. coli* presented in section 6.2. and 6.3. of this chapter. Two drugs were used for antimicrobial activity against *H. pylori* i). furazolidone and ii). NAC.

AIMS:

1. The aim of this chapter was to check the augmented effect of furazolidone and NAC on killing time of *H. pylori*
2. To check the minimum inhibitory concentration of furazolidone and NAC against *H. pylori*
3. To establish the assay protocol for free and liposomal bound furazolidone on *E. coli.*
4. To check the antimicrobial activity of all the formulations prepared in this study

6.2. Results and Discussion

6.2.1. Calibration growth curve of *E. coli*

There were no colonies in first and second dilutions. However, the colonies started to appear in third dilution after half an hour and continues till 4th dilution with in three and half hours. after 4.5 hours the absorbance was high therefore it was diluted before plating out and the colonies appear in 4th dilution. However, the calibration curve of *E. coli* is represented in figure 6.1.

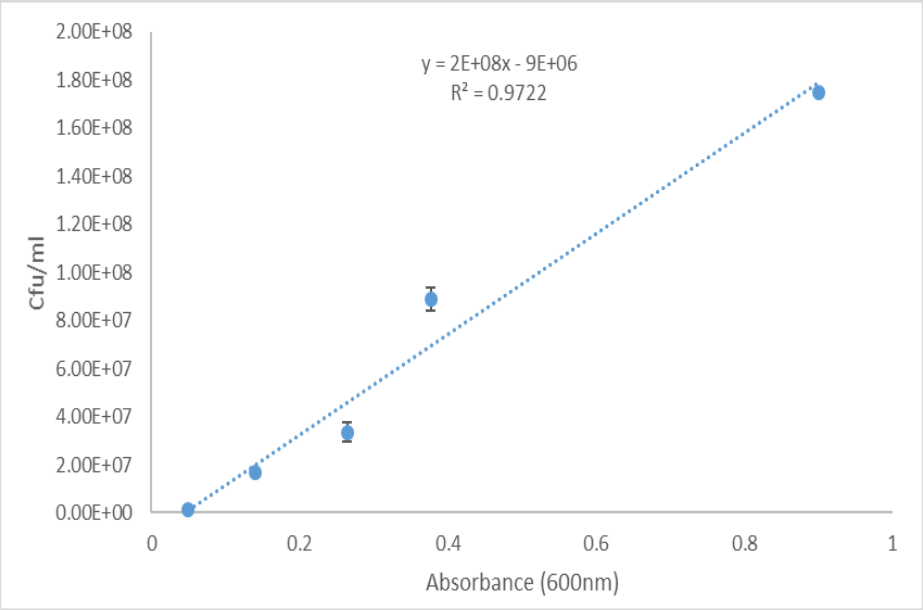


Figure 6.1: Absorbance v/s cfu/ml curve of actively growing *E. coli* culture at OD 600nm incubated in luria-Bertani broth at 35ºC.

6.2.2. Minimum inhibitory concentration of furazolidone against *E. coli*

Figure 6.2 shows the minimum inhibitory concentration curve of furazolidone against *E. coli*. The curve was based on the absorbance values of bacterial suspension after 24 hours of incubation. This curve revealed that there is only little drop in absorbance values from 1 µg/ml up to 8µg/ml and then substantial drop can be seen at 16µg/ml. Therefore, this reading was assumed as minimum inhibitory concentration and was used for further analysis for liposomal formulations.

According to one study the MIC value of furazolidone was also determined as 16µg/ml which shows the results in current study are in agreement with study conducted by Kobe in 1996 (Kobe *et al.,* 1996).

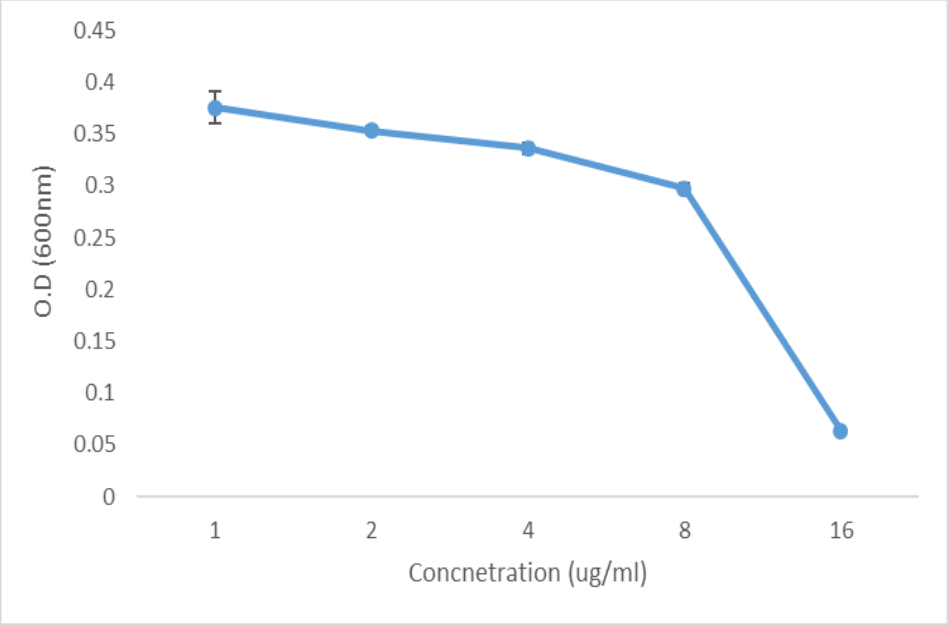


Figure 6.2: Minimum inhibitory curve of furazolidone against *E. coli* in LB broth using 96 well microtiter plate. The experiment was performed in at absorbance 600_{nm}

6.2.3. Break point of liposomal formulation (L5) against *E. coli*

The break point of formulation contains 16 µg/ml of furazolidone takes 4 hours to kill the bacterial cells. However, if the concentration of drug is reduced below MIC v the efficacy of formulation to kill the bacterial cell become linear after 3 hours as shown in figure 6.3. Most probably bacteria took three hours to withstand the effect of drug. After three hours the remaining bacterial cell started to regrow again from the live. The control in figure 6.3 shows the growth of the bacterial cell even after 4 hours of incubation that clearly shows killing effect is only due to the drug but not their natural death cycle after four hours.

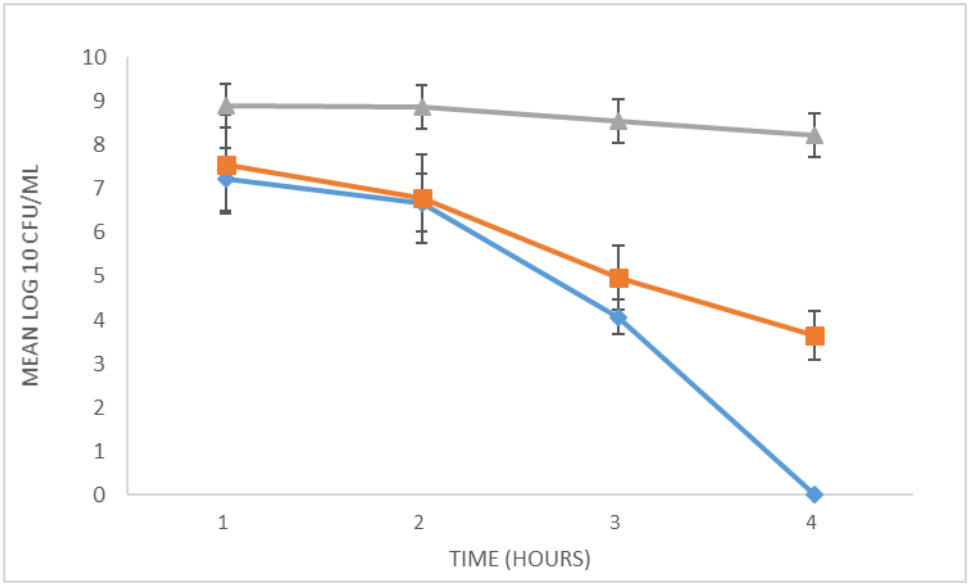
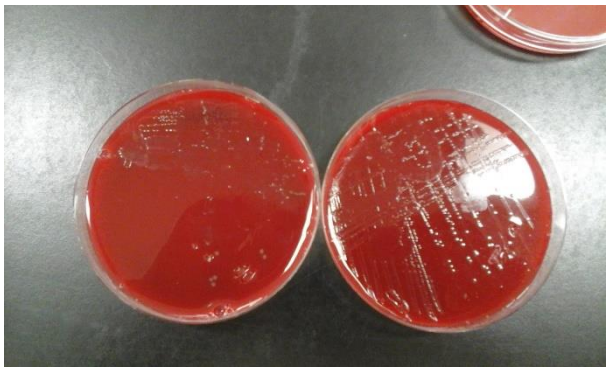


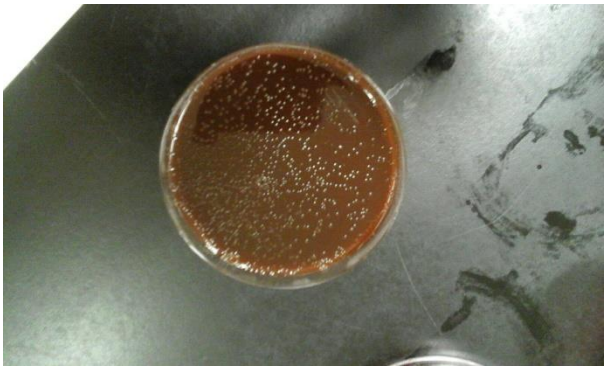
Figure 6.3. Time killed curve experiment of furazolidone against *E. coli inoculated in on agar plate and incubated at 37ºC for 24 hours before counting the colonies. mean value of log number of cfu per millilitre was potted against time. Representative results of five different experiment are shown. Symbol:* ▲control with no furazolidone, ■ 8µg/ml concentration of furazolidone and ♦ 16µg/ml of furazolidone**.**

6.2.4. Identification of *Helicobacter pylori*

Colony morphology, gram stained microscopic slides confirmed *H. pylori* in fig 6.4. In fig 6.4 (a and b) appearance of circular, convex and translucent colonies of 1-2 mm in diameter on dent supplemented blood and chocolate agar plates indicates the culture of *H. pylori*. Pink colour rods in microscopic slides followed by gram staining further confirms *H. pylori* in fig 6.4 (c)



a).



b).

c).

Figure 6.4. a) Colonies of *H. pylori* on blood agar, b) chocolate agar, c) microscopic gram stained slide.

6.2.5. Antimicrobial assay of furazolidone and NAC against *H. pylori*

6.2.5.1. Minimum inhibitory concentration of furazolidone and NAC against *H. pylori*

MIC values of free furazolidone and NAC were 4.1 µg/ml and 7mg/ml respectively against *H. pylori* (NCTC 12455). Figure 6.5 (a and b) shows the substantial drop in absorbance for furazolidone and NAC at concentration of 4µg/ml and 7mg/ml respectively therefore these values were considered as minimum inhibitory concentrations against *H. pylori* NCTC 12455.

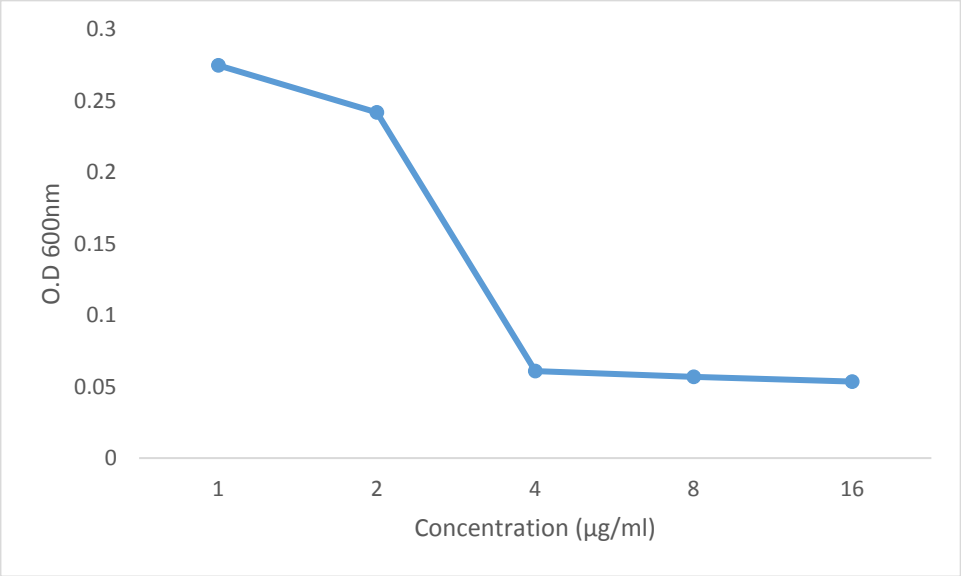


Figure 6.5: a). Minimum inhibitory curve of furazolidone against *Helicobacter pylori in* Brain heart infusion broth using 96 well microtiter plate incubated for 24 hours on shaker incubator in anaerobic jar system at 37ºC. The experiment was performed in triplicate at absorbance was reordered at $600_{nm.}$ OD 600nm values were plotted against concentration of furazolidone in µg/ml.

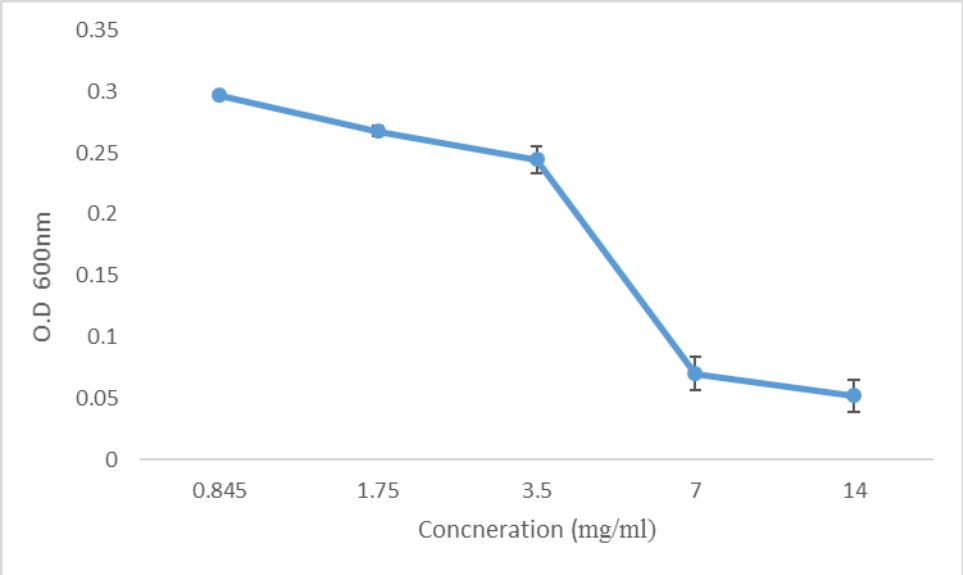


Figure 6.5: b). Minimum inhibitory curve of N-acetyl cystiene against *Helicobacter pylori in* Brain heart infusion broth using 96 well microtiter plate incubated for 24 hours on shaker incubator in anaerobic jar system at 37ºC. The experiment was performed in triplicate at absorbance was reordered at $600_{nm.}$ OD 600nm values were plotted against concentration of furazolidone in mg/ml.

6.2.5.2. Antibiotic augmentation and time killed curve of formulation (MP1)

Based on MIC values, time killed study was conducted to check the augmented effect of both drug in single formulation (MP5) against *H. pylori*. Figure 6.6 shows the results of time killed curve for NCTC 12455. At half of the MIC (2µg/ml) of furazolidone in combination with 1 % MIC of NAC demonstrated initial drop of 1 cfu ml-1 in the colony count up to 3 hours but then re growth was observed up to eight hours of incubation. Similar phase of regrowth was observed in the study conducted by (Rukholm *et al.,* 2006) in which antibiotic was unable to maintain the antimicrobial performance at its MIC after 6 hours. This suggests that the combined effect of sub inhibitory concentration of furazolidone with NAC was not sufficient to induce the significant cell killing. Higher concentration decreased the significant drop of log i.e. by increasing the concentration 4 x MIC of furazolidone complete killing was observed after eight hours of incubation. This shows the inhibition of *H. pylori* is concentration dependent. Ideia Maria *et al* reported similar results in their studies that increased in concentration by four times of initial MIC completely killed the bacterial cells. However, the same concentration of furazolidone when used in combination with 1 % NAC showed complete killing in 6 hours rather than 8 hours. NAC in lower concentration decreased the MIC of the furazolidone (<4 µg/ml) therefore, when furazolidone was used in same concentration in combination with 1 %NAC it reduced the time to kill the bacterial cells. The modulation effect of NAC when used in combination reduced the MIC of carbenicilline from 16 µg/ml to 1 µg/ml as reported in another study. (Zhao and liu 2010). Manish and Narendra reported that NAC could be used as modulator of different antibiotics (Manish and Narendra, 2010). Results in this study were also in agreement with hypothesis postulated that NAC augments the activity of antibiotic to reduce the *H. pylori* cells (Kian and Frank 2011). However, the kinetics of killing curve shows that use of increased

concentration of furazolidone by MIC x 8 effectively killed the bacterial cells as quick as the killing was observed by use furazolidone 4 x MIC in combination with 1 % NAC. These results are supported by another study in which use of increased concentration of amoxicillin or the combination of amoxicillin with glycine as modulator increased the rate of killing of H. Pylori (Masaaki *et al.,* 2004)

The mechanism of modulation is still not clear but it could be of two different reason, Thiol group present in cysteine could cause antibacterial activity. (Gowswami *et al.,* 2007) or the presence of sulphydryl group may react with cell proteins (Zaho and liu 2010).

However, when used in liposomal formulation 8X MIC of furazolidone and 1 % MIC of NAC achieved the complete killing of bacterial cell in 2.5 hours which is the resistant time of unmodified dosage form in stomach under normal physiological conditions (Rajak *et al.,* 2011).

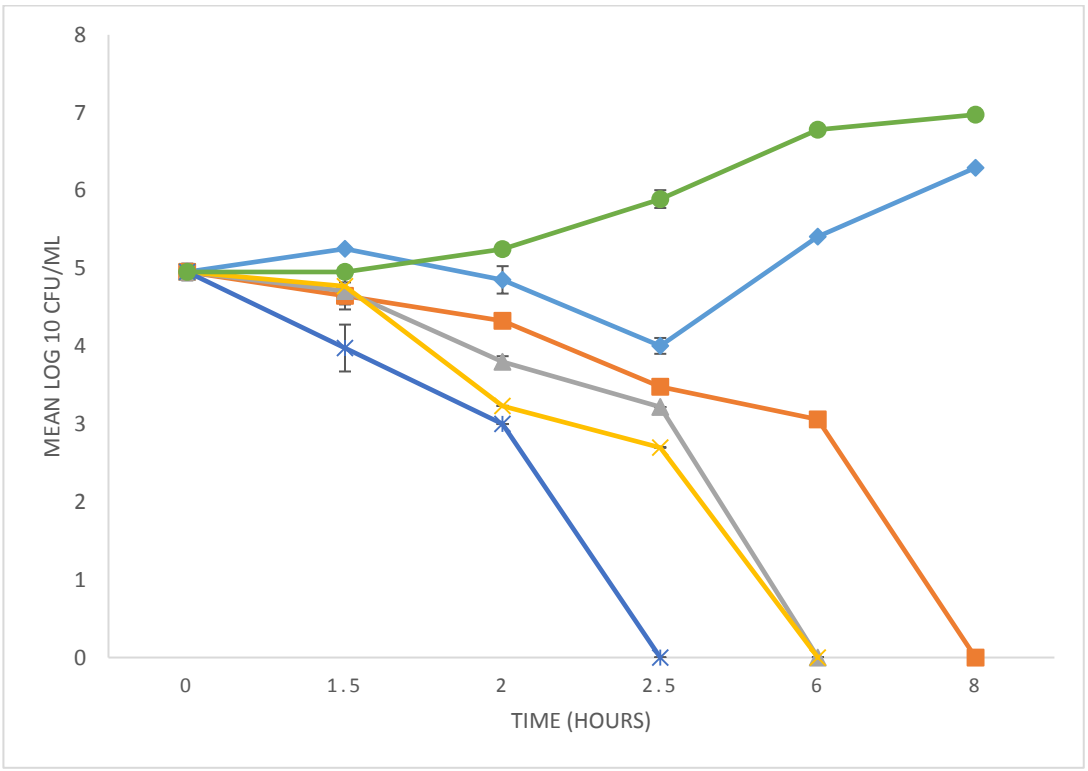


Figure 6.6. Time killed curve experiment of furazolidone augmented with NAC at concentration of 1 % of its MIC against *H. pylori* inoculated *on* blood agar plate and incubated at 37ºC for 24 hours in

anaerobic jar with campyGen gas packs before counting the colonies. Mean value of log number of cfu per millilitre was potted against time. Representative results of six different experiment are shown. Symbol: ▲ Fur+1 % NAC at 4 X MIC, ■ Fur at 4 X MIC, ♦ Fu r+1 % NAC at 1/2 X MIC, ● Control with no Fur and NAC, ✱ Fu r+1 % NAC at 8 X MIC, **X** Fur at 8 X MIC.

6.2.6. Time killing curve for mucoadhesive liposomal (L5) and microparticles (F2) formulations against *H. pylori*

As shown in figure 6.7 liposomal formulation with concentration equals to 8 x MIC of furazolidone takes six hours to completely kill the bacteria, which is almost similar to the test performed in previous section with free drug as shown in figure 6.6. This result shows that the liposomal bound furazolidone and free furazolidone has similar killing efficiency. Literature shows that chitosan has generic antimicrobial activity. However, chitosan in the formulations has no antimicrobial activity against *H. pylori* because the control used in mucoadhesive liposomal showed the normal increase in growth over time. The possible reason could be the low concentration of chitosan used while diluting down the liposomal formulation for antimicrobial assay procedure. When liposomal formulation with less concentration of furazolidone (MIC x 6) was used in the assay. There were still some colonies after 6 of incubation that means MIC less than MIC X 8 take more than 6 hours to kill the bacteria.

Microparticle formulation (8 X MIC FS_2) with glutaraldehyde showed sharp decline in the colony count and the killing was achieved in three hours instead of six hours. However, the same concentration MIC x 8 that previously took six hours to completely kill the bacteria with MP_1 (mucopenetrative liposomes) and L_5 (mucoadhesive liposomes) formulations. This could be due to presence of glutaraldehyde in the formulation because the killing was also observed when blank microparticles without any drug were used as control (Control FS2). However, the decline by using control was not as sharp as in the presence of furazolidone (8 X

MIC FS_2) shown in figure 6.7. The control for microparticles formulation in figure 6.10 decreased the bacterial count up to certain level but could not kill the bacteria completely while using the (8 X MIC FS_2) that is otherwise similar to control with the presence of furazolidone completely killed the bacteria in three hours. There is literature evidence which proves that glutaraldehyde has antimicrobial activity against *H. pylori* and similar effect can be seen in current study

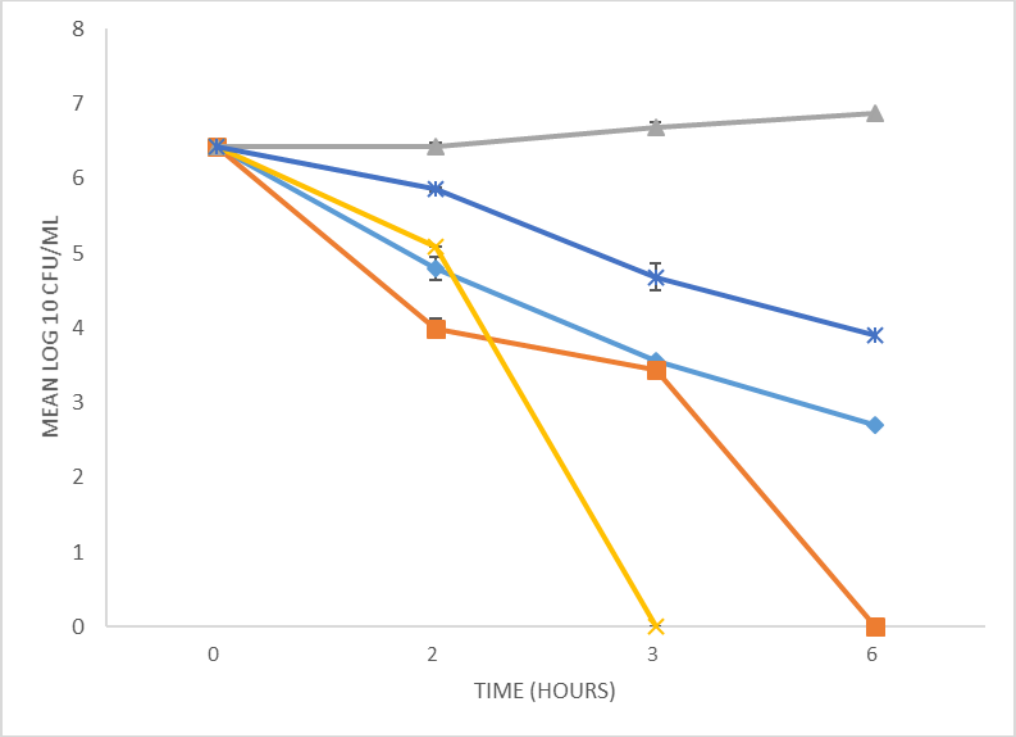


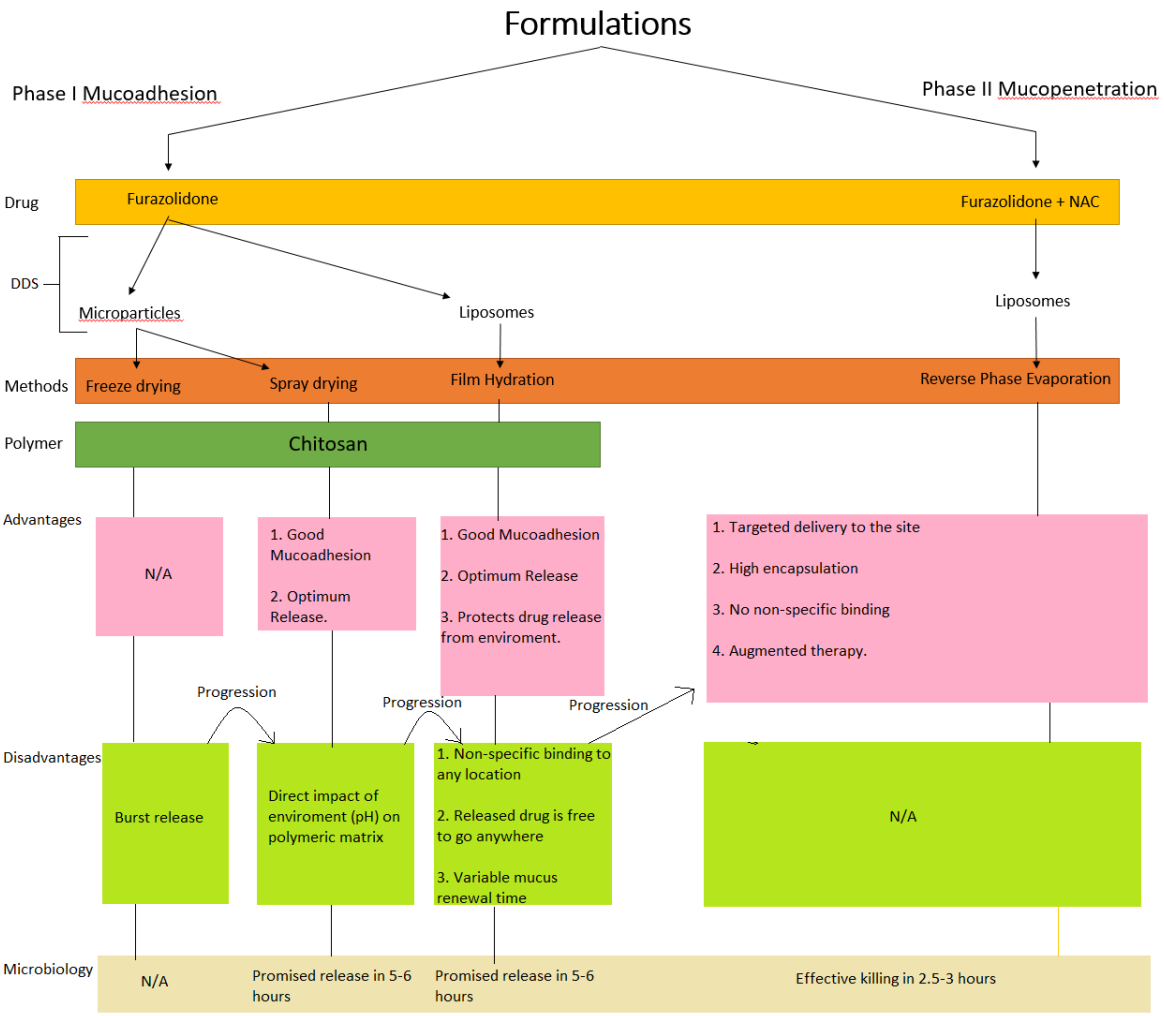
Figure 6.7. Time killed curve experiment of furazolidone against *H. pylori* inoculated *on* blood agar plate and incubated at 37ºC for 24 hours in anaerobic jar with campyGen gas packs before counting the colonies. Mean value of log number of cfu per millilitre was potted against time. Representative results of six different experiment are shown. Symbol: ▲ Control LC5, ✱ Control F2 with glutaraldehyde and chitosan but no Fur, ♦ L5 with Fur at 6 X MIC, ■ L5 with Fur at 8 X MIC, **X** F2 with glutaraldehyde, chitosan and Fur at 8 X MIC.

6.3. Conclusion

In conclusion, both mucopenetrative formulations and the drugs have shown the effective antibacterial activity against *H. pylori*. It can beconcluded by comparing the results of this chapter with literature that MIC of furazolidone is much lower than other conventionally used antibiotics against *H. pylori* hence the lower dose of furazolidone could be potentially used as primary anti helicobacter agent to overcome the side effects associated with the drug. The time required by the furazolidone to kill the *H. pylori* could be decreased by augmented effect of N- acetyl cysteine and hence the mucopenetrative formulation could be suitably used against *H. pylori* as the combination of furazolidone and NAC decreased the killing time from 6 hours to 2.5 hours which concedes with the time of stay of drug in stomach. Similarly, the use of furazolidone alone takes six hours to kill the bacteria completely. Therefore, it provides the basis of using mucoadhesive liposomal formulation against *H. pylori* as the formulation stays in stomach for six hours. N-acetyl cysteine used in the formulation could enhance the eradication by augmentation to reduce the time for killing, modulation to decrease the MIC and mucolytic effect to disrupt the biofilm and reduce the resistance of *H. pylori* against furazolidone and NAC itself.

CHAPTER SEVEN

CONCLUSION



H. pylori has evolved number of resistance mechanisms against different groups of antibiotics. The most common mechanisms against tetracycline and macrolides are mutation in ribosomal subunit, and alteration in penicillin binding protein against penicillin i.e. amoxicillin. Because of increased antimicrobial resistance complete eradication of H. pylori became one of the biggest challenge for clinicians and researches these days. Genetic mutation is considered as a major contributing factor for phenotypic variation that enables the organism to stress response and develop antibiotic resistance.

Microenvironment of *H. pylori* biological niche plays a critical role in colonization and pathogenesis. Most interesting fact about this bacteria is that it lives and thrive in

alkaline conditions but on the other hand its natural habitat is in the stomach where the condition is extremely acidic and no microorganism can withstand such harsh condition therefore stomach was considered as a sterile part of the body but presence of H. pylori has changed the concept. Therefore, in order to survive in stomach conditions this bacteria has developed number of survival mechanism. Its spiral shape helps it penetrate through mucus layer of stomach and reaches to epithelial surface where the pH is high (pH 7.0) as compared to lumen (pH 1.3-2.0). *H. pylori* is able to produce enzyme called urease that converts urea from food, gastric juice and saliva to ammonia and bicarbonate that are extremely alkaline. This create the alkaline cloud around bacteria and it can easily survive the hard conditions of the stomach. It is also extremely difficult for our natural defence mechanism to encounter *H. pylori* as it lives deep in mucus where the immune system is unable to send infection fighting natural killer cells and white cells. These natural eradicators cannot penetrate through mucus and start dumping that makes the situation more worst as their dead debris put oxidative stress by producing superoxide radicals. That leads to gastric ulcers and if untreated it transforms into gastric carcinoma.

Therefore, this study is aimed to solve two major challenges. First to find out antimicrobial agent or agents in combination that present nil or extremely low resistance to avoid the resistance problem. Second to design such drug delivery system that is compatible with microenvironment of *H. pylori* and deliver the antimicrobial formulations to the site where it resides at the junction of epithelium and mucus layer.

In order to overcome resistance furazolidone was selected as a major antibiotic in this study that presents no resistance against *H. pylori*. This antibiotic was coupled with another antimicrobial agent NAC, to potentiate the activity of furazolidone. The

selection of NAC in this study was based on its two important aspects. Firstly, it can be used as adjunct to furazolidone to enhance the antimicrobial activity.Secondly, it is used as a mucopenetrative agent to deliver the formulations (mucopenetrative liposomes) in close proximity to *H. pylori* across the mucus layer.

First phase of the study was dedicated to mucoadhesive drug delivery approach in which the resident time of the antibiotic (furazolidone) was increased to deliver the drug for prolonged period of time in order to give formulations more time to release the drug.

For the mucoadhesive drug delivery approach, two different delivery systems were used. The first one was mucoadhesive microparticles. In chapter number 3 mucoadhesive microparticles were prepared by using two different techniques.ie. spray and freeze drying. Freeze drying failed to form proper microparticles and showed burst release therefore they were not investigated further for mucin adsorption but spray dried particles were selected for further analyses.

For preparing mucoadhesive delivery system, chitosan was selected as polymer because of its biocompatibility and its Pka value that is 6.0. At acidic pH the amino group is protonated that makes it positive and increase the probability of mucoadhesion to negatively charged mucus layer. The study aimed to deliver the drug against *H. pylori* that creates the alkaline environment and proton pump inhibitor also increase the pH to alkaline side. Therefore, in mucoadhesion phase the pH selected was 4.5 and the results in chapter three revealed good mucin adhesion profile at pH 4.5. At pH lower than 4.5 the zeta potential become unstable and drastically overshoot to positive charge side that is good for mucus adhesion but on the other hand the chitosan at pH below 4.5 starts to solubilize that tends to dissolve the mucoadhesion assembly. However, at high pH it is not solubilized and retain the mucoadhesion assembly. However, increasing pH from 4 to 8 there is

slight increase in negative zeta potential and lesser degree of protonation but that does not affect the mucus adhesion because the degree of protonation already established is enough for mucus adhesion at pH 4.5 which is indicated by the results showing 87% of mucin adhesion at pH 4.5. The second pH level selected was pH 1.3 where the release of the drug was higher as compared to release at pH 4.5 that further confirmed that low pH solubilizes the chitosan and that in turn release the encapsulated drug at a faster rate.

Because in microparticles the drug was directly encapsulated in the chitosan matrix the pH of the environment has direct impact on the characteristics of microparticles formulations in terms of drug release and mucin adhesion which are linked together. In case of microparticles chitosan at low pH is solubilized but still protonated and could easily bind to mucus where it showed 60% of mucin adsorption.

Therefore, in chapter four liposomes were prepared to check the effect of pH on mucus adhesion and release of the drug separately. Similar pH 1.3 and 4.5 levels were selected for this chapter because the polymers used was chitosan as used in previous chapter. In this chapter liposomes were used as delivery vehicles to deliver furazolidone to the stomach for extended period of time. The results revealed that at lower pH the mucoadhesion was low because below 4.5 chitosan started to solubilize and release the liposomes as shown in results where stomach mucus can only retain 30% of mucoadhesive liposomes. However, in contrast up to 60% of mucoadhesive liposomes remain stick to stomach mucus at pH 4.5.

The release profile of mucoadhesive liposomes showed that decrease in pH results in less release in contrast to the results of previous chapter where release was enhanced by low pH. This finding indicates that if the drug is directly embedded into polymeric system, it behaves differently when the drug is encapsulated in a delivery vehicle that is coated with polymeric system. The results of release in this chapter

favors the high pH condition and therefore liposomes coated with chitosan is more promising approach when compared with chitosan microparticles.

Secondly, in this chapter mucus adhesion was determined directly in which the amount of liposomes remained adherent to mucus was determined and compared with those that are dropped from mucus. However, in chapter 3 the mucin adsorption was determined indirectly where the amount of mucin adsorbed was determined that does not indicate the difference in the adsorption of solubilized chitosan at low pH or the adsorption of chitosan microparticles.

The other major finding in chapter four was the amount of cholesterol in the liposomal formulation and the results suggested that low amount of cholesterol gives better mucus adhesion and better release as compared with the formulation having high cholesterol.

In chapter five mucopenetrative liposomal drug delivery approach was used. Because chitosan was not used in these formulations the prolonged release was not possible and therefore in order to provide appropriate amount of drug to kill the bacteria in a short time mucus penetrating approach was used. In this approach liposomes were designed in such a way that they penetrate deep in the mucus layer and deliver the drug in close proximity to bacteria. In mucus penetrating liposomes two major factors were controlled to optimize the formulation according to alkaline microenvironment of H. pylori.

First important factor was the surface charge of the liposomal formulation. Mucus being negatively charged repel the negative liposomes and on the other hand trap the cationic liposomes due to attractive forces. In this chapter electrically neutral liposomes showed maximum mucus penetration up to 55%. The pH selected for mucopenetration and release was 6.0 in this chapter because this pH favors H. pylori. One of the neutral formulation showed +35mV and second neutral

formulation showed -1.4mV which is almost neutral surface charge. These finding also indicate that pH >4 has only slight effect on zeta potential. However, in terms of release and encapsulation efficiency charged particles shown better results than neutral particles. Cationic particles gave better encapsulation efficiency followed by anionic particles and neutral particles comes to last. The release profile is somewhat similar to encapsulation efficiency profile between charged and uncharged particles. However, among charged particles in vitro release showed enhanced release in anionic particles as compared to cationic ones. Neutral particles released 50-60% of furazolidone and NAC respectively in three hours as compared to charged particles. NAC here is good for two main reasons, it enhances the penetration of liposomes through mucus and secondly, as the drug release of furazolidone is low therefore it needs some adjunct for augmented therapy of *H. pylori*. The released amounts of furazolidone and NAC in given time window have shown very good results in killing *H. pylori* as shown in results from chapter number 6.

Second factor is the encapsulation efficiency, because these formulations are designed in such a way that they carry furazolidone along with NAC for augmented therapy as well as for mucus penetration, increased encapsulation efficiency is major determinant for ultimate killing of bacteria.Therefore, reverse phase evaporation was selected as a method of liposme preparation.This method increases the encapsulation of hydrophilic drug and NAC being hydrophilic was successfully encapsulated in liposomes. Secondly, the selection of long alkyl chain lipid (DSPC) was used to accumulate maximum amount of furazolidone in lipids bilayer of liposomes.

In chapter six that deals with microbiology section of the overall study, the results reveled that mucopenetrative formulations gave comparable results of augmentation therapy when compared with augmentation therapy of free

furazolidone and NAC. When these drugs used in combination promising results were obtained but in order to achieve complete killing in desired time MIC X8 furazolidone when combined with 1% MIC of NAC irrespective of liposomal or free drug was the only effective concentration. Any concentration less than this couldnot achieve complete killing in 3 hours' time.

However, when furazolidone was used alone without augmented therapy it took eight hours to completely kill the bacteria at its concertation MIC X 4. In order to make it useful for mucoadhesive formulation the time for killing was narrow down to 6 hours by increasing the concentration of furazolidone i.e. MIC X 8. In the results it showed that liposomal bound or free furazolidone alone can completely kill the bacteria (Mucoadhesive microparticles and mucoadhesive liposomes) in six hours.

Therefore, it is concluded that the formulation made in this study whether they are mucoadhesive or mucopenetrative provides the good potential of using these formulations against H. pylori as they deliver appropriate amount of furazolidone to target the bacteria. Both of the approaches deliver controlled amount of furazolidone topically to the site of action that can potentially reduce the side effect associated with furazolidone.

Future work.

1. As all the formulations were kept in dry powder form they all showed good results during the course of study. However, further stability studies could be performed to check the long term stability of these two formulations.
2. Augmented therapy NAC and furazolidone could be performed in mucoadhesive liposomal systems to check the activity against H. pylori.

3. This pilot study could be designed on large scale and exact dose optimization could be done to deliver the minimum amount of furazolidone at the site of action that is sufficient to kill the bacteria.
4. Further, studying the effect of pluronic P-127 in different concentration to increase the drug encapsulation efficiency.

CHAPTER EIGHT
REFERENCES

Acar M, Bozkurt E, Meral K and Onganer Y (2015). The fluorescence quenching mechanism of aqueous suspension. *Journal of Luminescence*. 157:10-15.

Agarwal V, Himanshu P,Radha R (2016). Liposome and Their Applications in Cancer Therapy. *Brazilian archives of biology and technology*.59: 1-10

Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N et al., (2013). Liposome: classification, preparation, and applications. *Nanoscale Research* Letters. 8:102.

Akbarzadeh A, Rogaie RS, Soodabeh D, Sang WJ et al (2013). Liposome: classification, preparation, and applications*. Nanoscale Research Letters*.8:102

Aljayyoussi G, Muthanna A, Peter G, Mark Gumbleton (2012). Pharmaceutical Nanoparticles and the Mucin Biopolymer Barrier. *BioImpacts*.2(4):173-174

Almeida N, Romãozinho JM, Donato MM, Luxo C, Cardoso O, Cipriano MA, Marinho C, Sofia C (2014). Triple therapy with high-dose proton-pump inhibitor, amoxicillin, and doxycycline is useless for *Helicobacter pylori* eradication: a proof-of-concept study. *Helicobacter*. 19: 90-97.

Al-Zoubi N, Alkhatib HS, Bustanji Y, Aiedeh K, Malamataris S (2008). Sustainedrelease of buspirone HCl by co spray-drying with aqueous polymeric dispersions. *Eur J Pharm Biopharm*. 69:735–42.

Analava M and Baishakhi D (2011). Chitosan Microspheres in Novel Drug Delivery Systems. *Indian J Pharm Sci*. 73(4): 355–366.

Analava M and Baishakhi D (2011). Chitosan Microspheres in Novel Drug Delivery Systems. *Indian J Pharm Sci*.73:355–366.

Bansil R, Stanley E and LaMont JT (1995). Mucin biophysics. *Ann Rev Physiol*.57:635.

Baras B, Benoit MA, Gillard J (2000). Parameters influencing the antigen release from spray-dried poly(DL-lactide) microparticles*. Int J Pharm*.200:133–45.

Bathini ST, Durgaramani S and Shalini D (2011). Formulation and in vitro evaluation of clarithromycin floating microspheres for eradication of *Helicobacter Pylori. Der Pharmacia Lettre*.3 (6):90-101

Beck RCR, Lionzo MIZ, Costa TMH, Benvenutti EV et al., (2008). Surface morphology of spray-dried nanoparticle-coated microparticles designed as an oral drug delivery system. *Brazilian Journal of Chemical Engineering*. 25(2):389 – 398.

Begum YM, Abbulu K, Sudhakar M, Aneesa (2012). Celecoxib-encapsulated liposomes of long alkyl chain lipids: formulation, characterization and in vitro performance. Pharm. Sin.3:117–125. coumarin 120 with CdS nanoparticles inGuo J, Guo J,Ping Q, Jiang G, Huang L, Tong Y (2003). Chitosan-coated liposomes: characterization and interaction with leuprolide. *Int J Pharm*.260:167–73.

Bhaskar KR, Garik P, Turner BS, Bradley JD, Bansil R, Stanley HE, LaMont JT (1992). Viscous fingering of HCI through gastric mucin. *Nature.*360:458-461.

Bilancetti LL, Poncelet DD, Loisel CC, Mazzitelli SS and Nastruzzi CC (2010). A statistical approach to optimize the spray drying of starch particles.*AAPS PharmSciTech*.11 (3):1257–1267.

Bina JE, Alm RA, Uria-Nickelsen M, Thomas SR, Trust TJ, and Hancock RE (2000). Helicobacter pylori uptake and efflux: basis for intrinsic susceptibility to antibiotics in vitro. *Antimicrob. Agents Chemother*. 44:248–254.

Bindu MB, Zulkar NKM, Ravinder AN,David Banji (2010). Mucoadhesive drug delivery system: An overview. *Journal of Advanced Pharmaceutical Technology & Research*.4:381-387

Boer WA and Tytgat GN (2000). Regular review: treatment of *Helicobacter pylori* infection. *BMJ* .320: 31-34.

Bowey K, Swift BE, Flynn LE, Neufeld RJ (2013). Characterization of biologically active insulin-loaded alginate microparticles prepared by spray drying. *Drug Dev Ind Pharm*.39:457–65.

Bury-Moné S, Kaakoush NO, Asencio C, Mégraud F, Thibonnier M, De Reuse H, Mendz GL (2006). Is Helicobacter pylori a true microaerophile? *Helicobacter*.11:296–303.

Çağdaş M, Ali DS and Seyda B (2014). Liposomes as Potential Drug Carrier Systems for Drug Delivery.Application of Nanotechnology in Drug Delivery", book edited by Ali Demir Sezer

Cammarota G, Sanguinetti M, Gallo A & Posteraro B (2012). Review article: biofilm formation by *Helicobacter pylori* as a target for eradication of resistant infection. *Aliment Pharmacol Ther*. 36: 222-230.

Carlstedt I andSheehan JK (1989). Structure and macromolecular properties of cervical mucus glycoproteins. *Symp Soc Exp Biol*. 43:289-316.

Carvalho FC, Bruschi ML, Evangelista RC and Daflon MP (2010). Mucoadhesive drug delivery systems. *BJPS*. 46(1). 1-17

Caselli M, Zullo A, Maconi G, Parente F, Alvisi V, Casetti T. et al. (2007). Cervia II Working Group Report 2006': guidelines on diagnosis and treatment of *Helicobacter pylori* infection in Italy. *Dig Liver Dis*.39:782-789.

Celli JP, Turner BS, Afdhal NH, Ewoldt RH, McKinley GH, Bansil R, Erramilli S (2007). Rheology of gastric mucin exhibits a pH-dependent sol-gel transition. *Biomacromolecules*. 8:1580-6.

Channarong S, Wanpen C, Nuttanan S, and Ampol M (2010). Development and Evaluation of Chitosan-Coated Liposomes for Oral DNA Vaccine: The Improvement of Peyer's Patch Targeting Using a Polyplex-Loaded Liposomes. 12(1): 192–200.

Cho HJ, Prabagar B, Suk-Jae C, Chang KS, Dae-Duk K (2011). Evaluation of protein stability and in vitro permeation of lyophilized polysaccharides-based microparticles for intranasal protein delivery. *Int J Pharm*.416:77-84

Chowdary KPR and Rao YS (2003). Design and in vitro and in vivo evaluation of mucoadhesive microcapsules of glipizide for oral controlled release: A technical note, AAPS PharmSciTech. 39:

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Nineteenth informational supplement M100–S19. Wayne, PA: Clinical and Laboratory Standards Institute, 2009.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2–A10. Wayne, PA: Clinical and Laboratory Standards Institute, 2009

Coelho LG, Moretzsohn LD, Vieira WL, Gallo MA, Passos MC, Cindr JM, Cerqueira MC, Vitiello L, Ribeiro ML, Mendonca S, et al., (2005). New once-daily, highly effective rescue triple therapy after multiple *Helicobacter pylori* treatment failures: a pilot study. *Aliment Pharmacol Ther* .21(6):783-787.

Cortesi R, Esposito E, Gambarin S, Telloli P, Menegatti E, Nastruzzi C (1999). Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. *J Microencapsul*.16:251-6.

Dawson M,Eric K, Denis W,and Justin H (2004). Transport of Polymeric Nanoparticle Gene Carriers in Gastric Mucus. *Biotechnol.* Prog.20:851−857

De Francesco V, Ierardi E, Hassan C, Zullo A (2012). *Helicobacter pylori* therapy: Present and future. *World J Gastrointest Pharmacol Ther*. 3: 68-73.

Dent JC and McNulty CA (1988). Evaluation of a new selective medium for Campylobacter pylori. Eur. *J. Clin. Microbiol. Infect. Dis*.7:555–558.

Dodd S, Dean O, Copolov DL, et al (2008). N-acetylcysteine for antioxidant therapy: pharmacology and clinical utility*. Expert Opin Biol Ther*.8:1955–1962.

Dong H, Miae L, Kim JG, Kim FA. El-zaatari K, Osato S, and Graham DY (2001). Furazolidone- and Nitrofurantoin-resistant helicobacter pylori: prevalence and role of genes involved in metronidazole resistance. Antimicrobial agents and chemotherapy. 45:306-308.

DPPC liposomes*. Journal of Advanced Research*.1:187–191

Du HH,Liu MM Yang XX and Zhai GG (2015). The design of pH-sensitive chitosan-based for-mulations for gastrointestinal delivery.*Drug Discov Today*.20 (8):1004–1011.

Duffy J and Hill A (2012). Suspension stability: why particle size, zeta potential and rheology are important. Malvern Instruments Limited.

Eisig JN, Silva FM, Rodriguez TN, Hashimoto CL, Barbuti RC (2005). A furazolidone-based quadruple therapy for *Helicobacter pylori* retreatment, in patients with peptic ulcer disease. Clinics. 60(6):485-8.

Elversson J, Millqvist-Fureby A, Alderborn G, Elofsson U (2003). Droplet and particle size relationship and shell thickness of inhalable lactose particles during spray drying. *J Pharm Sci* .92:900–10.

Endo H, Yoshida H, Ohmi N, Ohta K, Higuchi S, Suga T (2002). Localization of [14C] clarithromycin in rat gastric tissue when administered with lansaprazole and amoxicillin*. J Antimicrob Chemother*. 50:285-8.

Endo H, Yoshida H, Ohmi N, Ohta K, Higuchi S. (2001). Localization of [14C] amoxicillin in rat gastric tissue when administered with lansoprazole and clarithromycin*. J Antimicrob Chemother*. 48:923-6.

Faizi M, N VM, Prasanjit P, Ravindra S and Pandey S (2010). Formulation and Evaluation of Mucoadhesive Microspheres of Amoxicillin Trihydrate by using Eudragit RS 100*. Int.J. ChemTech Res.* 2(1)

Farokhzad OC and Langer R (2006). Developing smarter therapeutic and diagnostic modalities. 58:1456–1459.

Fatnassi M, Tourné-Péteilh C, Peralta P, Cacciaguerra T, Dieudonné P, Devoisselle JM, et al. (2016) Encapsulation of complementary model drugs in spray-dried nanostructured materials. J Sol-Gel Sci *Techno*l.68:307–16

Filipović-Grcić J, Skalko-Basnet N, Jalsenjak I (2001). Mucoadhesive chitosan-coated liposomes: characteristics and stability*. J Microencapsul*. 18(1):3-12.

Flavia L and Andreas BS (2012). Development and in vitro evaluation of mucus inert nanoparticles for mucosal diseases. Nano con. Brno, Czech Republic, EU

Francesco P, Virginia F, Angelo A (1998). Treatment of Antibiotic-Resistant *Helicobacter pylori* Infection*. N Engl J Med*. 339:53.

Francesco VD, Zullo A , Hassan C, Giorgio F, Rosania R, Ierardi E (2011). Mechanisms of Helicobacter pylori antibiotic resistance: An updated appraisal. *World J Gastrointest Pathophysiol*. 2(3): 35-41.

Francis Mé graud (2012). The challenge of *Helicobacter pylori* resistance to antibiotics: the comeback of bismuth-based quadruple therapy. *Ther Adv Gastroenterol*. 5(2): 103 –109.

Fraternale A, Paoletti M, Casabianca A, Oiry J, Clayette P,et al (2006). Antiviral and immunomodulatory properties of new proglutathione (GSH) molecules*. Curr. Med. Chem*. 13:1749–1755.

Frézard MS, Michalick C, Soares F and Demicheli C (2000). Novel methods for the encapsulation of meglumine antimoniate into liposomes. *Braz. J. Med. Biol*. 33: 841-846.

Gartner LP and Hiatt JL. Color Textbook of Histology, 2nd edition, 2001. Philadelphia: W.B. Saunders

Gendler SJ and Spicer AP (1995). Epithelial mucin genes. *Ann Rev Physiol*. 57:607.

Genta PP and France PP (1998).Different molecular weight chitosan micro spheres, Iinfluence on drug loading and drug release. *Drug Dev. Ind. Pharm.* 24:779–784.

Gisbert JP and Morena F (2006c). Systematic review and meta-analysis: levofloxacin-based rescue regimens after *Helicobacter pylori* treatment failure*. Aliment Pharmacol Ther*. 23: 35-44.

Gisbert JP and Pajares JM (2002). Review article: *Helicobacter pylori* "rescue" regimen when proton pump inhibitor-based triple therapies fail*. Aliment. Pharmacol. Ther.*16:1047–1057.

Gisbert JP and Pajares JM. (2005) Helicobacter pylori ‘rescue’ therapy after failure of two eradication treatments. Helicobacter.10: 363-372

Giuseppina B and Agnese M (2015). Liposomes as Nano medical devices. *International Journal of Nanomedicine*.10:975–999

Gómez-Gaete C, Fattal E, Silva L, Besnard M, Tsapis N (2008). Dexamethasone acetate encapsulation into Trojan particles*. J Control Release*.128:41

Goodwin A, Kersulyte D, Sisson G, Veldhuyzen VZ, Berg DE, and Hoffman PS (1998). Metronidazole-resistance in *Helicobacter pylori* is due to null mutations in a gene (rdxA) that encodes an oxygeninsensitive NAD(P)H nitroreductase*. Mol. Microbiol*. 28:383–393.

Goswami M and Jawali N (2007). Glutathione mediated augmentation of beta lactam antibacterial activity against Escherichia coli. *J. Antimicrob. Chemother*. 60:184–18.

Goswami M and Jawali N (2010). N-Acetylcysteine-Mediated Modulation of Bacterial Antibiotic Susceptibility*. J. Antimicrob. Chemother*.54:3529–3530.

Goswami M, Suhas H. Mangoli and JawalI N (2007). Effects of Glutathione and Ascorbic Acid on Streptomycin Sensitivity of Escherichia coli*. Antimicrob. Agents Chemother*. 51:1119-1122

Graham DY (2012). Furazolidone in Helicobacter Pylori Therapy: Misunderstood and Often Unfairly Maligned Drug Told in a Story of French bread. *Saudi J Gastroenterol.*18(1):1-2.

Graham DY, Osato MS, Hoffman J, Opekun AR, Anderson SY, ElZimaity H (2000). Furazolidone combination therapies for Helicobacter pylori infection in the United States. *Aliment Pharmacol Ther.* 14(2):211-215.

Graham DY, Osato MS, Hoffman J, Opekun AR, Anderson SY, ElZimaity H (2000). Furazolidone combination therapies for *Helicobacter pylori* infection in the United States. *Aliment Pharmacol Ther.* 14(2):211-215.

Gürsoy A, Kut E, Ozkirimli S (2004). Co-encapsulation of isoniazid and rifampicin in liposomes and characterization of liposomes by derivative spectroscopy. *Int J Pharm*.115-23.

Haeria A, Alinaghiana B, Daeihameda M, Dadashzadeha S (2014). Preparation and characterization of stable nanoliposomal formulation of fluoxetine as a potential adjuvant therapy for drug-resistant tumors. *Iran. J. Pharm. Res.* 13:3–14.

Hall-Stoodley L, Stoodley P (2009). Evolving concepts in biofilm infections. *Cell Microbiol.*11: 1034–43.

Han HK, Shin HJ, Ha DH (2012). Improved oral bioavailability of alendronate via the mucoadhesive liposomal delivery system. *Eur. J. Pharm. Sci.* 46:500–507.

Handa T, Naito S, Hiramatsu M, Tsuboi M (2006). Thermal SiO and H13CO+ line observations of the dense molecular cloud G0.11-0.11 in the Galactic Center Region. *Astrophys* J.636:261–266.

Hanes J, Suk JS, Lai SK, Boylan NJ, Dawson MR, Boyle MP (2011). Rapid transport of muco-inert nanoparticles in cystic fibrosis sputum treated with N-acetyl cysteine. *Nanomedicine*. 6(2):365–375.

Hanes J. Dawson M. Har-el Y, Suh J, Fiegel J (2003). Gene Delivery to the Lung, Pharmaceutical Inhalation Aerosol Technology (ed). Marcel Dekker Inc; New York. Pp. 489-539

Hasanin A and Abdelwahab Omri (2014). Liposomal N-acetylcysteine modulates the Pathogenesis of P. Aeruginosa Isolated from the Lungs of Cystic Fibrosis Patient. *J Nanomed Nanotechnol*. 5:4

He P, a, Stanley SD, Lisbeth I (1999). Chitosan microspheres prepared by spray drying. *International Journal of Pharmaceutics*.30:53-65

Hejazi R and Amiji M (2002). Stomach-specific anti-*H. pylori* therapy. I: Preparation and characterization of tetracyline-loaded chitosan microspheres. *Int J Pharm*.235:87-94

Helicobacter pylori infection .*MJA*.204 (10):376-380

Henke MO and Ratjen F (2007). Mucolytics in cystic fibrosis. *Paediatr Respir Rev*.8:24–9.

Hidekazu S, Toshihiro N, Toshifumi H (2015). Helicobacter pylori eradication therapy. *Future Microbiol*.639–648.

Himanshu A, Sitasharan P, Singhai AK (2011). Liposomes as drug carriers. IJPLS .2:945–951.

Hong Cheng and Fu-Lian Hu (2009). Furazolidone, amoxicillin, bismuth and Rabeprazole quadruple rescue therapy for the eradication of *Helicobacter pylori*. *World Journal of Gastroenterology*. 15(7):860-864

Hong Cheng and Fu-Lian Hu (2009). Furazolidone, amoxicillin, bismuth and Rabeprazole quadruple rescue therapy for the eradication of *Helicobacter pylori*. *World Journal of Gastroenterology.* 15(7):860-864

Hong Z, Chasan B, Bansil R, et al (2005). Atomic force microscopy reveals aggregation of gastric mucin at low pH. *Biomacromolecules*.6:3458-3466.

Horiki N, Omata F, Uemura M, et al (2009). Annual change of primary resistance to clarithromycin among Helicobacter pylori isolates from 1996 through 2008 in Japan. Helicobacter. 14: 86–90.

Huang YC, Chiang CH, Yeh MK (2003). Optimizing formulation factors in preparing chitosan microparticles by spray-drying method*. J. Microencapsul*. 20:247–260.

Huynh HQ, Couper RTL, Tran CD, Moore L, Kelso R, and Butler RN (2004). N-acetylcysteine, a novel treatment for *Helicobacter pylori* infection. *Digestive Diseases and Sciences*. 49(11)1853–1861

IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Geneva: World Health Organization, International Agency for Research on Cancer.

Ieda MST, Etiene B, Larissa da CA, Luisa ZCMS, Eliana ML (2012). Preparation, characterization and in vitro antimicrobial activity of liposomal ceftazidime and cefepime against pseudomonas aeruginosa strains. *Brazilian Journal of Microbiology*.43:984-992

Inês C, Gonçalves , Ana M, Costa S, José R, Oliveira, Patrícia C, Henriques, Paula G, Celso A. Reis, Cristina L and Martins (2016). Bacteria-targeted biomaterials: Glycan-coated microspheres to bind Helicobacter pylori. *Acta Biomaterialia*.33: 40–50.

Inês C, Gonçalves , Ana M, Costa S, José R, Oliveira, Patrícia C, Henriques, Paula G, Celso A. Reis, Cristina L and Martins (2013). Bacterial-binding chitosan microspheres for gastric infection treatment and prevention. *Acta Biomaterialia*. 9:9370–9378

Ishaque MS, Kuan-Boone T, Anumita C, Yuanjie L, Bee-Jen T, Gigi NC (2013). Liposome co-encapsulation of synergistic combination of irinotecan and doxorubicin for the treatment of intraperitoneally grown ovarian tumour xenograft. *J.Control Release*.172 (3): 852–861

Jaime NE, Fernando MS, Ricardo CB, Tomás NR, Peter M, Moraes JF and Schlioma Z (2009). Furazolidone, levofloxacin, and lansoprazole after failed *Helicobacter pylori* eradication.*BMC Gastroenterology*. 9(38):1-5.

Jayakumar R, Menon D, Manzoor K, Nair SV, Tamura H (2010). Biomedical applications of chitin and chitosan based nanomaterials — a short review. Carbohydr.Polym. 82:227–232.

Jesorka A and Orwar O (2008). Liposomes: technologies and analytical applications. Annu

Jie M, Fangqin G, Haiyan X, Wei L, Chen W, Xian-Da Y (2016). Combination Therapy using Co-encapsulated Resveratrol and Paclitaxel in Liposomes for Drug Resistance Reversal in Breast Cancer Cells in vivo. *Sci Rep*.7(6):1-11

Jin X, Tang S, Chen Q, Zhang T, Liu F, Zhang S, et al., (2011). Furazolidone induced oxidative DNA damage via up-regulating ROS that caused cell cycle arrest in human hepatoma G2 cells. *Toxicol Lett*. 201:205–12

Jung SS, Samuel KL, Nicholas JB, Michelle RD, Michael PB, and Justin H (2011). Rapid transport of muco-inert nanoparticles in cystic fibrosis sputum treated with N-acetyl cysteine*. Nanomedicine*.6: 365–375.

Jyssum, K. (2012). Particle mobility in mucus (Master's thesis,Norwegian institute of science and technology,Norway)

Karbasi A, Hossein Hosseini S, Shohrati.M, Amini M., and Najafian B (2013). Effect of oral N-acetyl cysteine on eradication of *Helicobacter pylori* in patients with dyspepsia. *Minerva Gastroenterol Dietol*. 59(1):107-12.

Karina F and Devienne MS (2002). Screening for antimicrobial activity of natural products using a microplate photometer. *Braz. J. Microbiol*.33:166-168

Karn PR, Vanić Z, Pepi I, Skalko-Basnet, N (2011).Mucoadhesive liposomal delivery systems: the choice of coating material. *Drug Dev. Ind. Pharm*. 37:482–488.

Kataria S, Sandhu P, Bilandi A, Akanksha M, Kapoor B, Seth GL, Bihani SD (2011). Stealth liposomes: a review*. IJRAP*. 2:1534–1538.

Katharine Sanderson (2006). Designing liposomes to avoid chemotherapy side effects. *RSC.*

Kenneth R (2003). Fluorescence Microscopy. Encyclopedia of Optical Engineering. Pp 548-555.

Kharia AA and Singhai AK (2013). Formulation and Evaluation of Gastroretentive Drug Delivery System of Acyclovir as Mucoadhesive Nanoparticles. *International Journal of PharmTech Research.* 5:1538-1545

Kian M and Frank KF (2011). The Potential Role of N-Acetylcysteine for the Treatment of *Helicobacter pylori. J Clin Gastroenterol.*45(10):841-843.

Kobe A, Ebrecht A, Fries R (1996). Minimum inhibitory concentrations of intestinal Escherichia coli from broiler chickens after oral administration of apramycin. *J Appl Bacteriol*. 80:26-30.

Kotadiya RR, Patel VV, Patel HH and Koradiya HH (2009).Effect of cross-linking on physio-chemical properties of chitosan mucoadhesive microspheres: A factorial approach, IJGP 1:58–62.

Kreuter J, Muller U, Munz K (1989). Quantitative and microautoradiographic study on mouse intestinal distribution of polycyanoacrylate nanoparticles*. Int J Pharm.* 55:39–45

Kuo SH, Chen LT, Lin CW, Wu MS, Hsu PN, Tsai HJ, Chu CY, Tzeng YS, Wang HP, Yeh KH, Cheng AL (2013). Detection of the Helicobacter pylori CagA protein in gastric mucosa-associated lymphoid tissue lymphoma cells: clinical and biological significance. *Blood Cancer J* .3:e125

Kusters JG, Arnoud HMV and Ernst JK (2006). Pathogenesis of Helicobacter pylori Infection. Clin. Microbiol. Rev. 19(3):449–490

Kwon DH, El-Zaatari FAK, Kato M, Osato MS, Reddy R, Yamaoka Y, and Graham DY (2000). Analysis of an rdxA gene and involvement of additional genes encoding NADPH flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. *Antimicrob. Agents Chemother.* 44:2133–2142.

Kwon DH, Lee M, Kim JJ, Kim JG et al, (2001). Furazolidone-and nitrofurantoin-resistant Helicobacter pylori: prevalence and role of genes involved in metronidazole resistance. Antimicrob. Agents Chemother.45:306–308

Lai SK and Ying-Ying HJ (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev*.61(2): 158–171.

Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang YY, Cone R, et al (2007). Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc Natl Acad Sci USA*.104:1482-7

Lam SK and Talley NJ (1998). Report of the 1997 Asia Pacific Consensus Conference on the Management of *Helicobacter pylori* Infection. *J Gastroenterol Hepatol*. 13:1–12.

Langer R (1998). Drug delivery and targeting. *Nature.* 392:5–10

Lasic DD (1998). Novel applications of liposomes. Trends Biotechnol. 16:307–321.

Laura M, Ensigna B, Conea R, Hanes J (2012). Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Adv. Drug Deliv*. Rev. 557–570.

Lee SH, Heng D, Ng WK, Chan HK, Tan RB (2011). Nano spray drying: a novel method for preparing protein nanoparticles for protein therapy. *Int J Pharm*.403:192–200.

Lehr CM, Poelma FGJ, Junginger HE, Tukker JJ (1992). An Estimate of Turnover Time of Intestinal Mucus Gel Layer in the Rat Insitu Loop. *Int J Pharm. 70*:235–240

Li N, Zhuang C, Wang M, Sun X, Nie S, Pan W(2009). Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery. *Int. J. Pharm*. 379:131–138

Li X, Anton N, Arpagaus C, Belleteix F, Vandamme TF (2010). Nanoparticles by spray drying using innovative new technology: The Büchi nano spray dryer B-90. *J Control Release*.147:304–10.

Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A and Vance DE (2006). The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell Metab. 3(5):321-31.

Lin YH, Chang CH, Wu YS, Hsu YM, Chiou SF, Chen YJ (2009). Development of pH-responsive chitosan/heparin nanoparticles for stomach- specific anti-Helicobacter pylori therapy. *Biomaterials.* 30:3332-42

Linda MB (2000) .Helicobacter pylori: Epidemiology and Routes of Transmission. *Epidemiol Rev*. 22(2):283-297

Littringer EM, Zellnitz S, Hammernik K, Adamer V, Friedl H, Urbanetz NA (2013). Spray drying of aqueous salbutamol sulfate solutions using the nano spray dryer B-90— the impact of process parameters on particle size. *Dry Technol*.31:1346–53.

Liu M, Zhang J, Shan W, Huang Y(2015). Developments of mucus penetrating nanoparticles. *Asian J. Pharm. Sci.* 275–282

Liu Z, Lu W, Qian L, Zhang X, Zeng P, Pan J (2005). In vitro and in vivo studies on mucoadhesive microspheres of amoxicillin. *J Control Release.*102(1):135-44.

Luo P 2014. Izon q nano training modules. The Oxford Science Park, Magdalen Centre, Robert Robinson Avenue, Oxford, OX4 4GA, United Kingdom (www.izon.com.Accessed 25/06/2014).

Maa YF and Prestrelski SJ (2000). Biopharmaceutical powders: particle formation and formulation considerations. *Curr Pharm Biotechnol*. 1(3):283-302.

MacKay JA, Deen DF, Szoka JFC (2005). Distribution in brain of liposomes after convection enhanced delivery; modulation by particle charge, particle diameter, and presence of steric coating. *Brain Res.* 1035:139–153.

Madrigal-Bujaidar E, Ibañez JC, Cassani M, Chamorro G (1997). Effect of furazolidone on sister-chromatid exchanges, cell proliferation kinetics, and mitotic index in vivo and in vitro. *J Toxicol Environ Health*.51:89–96.

Mady MM and Darwish MM (2010). Effect of chitosan coating on the characteristics of

Majithiya R.J, Murthy R.S. (2005). Chitosan-based mucoadhesive microspheres of Clarithromycin as a delivery system for antibiotic to stomach. *Curr Drug Deliv*. 2(3):235-42.

Malfertheiner P., Megraud F., O'Morain C, Bazzoli F., El-Omar E., Graham D. et al. (2007). Current Concepts in the Management of *Helicobacter pylori* Infection: The Maastricht III Consensus Report. Gut. 56:772–781.

Mantle M, Basaraba L, Peacock SC and Gall DG (1989). Binding of Yersinia enterocolitica to rabbit intestinal brush border membranes, mucus, and mucin. *Infect Immun*.7:292-9

Marja-na Du ¨rrig, KK, Anita HH,Maja SS, egvic KK, Aleksandra DD, Biserka CC and Jelena F (2011).Spray dried microparticles for controlled delivery of mupirocin calcium: Process–-tailored modulation of drug release*. Microencap-sul*. 28 (2):108–121.

Marzio LD, Coraggio S, Capodicasa L, Grossi and Cappello G (2006). Role of the preliminary susceptibility testing for initial and after failed therapy of *Helicobacter pylori* infection with levofloxacin, amoxicillin, and esomeprazole. *Helicobacter*. 11(4): 237–242.

Masaaki M, Takafumi A, Shin-nosuke H, Keizo Torii, Tadao Hasegawa, Dawn AI , Kenji I, Kazuo K, Hidemi G and Michio O (2004).Effect of Glycine on Helicobacter pylori In Vitro. *Antimicrob. Agents* C*hemother*. 48:3782-3788

Mathiowitz E, Chickering DE, Lehr CM. (eds.). Bioadhesive drug delivery systems: fundamentals, novel approaches, and development. Drugs and the Pharmaceutical Sciences. New York: Marcel Dekker, 1999. 696 p.

Mégraud F and Lamouliatte H (2003). Review article: The treatment of refractory *Helicobacter pylori* infection. *Alimentary Pharmacology and Therapeutics*. 17:1333-43

Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y (2013). Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. Gut.62:34

Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y (2013). Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut*.62:34

Menikarachchi MASK, Katuwavila KANP, Ekanayake EWMA, Thevanesam and Karunaratne DN (2016). Release behaviour of amoxicillin from chitosan coated liposomes

Min L, Jian Z, Wei S, Yuan H (2015). Developments of mucus penetrating nanoparticles. Asian Journal of Pharmaceutical Sciences.10:275–282

Mitchell H and Katelaris P (2016). Epidemiology, clinical impacts and current clinical management of

Mohamed MN,Labiba kE,Nawal AK,Said AK (2006). In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels*. Acta Pharm*. 56 :311–324

Mohsen M, Mady, Mirhane M. Darwish. (2010). Effect of chitosan coating on the characteristics of DPPC liposomes. *Journal of Advanced Research*. 1 (3):187-191

Mu L, TeoMM, Ning HZ, Tan CS, Feng SS (2005). Novel powder formulations for controlled delivery of poorly soluble anticancer drug: application and investigation of TPGS and PEG in spray-dried particulate system. *J Control Release*.103:565–75.

Mujumdar AS, editor. Handbook of industrial drying. Third ed. US: Taylor & Francis Group, CRC Press; 2006.

Nishizawa T and Suzuki H (2014). Mechanisms of *Helicobacter pylori* antibiotic resistance and molecular testing. *Front. Mol. Biosci*. 1(9): 1-7

Nishizawa T, Suzuki H, Tsugawa H, Muraoka H, Matsuzaki J, Hirata K et al. (2011a). Enhancement of amoxicillin resistance after unsuccessful Helicobacter pylori eradication*. Antimicrob. Agents Chemother*. 55:3012–3014.

Nounou MM, El-khordagui LK, Khalafallah N (2005). Effect of various formulation variables on the encapsulation and stability of dibucaine base in multilamellar vesicles. Acta Pol. Pharm. 62:369–379.

O'Gara EA, Maslin DJ, Nevill AM, Hill DJ (2008). The effect of simulated gastric environments on the anti-Helicobacter activity of garlic oil. *J. Appl. Microbiol*. 104:1324–1331.

Oliveira BF, Santana MHA and Re MI (2005). Spray-dried chitosan microspheres cross-linked with d, l-glyceraldehyde as a potential drug delivery system: Preparation and characterization. *Braz. J. Chem. Eng*.22:353 – 360.

Olmsted SS, Padgett JL, Yudin AI, Whaley KJ, Moench TR and Cone RA (2011).Diffusion of macromolecules and virus-like particles in human cervical mucus*. Biophys* J.81:1930-7

Osato MS, Reddy R, Reddy SG, Penland RL, Malaty HM, Graham DY (2001). Pattern of primary resistance of *Helicobacter pylori* to metronidazole or clarithromycin in the United States. *Arch Intern*. 161: 1217–20.

P He, Davis SS, Illum L (1999).Chitosan microspheres prepared by spray drying. *Int J Pharm*.187:53–65.

Park CW, Li X, Vogt FG, Hayes Jr D, Zwischenberger JB, Park ES, et al (2013). Advanced spray-dried design, physicochemical characterization, and aerosol dispersion performance of vancomycin and clarithromycin multifunctional controlled release particles for targeted respiratory delivery as dry powder inhalation aerosols. *Int J Pharm*.455:374–92.

Paruvathanahalli SR, Lakshmi NK, Jagdish B and Brahmeshwar M (2008). Formulation and Evaluation of Clarithromycin Microspheres for Eradication of *Helicobacter pylori. Chem. Pharm. Bull.* 56(12):1658-1664.

Patel JK and Chavda JR (2009). Formulation and evaluation of stomach-specific amoxicillin-loaded carbopol-934P mucoadhesive microspheres for anti-Helicobacter pylori therapy. *J Microencapsul.* (4):365-76.

Patel JK and Patel MM (2007). Stomach specific anti-helicobacter pylori therapy: preparation and evaluation of amoxicillin-loaded chitosan mucoadhesive microspheres. *Curr Drug Deliv.* 4(1):41-50.

Patel RP, Patel MP and Suthar AM (2009).Spray drying technology: an overview. *Indian J.Sci.Technol*. 2(10):44-47

Paul W. Toole O and Clyne M (eds.). Helicobacter pylori: *Physiology and Genetics*. Chapter 7Cell Envelope. 2001

Pedrazzoli JJ, Calafatti SA, Ortiz RA, Dias FE, Deguer M, Mendes FD, et al. (2001). Transfer of clarithromycin to gastric juice is enhanced by omeprazole in Helicobacter pylori-infected individuals. *Scand J Gastroenterol*. 36:1248-53

Perri F, Villani MR, Quitadamo M, Annese V, Niro GA, Andriulli A (2001b). Ranitidine bismuth citrate-based triple therapies after failure of the standard 'Maastricht triple therapy': a promising alternative to the quadruple therapy? *Aliment Pharmacol Ther* .15:1017–1022

Pierre-Louis B, Vincent F, Paul B, Jean-Claude P, Francoise F.(2008). Pre-formulation of liposomes against Helicobacter pylori: Characterization and interaction with the bacteria. Eur. J. Pharm. Biopharm. 69: 908–922.

Ponchel G and Irache J (1998). Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv Drug Deliv Rev*. 34:191–219.

Portero A, Remuñán-López C, Criado MT, Alonso MJ (2002). Reacetylated chitosan microspheres for controlled delivery of anti-microbial agents to the gastric mucosa. *J Microencapsul*.19 (6):797-809.

Poyner EA, Alpar HO and Brown MRW (1993). Preparation, properties and the effects of free and liposomal tobramycin on siderophore production by Pseudomonas aeruginosa. *Antimicrob. Chemother.*34 (1): 43-52

Prego C, Garcia M, Torres D, Alonso MJ (2005). Transmucosal macromolecular drug delivery. *J Control Release*. 101:151–62.

Qasim A, Sebastian S, Thornton O, Dobson M, McLoughlin R, Buckley M, O'Connor H, O'Morain C (2005). Rifabutin- and furazolidone-based *Helicobacter pylori* eradication therapies after failure of standard first- and second-line eradication attempts in dyspepsia patients. *Aliment Pharmacol Ther*.21(1):91-96.

Rabbani NR, Seville PC (2005). The influence of formulation components on the aerosolisation properties of spray-dried powders. *J Control Release*.110:130–40.

Raffin RP, Jornada DS, Ré MI, Pohlmann AR, Guterres SS (2006). Sodium pantoprazole loaded enteric microparticles prepared by spray drying: effect of the scale of production and process validation. *Int J Pharm.*324:10–8.

Rajak P, Bhattacharya,Sharm N ,Sarma M kataki, Rajkumari A (2011). Gastro-retentive floating drug delivery system- an approach in gastroretentive drug delivery. *Int J Pharm Pharm Sci*.3:9-16

Ramteke S, Ganesh N, Bhattacharya S, Jain NK (2008). Triple therapy-based targeted nanoparticles for the treatment of Helicobacter pylori. *J Drug Target*.16 (9):694-705.

Ramteke S, Ganesh N, Bhattacharya S, Jain NK (2009). Amoxicillin, clarithromycin, and omeprazole based targeted nanoparticles for the treatment of H. pylori. *J Drug Target*. 17(3):225-34.

Rege PR, Garmise RJ and Block LH (2003). Spray-dried chitosan. Part I: preparation and characterization*. Int. J. Pharm*. 252.

Ren L, Zhou Z, Lu D, Wang S (2013). Design and evaluation of biodegradable enteric microcapsules of amifostine for oral delivery. Int J Pharm.453:441–7.

Richard JS and William DC (2008). Persistent *Helicobacter pylori* Infection after a Course of Antimicrobial Therapy -- What's next? Clin Gastroenterol Hepatol. 6(10):1086-1090.

Roger E, Lagarce F, Garcion E, Benoit JP (2010). Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery*. Nanomedicine* 5:287–306.

Romero EL, Morilla MJ (2011). Topical and mucosal liposomes for vaccine delivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 3(4): 356–375.

Rosen H and Abribat T (2005). The rise and rise of drug delivery. *Nat Rev Drug Discov* 4:381-5

Rukholm G, Mugabe C, Azghani AO, Omri A (2006) Antibacterial activity of liposomal gentamicin against Pseudomonas aeruginosa: a time-kill study*. Int J Antimicrob Agents*.27: 247-252.

Saad RJ, Schoenfeld P, Kim HM and Chey WD (2006). Levofloxacin-based triple therapy versus bismuth-based quadruple therapy for persistent *Helicobacter pylori* infection: a meta-analysis*.Am J Gastroenterol.*101: 488-496.

Sader HS and Pignatari AC (1994). E test: a novel technique for antimicrobial susceptibility testing. *Sao Paulo Med J*.112:635-8.

Sader HS, Pignatari AC (1994). E test: a novel technique for antimicrobial susceptibility testing. *Sao Paulo Med J*.112:635-8.

Safavi M, Sabourian R, Foroumadi A (2016). Treatment of *Helicobacter pylori* infection: Current and future insights. *World J Clin Cases*. 4(1): 5-19

Samuel K, Lai, Ying-Ying W, Justin H (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv*. 61(2): 158–171

Samuel KL, Ying-Ying W, and Justin H (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev.61: 158–171.

Sanches B, Coelho L, Moretzsohn L et al., (2008). Failure of *Helicobacter pylori* treatment after regimes containing clarithromycin: New practical therapeutic options. *Helicobacter*.13:572-6

Schoubben A, Blasi P, Giovagnoli S, Rossi C, Ricci M (2010). Development of a scalable procedure for fine calcium alginate particle preparation. *Chem Eng J* .160:363–9.

Schreiber S and Scheid P (1997). Gastric mucus of the guinea pig: proton carrier and diffusion barrier. *Am J Physiol*. 272:63–70.

Scott D, Weeks D, Melchers K, Sachs G (1998). The life and death of Helicobacter pylori. Gut .43(1):S56–S60

Shah S, Qaqish R, Patel V, Amiji (1999). Evaluation of the factors influencing stomach-specific delivery of antibacterial agents for Helicobacter pylori infection. *J Pharm Pharmacol*. 51(6):667-72.

Shazly G,Nawroth T and Peter L (2008).Comparison of Dialysis and Dispersion Methods for In Vitro Release Determination of Drugs from Multilamellar Liposomes. *Dissolution Technologies*.10:7-10

Sheffner AL, Medler EM, Jacobs LW, Sarett HP (1964). The in Vitro Reduction in Viscosity of Human

Shin JM and Sachs G (2008). Pharmacology of Proton Pump Inhibitors. *Curr Gastroenterol Rep*. 10(6): 528–534.

Shiota S, Reddy R, Alsarraj A, ElSerag HB, Graham DY (2015). Antibiotic resistance of *Helicobacter pylori* among male United States Veterans. *Clin Gastroenterol Hepat*ol. 13: 1616–24.

Shiva KY, Jeet S, Jawad AS, Rajkamal B, Sharada G and Naveen KN (2010). Design and Characterization of Amoxicillin trihydrate Mucoadhesive Microspheres for Prolonged Gastric retention*. Int. J. Pharma. Sci. Drug Res*. 2(2):112-114

Shmulewitz A, Langer R, Patton J (2006). Convergence in biomedical technology. *Nat Biotech.* 24:277-277.

Siepmann J et al., (2012). Fundamentals and applications of controlled release drug delivery. (Eds.) Advances in delivery science and technology. Controlled release society.

Smart JD (2005). The basics and underlying mechanisms of mucoadhesion. *Adv.Drug Del. Rev.* 57(11):1556-1568.

Smith JI,Brendan D, Wilhelm NA,Zdenka P and Philip MS (1990). In Vitro Surface Properties of the Newly Recognized Gastric Pathogen Helicobacter pylori. *Infect Immun*. 58(9):3056-3060

Smith MA and Edwards DI (1995). Redox potential and oxygen concentration as factors in the susceptibility of Helicobacter pylori to nitroheterocyclic drugs. *J Antimicrob Chemother*. 35: 751-764.

Sriwongsitanont S and Ueno M (2011). Effect of Freeze-Thawing Process on the Size and Lamellarity of PEG-Lipid Liposomes. *The Open Colloid Science Journal.* 4:1-6

Stulzer HK, Tagliari MP, Parize AL, Silva MA, Laranjeira MC (2009). Evaluation of crosslinked chitosan microparticles containing acyclovir obtained by spray-drying.*Mater Sci Eng C* .29:387–92.

Suhas H, Phadnis, Mary H, Parlow et al., (1996). Localization of Helicobacter pylori Urease and a Heat Shock Protein Homolog Requires Bacterial Autolysis*. Infection and Immunity*.64 (3):905-912.

Sunee C, Wanpen C, Nuttanan S, and Ampol M (2010). Development and Evaluation of Chitosan-Coated Liposomes for Oral DNA Vaccine: The Improvement of Peyer's Patch Targeting Using a Polyplex-Loaded Liposomes*. AAPS PharmSciTech*.12: 192–200.

Suzuki H, Nishizawa T, Hibi T (2010). Helicobacter pylori Eradication Therapy. Future Virology. 5(4):639-648.

Suzuki H, Nishizawa T, Hibi T (2010). *Helicobacter pylori* Eradication Therapy. *Future Virology*. 5(4):639-648.

Szoka FJ and Papahadjopoulos D (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci U S A.* 75(9):4194-8.

Takeuchi H and Yamamoto KY (2001). Mucoadhesive nanoparticulate systems for peptide drug delivery. *Adv Drug Deliv Rev.* 47:39–54.

Tang BC, Dawson M, Lai SK, Wang YY, Suk JS, Yang M, Zeitlin P, Boyle MP, Fu J, Hanes J (2009). Biodegradable polymer nanoparticles that rapidly penetrate the human mucus barrier. Proc Natl Acad Sci. 106(46):19268-73.

Tempone AG, Mortara RA, de Andrade HF Jr, Reimão JQ (2010). Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis. *Int J Antimicrob Agents*. 36(2):159-63

Testerman TL and Morris J (2014).Beyond the stomach: An updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. *World J Gastroenterol* .20(36): 12781-12808

Tewes et al, 2006. Microencapsulation: Methods and Industrial Applications. 2nd ed. Drugs and the Pharmaceutical Sciences. Pp: 106-113

Thung H, Aramin V, Vavinskaya S. Gupta JY, Park SE, Crowe and Valasek MA (2016). Review article: the global emergence of Helicobacter pylori antibiotic resistance. *Aliment Pharmacol Ther*. 43: 514–533.

Tracheobronchial Secretions by Acetylcysteine. *Am Rev Respir Dis*.90:721–9.

Tursi A, Picchio M and Elisei W (2012). Efficacy and tolerability of a third-line, levofloxacin-based, 10-day sequential therapy in curing resistant *Helicobacter pylori* infection. *J Gastrointestin Liver Dis*.21: 133-138.

Uygun A, Kadayifci A, Yesilova Z, Ates Y, Safali M, Ilgan S et al. (2007). Poor efficacy of ranitidine bismuth citrate - based triple therapies for eradication. *Indian J Gastroenterol*. 26:174-7.

Vaira D, Ricci C, Lanzini A, Perna F, Romano A and Corinaldesi R (2007). How to proceed in *Helicobacter pylori*-positive chronic gastritis refractory to first- and second-line eradication therapy. *Dig Dis*. 25: 203-205

Valeur B (2001).Characteristics of Fluorescence Emission, in Molecular Fluorescence: Principles and Applications, Wiley-VCH Verlag GmbH, Weinheim.

Venkateswaramurthy N, sambathkumar R, Vijayabaskaran M and perumal P (2010). Formulation and in vitro evaluation of furazolidone mucoadhesive microsphere*. Int J Pharmacy Pharm Sci.*2 (3):104-106

Versalovic J, Shortridge D, Kibler K, Griffy MV, Beyer J, Flamm RK, Tanaka SK, Graham DY, Go MG (1996). Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori. Antimicrob Agents Chemother*. 40: 477-80.

Vishal B, Nirmala SL, Harikumar (2013). Floating drug delivery system: a review. Pharmacophore. 4 (1):26-38

Wagner B (2009). The Use of Coumarins as Environmentally-Sensitive Fluorescent Probes of Heterogeneous Inclusion Systems. Molecule: 14(1):210-237

Wan F, Bohr A, Maltesen MJ, Bjerregaard S, Foged C, Rantanen J, et al (2013). Critical solvent properties affecting the particle formation process and characteristics of celecoxib-loaded PLGA microparticles via spray-drying. *Pharm Res*.30:1065–76

Wan LS, Heng PW, Chia CG and Cececilia GH (1992). Spray drying as a process for encapsulation and the effect of different coating polymers. *Drug. Dev. Ind .Pharm*. 18: 997-1011.

Werle M, Takeuchi H. (2009). Chitosan-aprotonin coated liposomes for oral peptide delivery: Development, characterization and in vivo evaluation. *Int J Pharm.* 370:26–32.

Whiteway J, Koziarz P, Veall J, Sandhu N, Kumar P, Hoecher B, and Lambert IB (1998). Oxygen-insensitive nitroreductases: analysis of the roles of nfsA and nfsB in development of resistance to 5-nitrofuran derivatives in Escherichia coli*. J. Bacteriol*. 180:5529–5539.

Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I (1998). MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage*. Biochem J*. 334:685-93.

William DC, Benjamin CY and Wong (2007). American College of Gastroenterology Guideline on the Management of Helicobacter pylori Infection. *Am J Gastroenterol*.102:1808–1825

Wong WM, Gu Q, Chu KM et al., (2006). Lansoprazole, levofloxacin and amoxicillin triple therapy vs. quadruple therapy as second-line treatment of resistant *Helicobacter pylori* infection. *Alimentary Pharmacology and Therapeutics.* 23(3) 421–427.

Xia HX, Keane CT, and O'Morain CA (1994). Culture of Helicobacter pylori under aerobic conditions on solid media*. Eur. J. Clin. Microbiol. Infect. Dis*. 13:406–409.

Xiuying Li, Dan C, Chaoyi Le, Chunliu ZYG, Lars H, Mingshi Y (2011). Novel mucus-penetrating liposomes as a potential oral drug delivery system: preparation, in vitro characterization, and enhanced cellular uptake.International Journal of Nanomedicine. 6: 3151–3162

Yandrapati, Rajiv Kumar (2012). "Effect of lipid composition on the physical properties of liposomes: a light scattering study". MastersTheses. Paper 6864

Yang M, Lai SK, Wang YY, et al (2010). Biodegradable nanoparticles composed entirely of safe materials that rapidly penetrate human mucus. *Angew Chem Int Ed Engl*. 50(11): 2597–2600

Yang M,Samuel KL,Ying-Ying W, Weixi Z, Christina H, Michael Z, Jie F, and Justin H (2011). Biodegradable Nanoparticles Composed Entirely of Safe Materials that Rapidly Penetrate Human Mucus. *Angew Chem Int Ed Engl.* 50: 2597–2600.

Yen Cu and W. Mark Saltzman (2009). Drug delivery: Stealth particles give mucus the slip. *Nature Materials*.8:11 – 13.

Ying-Ying W, Samuel KL,Laura E, Weixi Z, Richard Cone, and Justin H (2013). The Microstructure and Bulk Rheology of Human Cervicovaginal Mucus are remarkably resistant to changes in pH. *Biomacromolecules.* 12: 4429–4435

Yonezawa H, Osaki T, Kurata S, et al (2010). Assessment of in vitro biofilm formation by Helicobacter pylori. *J Gastroenterol Hepatol*. 25(1):90–94.

Yu N, Li J, Hong D, Li X, Li L, Bin H, Yao W, Zhongwei G (2012). Cholesterol Derivatives Based Charged Liposomes for Doxorubicin Delivery: Preparation, In Vitro and In Vivo Characterization. *Theranostics.* 2:1092-1103

Zamboni WC (2008). Concept and clinical evaluation of carrier mediated anticancer agents. *Oncologist*. 13:248–260.

Zgoulli S, Grek V, Barre G, Goffinet G, Thonart P, Zinner S (1999). Microencapsulation of erythromycin and clarithromycin using a spray-drying technique. *J Microencapsul*. 16(5):565-71.

Zgoulli S, Grek V, Barre G, Goffinet g, thonart PH, Zinner S (2008). Microencapsulation of erythromycin and clarithromycin using a spray-drying technique. Journal of Microencapsulation.16(5):565-571

Zhao T and Liu Y (2010). N-acetylcysteine inhibit biofilms produced by Pseudomonas aeruginosa. *BMC Microbiology*.10:140

Zheng-hong WU, Qi-neng P, Yi WEI, Jia-ming L (2004). Hypoglycemic efficacy of chitosan-coated insulin liposomes after oral administration in mice. *Acta Pharmacol Sin*.25(7):966-72

Zullo AV, de Francesco G, ManesG, Scaccianoce F, Cristofari and C Hassan (2010). Second-line and rescue therapies for *Helicobacter pylori* eradication in clinical practice. *Journal of Gastrointestinal and Liver Diseases*.2: 131– 134

Zwet AAV,Thijs JC, Kooistra-Smid AMD, and Snijder JAM (1994). Use of PCR with feces for detection of Helicobacter pylori infection in patients*. J. Clin. Microbiol*. 32:1346–1348.

APPENDIX

1. Calibration curve.

1.1. Mean values of concentrations for calibration curves

1. Furazolidone

Concentrations (µg/ml)	Mean value of AUC
10	53.46267
50	284.6517
100	568.7137
250	1506.592
500	3114.368

2. Coumarin-6

Concentration (PPM)	Mean value of Intensity
1	979.719
0.75	738.314
0.5	545.632
0.25	334.403
0.1	160.579
0.05	71.425

3. N-acetyl Cysteine

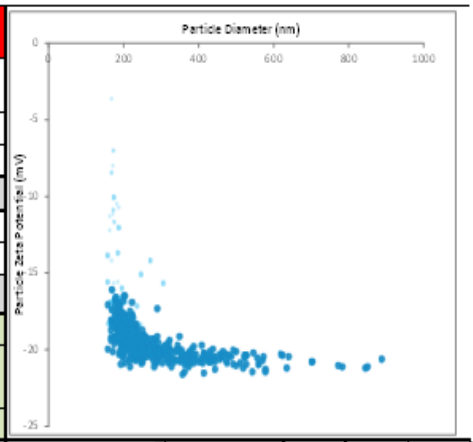
Concentration (mg/ml)	Mean value of AUC
40	296.3333
80	510.3333
120	1042.333
160	1560
200	1876.667

4. Mucin type I sigma Aldrich

Concentration (mg/2ml)	Mean values of absorbance
0.1	0.287
0.25	0.461
0.5	0.633
0.75	0.749667
1	0.935

2. Izon q-nano template for measuring zeta potential (Sample for MP2 and MP5)

Tick Boxes to Show or Hide Data Sets	Sample 1 ☑	Sample 2 ☑	Sample 3 ☑	Sample 4 ☑	Sample 5 ☑
Sample ID:	Ipo				
Mean Filtered Dia (nm)	275	Not Plotted	Not Plotted	Not Plotted	Not Plotted
Mean Filtered Zeta (mV)	-19.1	Not Plotted	Not Plotted	Not Plotted	Not Plotted
SAMPLE MEASUREMENT SETTINGS - check pressures (+) and vacuums (-) are correctly recorded					
Voltage (V)	0.1				
Pressure (Low Range)	0				
DATA FILTERS - enter top and bottom limits in the green cells to display a part of the population					
Diameter Upper (nm)					
Diameter Lower (nm)					
Zeta Upper (mV)					
Zeta Lower (mV)					
% of population displayed	100	Not Plotted	Not Plotted	Not Plotted	Not Plotted



SYSTEM CALIBRATION - enter the electrolyte and calibration particle details into the green cells

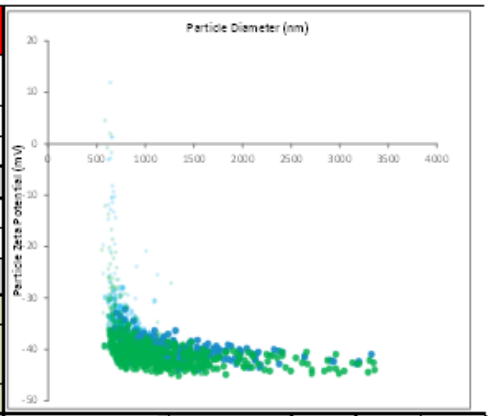
Electrolyte*:	Particles*:	Calibration	Sample ID	Voltage	Pressure
PBS (137 mM)	CPC200	V1P2	V1P2	0.10	2.0
		V1P1	V1P1	0.10	0.0
		V2P1	V2P1	0.08	0.0
		V3P1	V3P1	0.06	0.0

PBS (137 mM) / KCl Tris 100 mM / KCl Hepes 100 mM / KCl Tris 10 mM / User Electrolyte

CPC70 / CPC100 / CPC200 / CPC400

iZON	VARIANCE (mV) Less Than:	1	5	Unlimited
Sample 1	Total Filtered Count = 499	442	39	18
Sample 2	Total Filtered Count = 0	0	0	0
Sample 3	Total Filtered Count = 0	0	0	0
Sample 4	Total Filtered Count = 0	0	0	0
Sample 5	Total Filtered Count = 0	0	0	0

Tick Boxes to Show or Hide Data Sets	Sample 1 ☑	Sample 2 ☑	Sample 3 ☑	Sample 4 ☑	Sample 5 ☑
Sample ID:	Ipo	Upo negative			
Mean Filtered Dia (nm)	1033	1103	Not Plotted	Not Plotted	Not Plotted
Mean Filtered Zeta (mV)	-36.1	-38.9	Not Plotted	Not Plotted	Not Plotted
SAMPLE MEASUREMENT SETTINGS - check pressures (+) and vacuums (-) are correctly recorded					
Voltage (V)	0.1	0.1			
Pressure (Low Range)	0	0			
DATA FILTERS - enter top and bottom limits in the green cells to display a part of the population					
Diameter Upper (nm)					
Diameter Lower (nm)					
Zeta Upper (mV)					
Zeta Lower (mV)					
% of population displayed	100	100	Not Plotted	Not Plotted	Not Plotted



SYSTEM CALIBRATION - enter the electrolyte and calibration particle details into the green cells

Electrolyte*:	Particles*:	Calibration	Sample ID	Voltage	Pressure
PBS (137 mM)	CPC800	V1P2	V1P2	0.10	2.0
		V1P1	V1P1	0.10	0.0
		V2P1	V2P1	0.08	0.0
		V3P1	V3P1	0.06	0.0

PBS (137 mM) / KCl Tris 100 mM / KCl Hepes 100 mM / KCl Tris 10 mM / User Electrolyte

CPC200 / CPC400 / CPC500 / CPC800

iZON	VARIANCE (mV) Less Than:	1	5	Unlimited
Sample 1	Total Filtered Count = 499	254	146	99
Sample 2	Total Filtered Count = 500	334	101	85
Sample 3	Total Filtered Count = 0	0	0	0
Sample 4	Total Filtered Count = 0	0	0	0
Sample 5	Total Filtered Count = 0	0	0	0

3. Izon q-nano report sheet for measuring particle size (Sample for MP2 and MP5)

Standard Analysis Report

06/03/201
Page 1 of

Sample Details

Investigation: irfan MLV1
Sample: Irfan MLV1
Electrolyte ID: Tris
Dilution: 1.00
Record: *3.Irfan MLV1.ibfx*
Notes:
MLV1, 400...

Sample Statistics

Size

Particle Diameter - Mean (nm):	695.9	d50 (nm):	600.5		
Particle Diameter - Mode (nm):	482.1	d10 (nm):	417.6		
Particle Diameter - Max. (nm):	2236.0	d90 (nm):	1101.3		
Particle Diameter - Min. (nm):	328.8	d90/d10:	2.6	Span:	1.1

Concentration

Measured Mean Concentration (particles/mL):	7.1E+012
Raw Mean Concentration (particles/mL):	7.1E+012

Duration

Baseline Duration - Mean (ms):	3.56	FWHM Duration - Mean (ms):	0.10
Baseline Duration - Mode (ms):	0.91	FWHM Duration - Mode (ms):	0.09
Baseline Duration - Max. (ms):	94.62	FWHM Duration - Max. (ms):	0.31
Baseline Duration - Min. (ms):	0.22	FWHM Duration - Min. (ms):	0.06

Run Statistics

Run Time (s):	36	Av. Current (nA):	149.51
Particle Count:	1070	Av. RMS Noise (pA):	121.25
Particle Rate (particles/min):	1765.3		

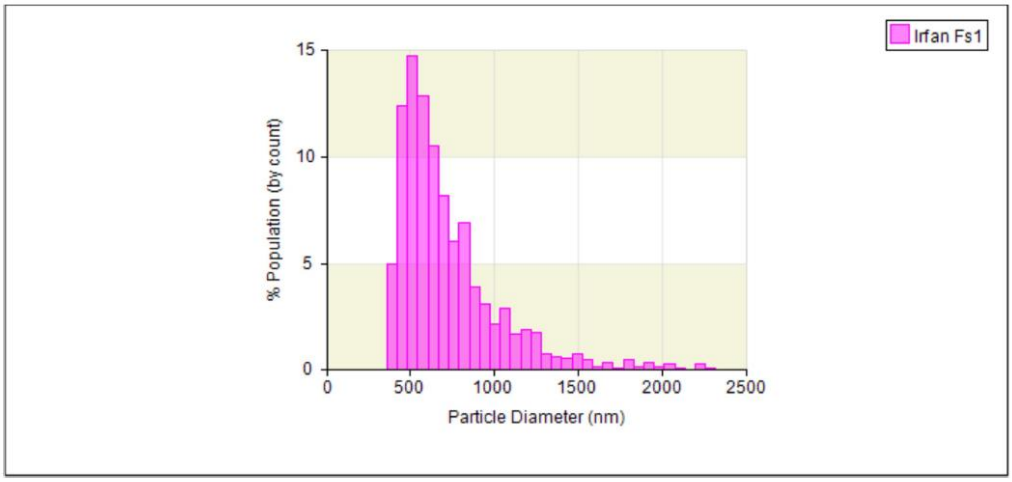
Measurement Settings

Measurement Date: 03 March 2015 16:08
Nanopore ID: A25577 Part #: 400
Stretch (mm): 47.00
Pressure (cm H20): 7
Voltage (V): 0.32
Bandwidth Filter (kHz): Not Applied

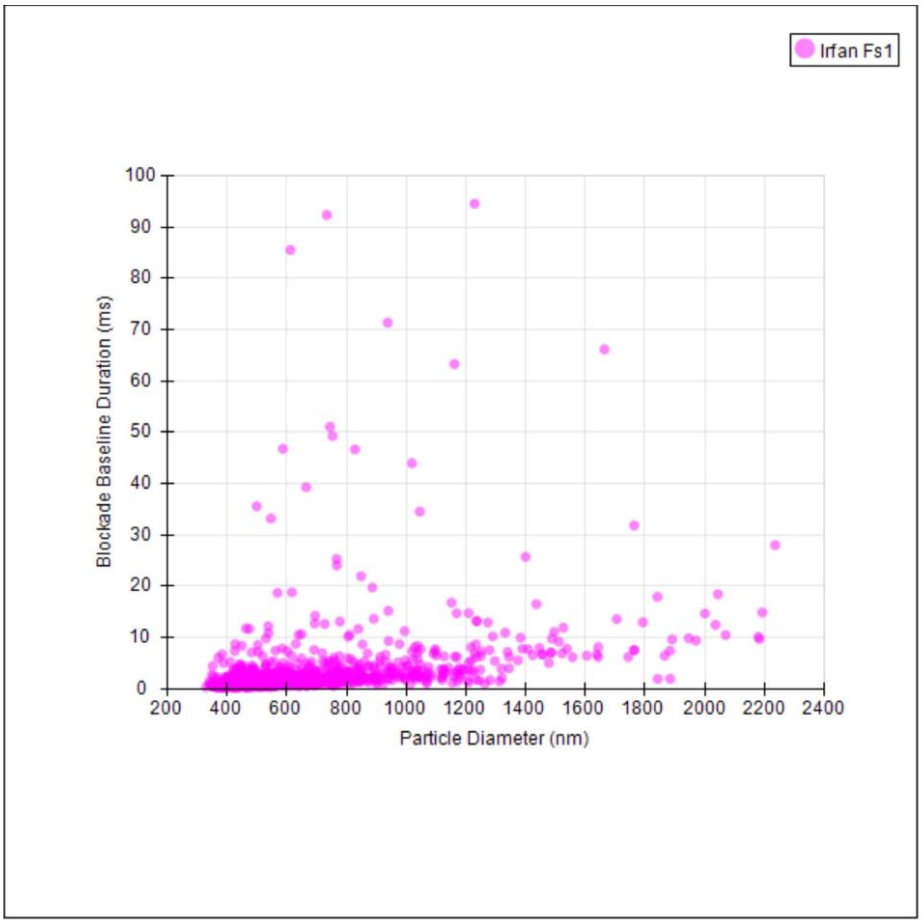
Calibration Files

Sample ID	P (cm H2O)
Irfan Fs1 400	7.00
Irfan Fs1 400	7.00

Size Histogram



Baseline Duration v Size Scatter Plot



Standard Analysis Report

Sample Details

Investigation: Irfan MLV1. 200
Sample: Irfan MLV1.200 (1)
Electrolyte ID: Tris
Dilution: 1.00
Record: *Irfan MLV1.200 (1).ibfx*
Notes:
200 NP, 200 CPC, MLV1 (1)

Sample Statistics

Size

Particle Diameter - Mean (nm):	535.2	d50 (nm):	432.9		
Particle Diameter - Mode (nm):	374.0	d10 (nm):	306.5		
Particle Diameter - Max. (nm):	2082.5	d90 (nm):	893.2		
Particle Diameter - Min. (nm):	266.6	d90/d10:	2.9	Span:	1.4

Concentration

Measured Mean Concentration (particles/mL): 4.3E+012
Raw Mean Concentration (particles/mL): 4.3E+012

Duration

Baseline Duration - Mean (ms):	3.02	FWHM Duration - Mean (ms):	0.16
Baseline Duration - Mode (ms):	0.51	FWHM Duration - Mode (ms):	0.08
Baseline Duration - Max. (ms):	102.98	FWHM Duration - Max. (ms):	13.35
Baseline Duration - Min. (ms):	0.24	FWHM Duration - Min. (ms):	0.06

Run Statistics

Run Time (s):	117	Av. Current (nA):	94.46
Particle Count:	435	Av. RMS Noise (pA):	72.15
Particle Rate (particles/min):	222.5		

Measurement Settings

Measurement Date: 06 March 2015 10:49
Nanopore ID: A26125 Part #: 200
Stretch (mm): 46.99
Pressure: 7
Voltage (V): 0.32
Bandwidth Filter (kHz): Not Applied

Calibration Details

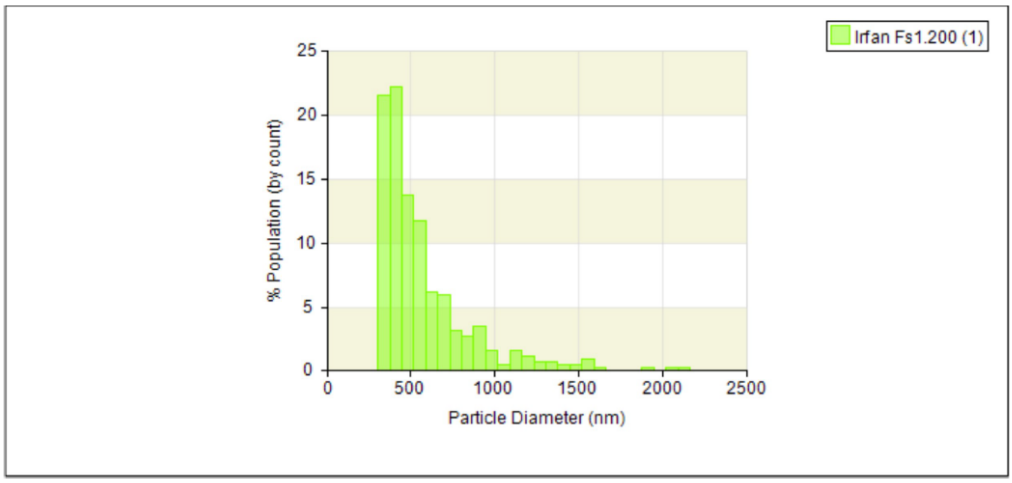
Sample ID: Irfan Fs1
Diameter (nm): 300.0
Raw Concentration (particles/mL): 1.0E+012
Dilution: 1.00
Electrolyte ID: Tris
Record: *Irfan MLV1.200 (1).ibfx*

Calibration Statistics

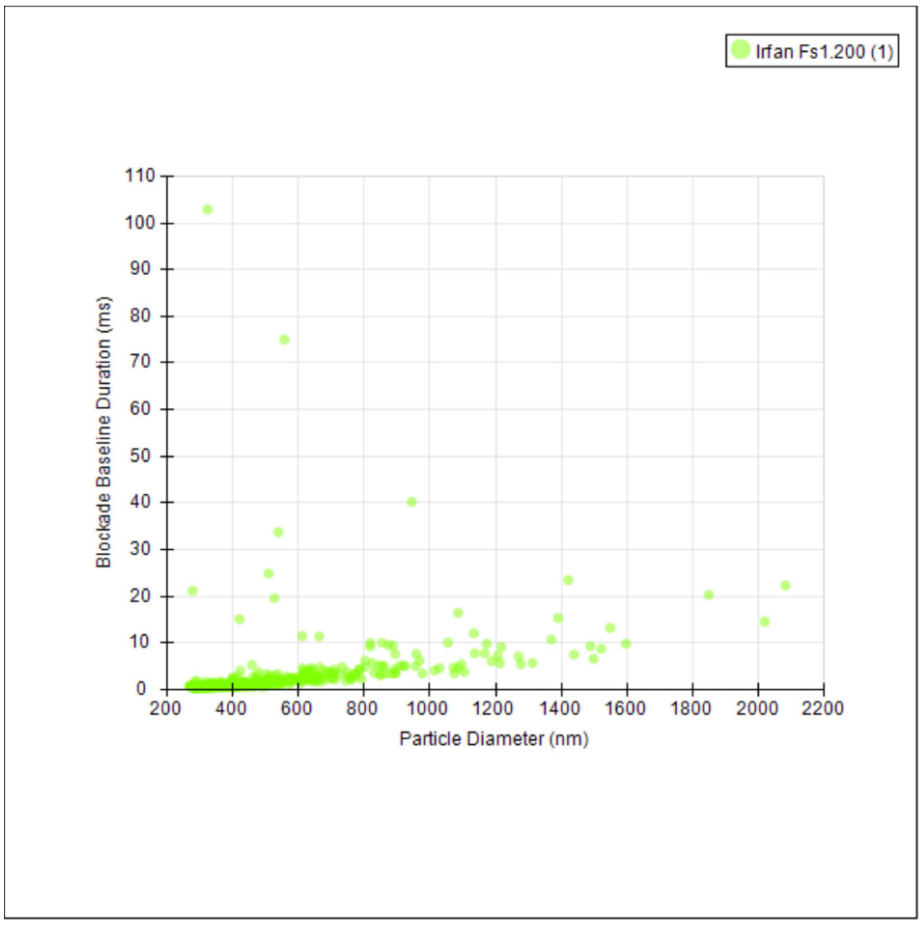
Run Time (s):	121
Particle Count:	104
Particle Rate (particles/min):	51.6
Av. Current (nA):	111.32
Av. RMS Noise (pA):	18.52

Notes:
Irfan Fs1. CPC. 200, (1) Np 200

Size Histogram



Baseline Duration v Size Scatter Plot



4.1. Mucin adsorption type I calculation templet, highlighted portion shows 1st row calculation that applies to all following rows

Mean value of absorbance	**Free mucin (2ml) (0.9-0.2584)/0.6821**	**Free mucin (%) (0.96*100)/1**	**Mucin adsorbed (%) (100-96.35)**
0.915667	0.963592826	96.35928261	3.640717392
0.890333	0.926452622	92.64526218	7.354737819
0.764667	0.742217661	74.22176611	25.77823389
0.712333	0.665493818	66.54938181	33.45061819
0.598667	0.498851586	49.88515858	50.11484142
0.526	0.392317842	39.2317842	60.7682158

4.2. Mucin adsorption type I for spray dried formulation at pH 1.3 and 4.5

Time (hours)	F1	F2	F3
1	3.640717	3.494111	3.103162
2	7.354738	5.155647	6.572839
3	25.77823	17.56829	19.96286
4	33.45062	32.81533	28.51488
5	50.11484	44.78815	36.9203
6	60.76822	54.02434	41.46508

4.2.1. pH: 1.3

Time (hours)	F1	F2	F3
1	22.89498	14.78278	8.820799
2	57.73836	29.39452	26.70674
3	75.81977	59.25329	42.39359
4	95.7582	79.87587	57.24967
5	95.51385	84.90935	71.7148
6	97.07765	87.84147	75.42882

4.2.2. pH: 4.5

5.1. Mucus penetration assay calculations, highlighted portion shows 1st row calculation that applies to all following rows

Time (Hours)	Slice (1mm)	Mean value of intensity (coumarin-6)	Equation (288.238-65.447)/919.77	Dilution factor (25) x 0.24223
1	1	288.238	0.24223	6.05563
2	1	253.477	0.20443	5.11079
3	1	207.62	0.15457	3.86435

5.2. Standard based on calculation of 20µl= 8.022

Time (Hours)	Coumarin-6 present in sample after specified time (1mm)	Standard – 1mm= 2mm (8.022-6.0556)
1	6.05563	1.96637
2	5.11079	2.91121
3	3.86435	4.15765

5.3. Percentage calculated based on table 5.2.

Time (Hours	1st mm (1mm x100)/(standard) 6.0556 x 100/ (8.022)	2nd mm (100% - %1mm) 100-75.48
1	75.4877	24.5123
2	63.7097	36.2903
3	48.1719	51.8281

5.4. Mean values of mucopenetration based on calculations for all formulation at different time intervals

Time (Hours)	MP1	MP2	MP3	MP4	MP5	MP6
1	24.09025042	3.847	4.6302	6.3	3.42224	5.34081
2	36.16302065	3.75309	11.8116	18.5	4.81432	8.94898
3	52.26944057	9.62434	16.4814	21.7	9.82518	14.359

Time Point 1.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D1				D1				D1	21	23	17
D2	36	34	37	D2	15	16	12	D2			
D3				D3				D3			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D1				D1							
D2	55	50	49	D2							
D3				D3	42	37	39				

6.1. Number of colonies at ½ X MIC of 1%NAC+Fur at five different time points

6.1.1. Calculation of log $_{10}$ cfu/ml for experiment at each time point based on number of colonies

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	36	100	18000	1.80E+05	5.255272505
1.5	34	100	17000	1.70E+05	5.230448921
1.5	37	100	18500	1.85E+05	5.267171728
2	15	100	7500	7.50E+04	4.875061263
2	16	100	8000	8.00E+04	4.903089987
2	12	100	6000	6.00E+04	4.77815125
3	21	10	1050	1.05E+04	4.021189299
3	23	10	1150	1.15E+04	4.06069784
3	17	10	850	8.50E+03	3.929418926
6	55	100	27500	2.75E+05	5.439332694
6	50	100	25000	2.50E+05	5.397940009
6	49	100	24500	2.45E+05	5.389166084
8	42	1000	210000	2.10E+06	6.322219295
8	37	1000	185000	1.85E+06	6.267171728
8	39	1000	195000	1.95E+06	6.290034611

6.1.2. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	5.250964385	0.018736624
2	4.852100834	0.065557669
3	4.003768688	0.067350921
6	5.408812929	0.026792489
8	6.293141878	0.027655017

6.2. Number of colonies at 4 X MIC of 1%NAC+Furazolidone at five different time points

Time Point 1.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D1				D1				D1	3	4	3
D2	10	12	9	D2	2	1	1	D2			
D3				D3				D3			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D1				D1							
D2				D2							
D3				D3							

6.2.1. Calculation of log $_{10}$ cfu/ml for experiment at each time point based on number of colonies

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	10	100	5000	5.00E+04	4.698970004
1.5	12	100	6000	6.00E+04	4.77815125
1.5	9	100	4500	4.50E+04	4.653212514
2	2	100	1000	1.00E+04	4
2	1	100	500	5.00E+03	3.698970004
2	1	100	500	5.00E+03	3.698970004
3	3	10	150	1.50E+03	3.176091259
3	4	10	200	2.00E+03	3.301029996
3	3	10	150	1.50E+03	3.176091259
6	0	0	0	0.00E+00	0
6	0	0	0	0.00E+00	0
6	0	0	0	0.00E+00	0
8	0	0	0	0.00E+00	0
8	0	0	0	0.00E+00	0
8	0	0	0	0.00E+00	0

6.2.3. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	4.710111256	0.063210107
2	3.799313336	0.173799749
3	3.217737505	0.072133413
6	0	0
8	0	0

6.3.1. Number of colonies at 8 X MIC of 1%NAC+Furazolidone at five different time points

Time Point 1.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D1	17	19	21	D1	4	2	1	D1			
D2				D2				D2			
D3				D3				D3			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D1				D1							
D2				D2							
D3				D3							

6.3.2. Calculation of log $_{10}$ cfu/ml for experiment at each time point based on number of colonies

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	17	10	850	8500	3.929418926
1.5	19	10	950	9500	3.977723605
1.5	21	10	1050	10500	4.021189299
2	4	10	200	2000	3.301029996
2	2	10	100	1000	3
2	1	10	50	500	2.698970004
3	0	0	0	0	0
3	0	0	0	0	0
3	0	0	0	0	0
6	0	0	0	0	0
6	0	0	0	0	0
6	0	0	0	0	0
8	0	0	0	0	0
8	0	0	0	0	0
8	0	0	0	0	0

6.3.3. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	3.97611061	0.045906445
2	3	0.301029996
3	0	0
6	0	0
8	0	0

Time Point 0.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D1				D1	42	45	40	D1	6	9	4
D2	9	7	11	D2				D2			
D3				D3				D3			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D1	3	2	2	D1	0	0	0				
D2				D2							
D3				D3							

6.4. Number of colonies at 4 X MIC of Furazolidone at five different time points

6.4.1. Calculation of log $_{10}$ cfu/ml for experiment at each time point based on number of colonies

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	9	100	4500	4.50E+04	4.653212514
1.5	7	100	3500	3.50E+04	4.544068044
1.5	11	100	5500	5.50E+04	4.740362689
2	42	10	2100	2.10E+04	4.322219295
2	45	10	2250	2.25E+04	4.352182518
2	40	10	2000	2.00E+04	4.301029996
3	6	10	300	3.00E+03	3.477121255
3	9	10	450	4.50E+03	3.653212514
3	4	10	200	2.00E+03	3.301029996
6	3	10	150	1.50E+03	3.176091259
6	2	10	100	1.00E+03	3
6	2	10	100	1.00E+03	3
8	0	10	0	0.00E+00	0
8	0	10	0	0.00E+00	0
8	0	10	0	0.00E+00	0

6.4.2. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	3.892535651	0.043139871
2	3.232990001	0.207511791
3	2.698970004	0
6	0	0
8	0	0

6.5. Number of colonies at 8 X MIC of Furazolidone at five different time points

Time Point 0.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D1				D1	5	4	2	D1	1	1	1
D2	10	11	15	D2				D2			
D3				D3				D3			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D1	0	0	0	D1							
D2				D2							
D3				D3							

6.5.1. Calculation of log $_{10}$ cfu/ml for experiment at each time point based on number of colonies

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	16	10	800	8.00E+03	3.903089987
1.5	14	10	700	7.00E+03	3.84509804
1.5	17	10	850	8.50E+03	3.929418926
2	5	10	250	2.50E+03	3.397940009
2	4	10	200	2.00E+03	3.301029996
2	2	10	100	1.00E+03	3
3	1	10	50	5.00E+02	2.698970004
3	1	10	50	5.00E+02	2.698970004
3	1	10	50	5.00E+02	2.698970004
6	0	10	0	0.00E+00	0
6	0	10	0	0.00E+00	0
6	0	10	0	0.00E+00	0
8	0	0	0	0.00E+00	0
8	0	0	0	0.00E+00	0
8	0	0	0	0.00E+00	0

6.5.2. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	3.892535651	0.043139871
2	3.232990001	0.207511791
3	2.698970004	0
6	0	0
8	0	0

6.6. Number of colonies in control with no drug at five different time points

Time Point 1.5				Time Point 2				Time Point 3			
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**
D2	18	14	23	D2	34	35	37	D2			
D3				D3				D3	16	13	18
D4				D4				D4			
Time Point 6				Time Point 8							
Column1	**P1**	**P2**	**P3**	**Column1**	**P1**	**P2**	**P3**				
D2				D2							
D3				D3							
D4	13	15	9	D4	22	17	18				

6.6.1. Number of colonies in control with no drug at five different time points

Time Points	Colonies	Dilution	Aliquot	cfu/ml	Log Base 10
1.5	18	100	9000	9.00E+04	4.954242509
1.5	14	100	7000	7.00E+04	4.84509804
1.5	23	100	11500	1.15E+05	5.06069784
2	34	100	17000	1.70E+05	5.230448921
2	35	100	17500	1.75E+05	5.243038049
2	37	100	18500	1.85E+05	5.267171728
3	16	1000	80000	8.00E+05	5.903089987
3	13	1000	65000	6.50E+05	5.812913357
3	18	1000	90000	9.00E+05	5.954242509
6	13	10000	650000	6.50E+06	6.812913357
6	15	10000	750000	7.50E+06	6.875061263
6	9	10000	450000	4.50E+06	6.653212514
8	22	10000	1100000	1.10E+07	7.041392685
8	17	10000	850000	8.50E+06	6.929418926
8	18	10000	900000	9.00E+06	6.954242509

6.6.2. Average of log $_{10}$ cfu/ml at each time point

Time Point	Average	Standard Deviation
1.5	4.95334613	0.107802695
2	5.246886233	0.018661391
3	5.890081951	0.071556894
6	6.780395711	0.114443282
8	6.97501804	0.058806868

6.7. Log $_{10}$ cfu/ml at different concentration of liposomal bound and free furazolidone and NAC

Time (Hour)	FUR +1% NAC	FUR	FUR +1% NAC	FUR	FUR +1% NAC	Control
1.5	5.250964385	4.645881083	4.710111256	4.771464652	3.97611061	4.95334613
2	4.852100834	4.325143936	3.799313336	3.232990001	3	5.246886233
2.5	4.003768688	3.477121255	3.217737505	2.698970004	0	5.890081951
6	5.408812929	3.058697086	0	0		6.780395711
8	6.293141878	0				6.97501804