Development and Characterization of Biomedical Textile through Layer by Layer Deposition of Erythromycin Loaded Antibacterial Nanoparticles

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Development and Characterization of Biomedical Textile through Layer by Layer Deposition of Erythromycin Loaded Antibacterial Nanoparticles

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CERTIFICATE OF APPROVAL

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We hereby recommend that the dissertation prepared under our supervision by: **Misbah Nazir** (NUST201362082MSMME62413F) Titled: **"DEVELOPMENT AND CHARACTERIZATION OF BIOMEDICAL TEXTILE THROUGH LAYER BY LAYER DEPOSITION OF ERYTHROMYCIN LOADED ANTIBACTERIAL NANOPARTICLES"** be accepted in partial fulfilment of the requirements for the award of MS degree with grade ___.

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I hereby declare that this research study has been done for partial fulfilment of requirements for the degree of Master of Science in Biomedical Sciences. The intellectual content of this thesis is a product of my own work and no portion of the work referred to in this thesis has been submitted in any other degree or other institute of learning. I also certify that the thesis has been written by me. The help I received during my research work and preparation of the thesis, itself has been acknowledged. Moreover, I certify that all sources and literature used have been indicated in the thesis.

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DEDICATION

Dedicated to my parents, to whom I owe everything!

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Misbah Nazir

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LIST OF ABBREVIATIONS

NPs	Nanoparticles
HPMCP	Hydroxypropyl methylcellulose phthalate
EM	Erythromycin
Nps	Nanoprecipitations
BL	Bilayers
LbL	Layer by Layer
S. aureus	*Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
°C	Degree centigrade
Rpm	Rotations per minute
OM	Optical Microscopy
OP	Optical Profilometry
SEM	Scanning Electron Microscopy
AFM	Atomic Force Microscopy
Nm	Nanometre
µm	Micrometre
Mm	Millimetre
mV	Millivolts
mM	Milimolar
Mg	Milligram
µg	Microgram
+ve	Positive
-ve	Negative
PDI	Polydispersity index
Mins	Minutes
APTMS	3-aminopropyltrimethoxysilane toluene
PDAC	Poly (diallyldimethyl ammonium chloride
ZOI	Zone of inhibition

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ABSTRACT

Skin infections are an important concern in human health. Fabrics as they are worn next to skin have an important role in the occurrence and spread of skin infections. The aim was to render antibacterial properties to the textile so that it can be used to prevent and/or treat skin infections. Nanotechnology has great potential for effective antibacterial therapy along with reduced side effects and better patient compliance. Nanoprecipitation technique was employed to prepare antibiotic loaded polymeric nanoparticles. For preparing nanoprecipitations erythromycin and the hydroxypropyl methyl cellulose phthalate were dissolved in acetone and added to surfactant stabilized water phase drop by drop under constant stirring. Nanoprecipitations were optimized for stability by varying different parameters. The most stable blank nanoprecipitations had nanoparticles of 189 nm in size with a poly dispersity index of 0.177 while drug loaded nanoprecipitations were found to have nanoparticles of 65 nm with a PDI of 0.169. Zeta potential values were -37mV and -17.2 mV for blank and drug loaded samples respectively. Size and shape of nanoparticles was also determined through SEM. Antibacterial activity was tested against *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027). Antibacterial nanoprecipitation was used to coat polyester fabric through Layer by Layer deposition method. 1,5,10 and 15 bilayers were produced. Antimicrobial activity of treated fabrics was evaluated against *Staphylococcus aureus* (ATCC 6538) using standard parallel streak method (AATCC147-2011). Treated fabrics showed good antimicrobial activity that increased with increasing number of bilayers.

Keywords: Nanoprecipitation, Erythromycin, Antibacterial nanoparticles, Biomedical Textile, Antibacterial coatings, Layer by Layer method, LbL antibacterial coatings

Chapter 1

Introduction

1.1 Background

Infectious diseases impose an important health challenge and are one of the prominent cause of deaths equally for children and adults worldwide [1].

Skin and soft tissue infections (SSTIs) is one of the important infectious diseases and includes microbial attack on the skin and the soft tissues underneath. Skin possess extremely diverse microbiota that may produce infection [2].

Due to variable presentation of SSTIs, it is difficult to calculate their incidence and prevalence. Incidence rate of SSTIs is estimated to be 24.6 per 1000 person [3]. Prevalence estimate is highly variable as majority of SSTIs appear to resolve in 7-10 days. SSTIs prevalence is 7% to 10% among hospitalized patients [4] [2].

Among all infection patients in hospital, SSTIs are the prominent ones and they are third most common diagnosis in emergency care settings [5].

Staphylococcus aureus (*S. aureus*) and *Streptococcus pyogenes* are notoriously pathogenic in the skin. Gram-negative organisms such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Pasteurella multocida*, Capnocytophaga canimorsus, *Bartonella* sp., *Klebsiella rhinoscleromatis*, and *Vibrio vulnificus* are not typical resident skin microflora but may cause cutaneous infections [6].

S. aureus is the most common cause of cutaneous infections [7] while *S. aureus* and *P. aeruginosa* are the commonly found bacteria in chronic wounds and on the skin [8].

These bacteria find their way to the host through contaminated environment and contaminated surfaces [9] and are a major threat to the immune compromised individuals especially [10] .

Textiles can be a carrier of microorganisms and can provide ideal environment for survival and growth of microorganisms as well. Dissemination of these microorganisms poses a health risk. When the fabric is worn next to skin, infestation of

microorganisms may cause cross infections by pathogens and odour development. So it is desirable to render antimicrobial properties to the fabrics [11] [12] [13].

1.2 Biomedical Textile

Textile products and constructions devised for biological and medical applications are categorized as biomedical textiles and find applications for first aid, clinical and hygienic purposes. Biomedical textiles are used in protective and healthcare textiles (operating drapes, wound dressings, surgeon's wear, bandages and sanitary towels), implantable materials (artificial ligaments, sutures, vascular grafts suture), external devices and extracorporeal devices [14].

1.2.1 Antibacterial Textile

Antibacterial textile is nested under biomedical textile and can be defined as textile materials or fabrics that are capable of killing the bacteria and/or inhibiting their growth. As already mentioned, textiles can be a carrier of microorganisms and can also provide ideal environment for survival and growth of microorganisms. Dissemination of these microorganisms poses a health risk. When the fabric is worn next to skin, infestation of microorganisms may cause cross infections by pathogens and odour development. So it is necessary to render antimicrobial properties to the fabrics. An ideal antimicrobial treatment should not only destroy undesirable microorganisms, but also satisfy a number of requirements concerning their effectiveness against a broad spectrum of microorganisms and minimum side effects or toxicity towards consumers [11] [12] [13] .

Consumer attitudes towards health and active lifestyle have created a rapidly growing market of antimicrobial textiles in order to protect the user from contamination by microorganisms. Fabrics can be functionalized for antibacterial activity through various procedures and antimicrobial finishing is one of them. Antimicrobial functional textile finishing adds a therapeutic value to the material intended, for example, for wound healing [15] [12] [16].

Several classes of antimicrobial agents have been used for antimicrobial functionalization of textiles. Most prevalent antimicrobials used include quaternary ammonium compounds (QAC), metal–organic complexes (such as zinc pyrithione), halogenated phenols (such as triclosan), polybiguanides (such as polyhexamethylene

biguanides, PHMB), N-halamine compounds, metals and their corresponding oxides and salts, regenerable peroxides [17] [18] [19] [20] [21].

For a variety of applications antimicrobial coatings have been developed with the aim of reducing the proliferation of bacteria, fungi, and viruses on different surfaces. Due to the increased risk of healthcare associated infections, scientific research involving antimicrobial coatings for textiles is gaining attention in healthcare industry [9].

1.3 Antibiotics as a Treatment Modality for Infectious Disease

The treatment of infectious diseases was revolutionized by discovery of antibiotics, and associated morbidity and mortality reduced significantly [22]. In every critical therapeutic area antibiotics are considered indispensable. General surgeries, organ transplants, chemotherapy in cancer patients and treatment of premature babies cannot be accomplished without efficiently preventing and treating bacterial infections [23]. Antibiotic drug therapy is compromised by numerous limitations. i.e. inadequate concentration of drug at target infection site, frequent administration, severe side effects and poor patient compliance [24] [22].

1.4 Potential of Nanotechnology for infectious disease treatment

Nanotechnology refers to the design as well as production and application of nano-sized materials.

Nanotechnology is being considered as a new paradigm to revolutionize the end results in the treatment of infectious diseases [22] . Due to novel physicochemical properties including small size, large surface area to mass ratio, unique interactions with cells of the host and microorganisms, and the ability to be structurally and functionally modified, nano-sized drug delivery systems could be a promising approach to overcome the current challenges of antibiotic therapy [25] [26]. Nano-sized antibiotic drug delivery systems bag the advantages of targeted drug delivery, uniform drug distribution at target site, better cellular internalization and solubility and sustained drug release. They also minimize the side effects related to antibiotic drug therapy and improve the patient compliance [27]. Antibacterial nanoparticles with applications in plastics, textile and other products have been produced in past and have been applied as antibacterial coatings as well [8].

1.5 Research Objectives

The aim of the project is to develop an antibacterial fabric (using antibiotic loaded nanoparticles as a coating) that can be used for prophylactic and/or treatment purpose for bacterial skin infections.

Chapter 2

Literature Review

2.1 Erythromycin

2.1.1 Origin and Classification

Erythromycin (EM) is a macrolide antibiotic derived from *Streptomyces erythreus*. It consists of a 14-member macrocyclic lactone ring, to which two sugar moieties cladinose and desosamine are attached [28]. Figure 2.1 shows the chemical structure of EM. Erythromycin is a hydrophobic [29], positively charged antibiotic [30]. It is basically a lipophilic molecule with a limited solubility in aqueous media [31].

Erythromycin belongs to class 4 drugs according to Biopharmaceutics Classification System and has low solubility and low permeability. So in clinics it is used in the form of esters and salts [32].

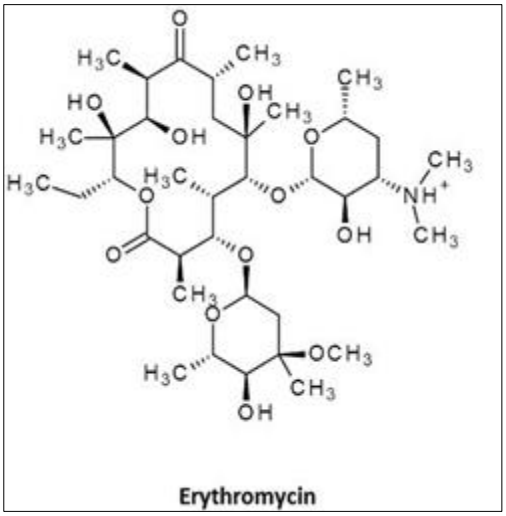

Figure 2.1: Chemical structure of Erythromycin [30].

2.1.2 Mode of Action

Depending upon the sensitivity of the organism and concentration of the antibiotic, EM may exhibit bacteriostatic or bactericidal effect [33]. In the susceptible

organism EM exhibits its antibacterial effect by inhibiting RNA-dependant protein synthesis. It binds to the 50s subunit of ribosome and during elongation phase of the protein synthesis, induces dissociation of peptidyl transfer RNA from the ribosome. Hence RNA-dependant protein synthesis is blocked and bacterial growth stops [28][34].

2.1.3 Efficacy against Infectious Bacteria

Erythromycin , a leading member of macrolide antibiotics (with broad spectrum activity against gram-positive and some gram negative bacteria [35]**)** is effective against a wide range of pathogens including *Bordetella pertussis*, *Brucella* sp, *Mycoplasma pneumoniae*, and intracellular microorganisms like *Chlamydia* [34].

It is a drug of choice in respiratory, ocular, corynebacterial, neonatal or genital chlamydial infections. EM is prescribed as a prophylactic treatment against endocarditis during dental procedures in patients with valvular heart disease. It is first drug of choice against pneumonia caused by *Legionella pneumophila* as well as *Mycoplasma pneumoniae* infections. EM is effective against skin infection causing bacteria *S. aureus, Streptococcus pyogenes, Escherichia coli* and *P. aeruginosa* [28]. *S. aureus*, *Streptococcus agalactiae* and *Streptococcus pyogenes* are the pathogens most commonly isolated from skin and soft tissue infections and EM exhibits effective antibacterial properties against these bacteria. EM has been found to effectively treat skin infections and eradicate *S. aureus* [36].

2.1.4 Advantages

EM is a safe antibiotic and most troublesome reactions are not life threatening. Rarely allergic reactions with rash can occur [28].

EM is a macrolide antibiotic and penetration feature of macrolides across cellular membranes is superior to many other antibiotics [37].

EM has been used to treat infectious disease for more than 50 years and has attracted a lot of attention in past decade due to its unique anti-inflammatory effects far beyond antibiotics[38] [39] [40] [41].

It exhibits a distinctive property of “phagocyte-targeted delivery” and concentrates in macrophages and monocytes [42] [43] [44] [45].

Through targeting to NF-κB signalling, EM produces anti-inflammatory effects [46] [47] [48].

2.1.5 Limitations

Several challenges associated with oral administration of EM include acid instability, bitter taste and gastrointestinal disturbances. Although EM is absorbed well from gastrointestinal tract but it is susceptible to degradation by gastric acid. EM is available in market in the form of enteric coated tablets to prevent degradation by gastric acid and release the active contents into the intestines. Esters and ester salts of EM are also available that are tasteless and more acid stable [49]. Gastrointestinal side effects of EM include nausea, vomiting, abdominal pain, diarrhoea, and anorexia [28] [50].

2.2 Antibacterial Nanoparticles

Nanotechnology is being considered as a new paradigm to revolutionize the end results in the treatment of infectious diseases [22]. Various biodegradable nanoparticles including solid lipid nanoparticles (SLN), liposomes and polymeric nanosystems for example; polymer micelles, polymeric nanoparticles, niosomes and dendrimers have been discovered for intracellular delivery of such antimicrobial agents that exhibit efficient bactericidal activity in vitro [51] [52] [53].

2.2.1 Applications of Antibacterial Nanoparticles

Antibacterial nanoparticles have found many applications including nanomedicine, disinfectants, antibacterial coatings and preservatives (Figure2.2) [8].

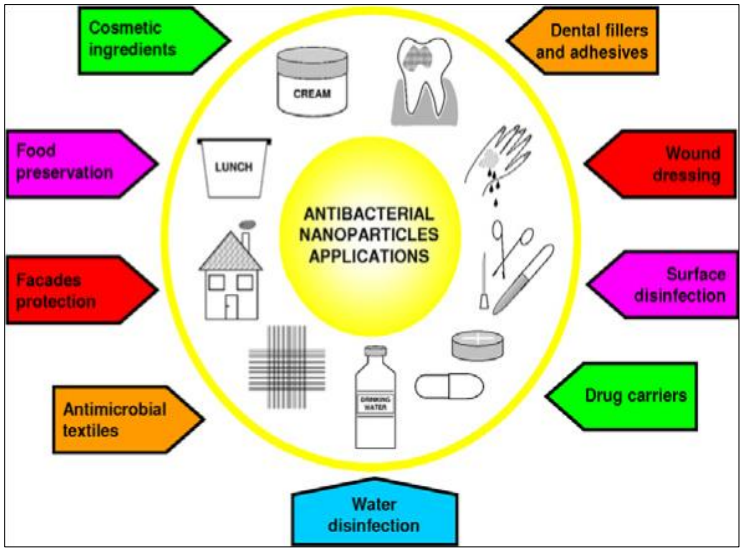

Figure 2.2: Versatile applications of antibacterial nanoparticles [8].

Nanoparticles (NPs) offer new promise for the development of nanomedicine. [54]. Nanoparticles are often used as carriers of antimicrobial drugs. Building Nano-based drugs of several antibiotics have been shown to improve pharmacokinetics or efficacy of antibiotics by the release of drug directly to the site of infection. Drug delivery and encapsulation through nanoparticles can help prevent side effects [55] [56].

Antibacterial NPs have been developed in past using various antibacterial agents including zinc oxide [57], silver [58] [59], and antibiotics like vancomycin [60] [61] violacein [62] gentamicin [63] ofloxacin and levofloxacin [64] doxycycline [55] cefotaxime, ampicillin, ceftriaxone and cefepime [65].

Antibacterial NPs have been applied as the components of dressing materials, inhibiting bleeding, protecting the wound from environmental irritants and preventing the invasion of microorganisms [66].

For application as preservatives, antibacterial silver NPs have been developed to not to penetrate the human skin but to protect the cosmetics against bacteria and fungi [67] antibacterial solid lipid nanoparticles (SLN) have been developed with potential application as food preservatives [68].

Antibacterial NPs with applications in plastics, textile and other products have also been produced [69] [70].

Some antibacterial NPs have been developed to be used as coating to plastics, surgical tools and catheters [71] [72]. Antibacterial surfaces have been produced using silver NPs [73].

For dentistry and orthopaedic applications, antibacterial nano-structured coatings for implants have been developed to prevent post-operative infections and enhance tissue integration [74].

Self-disinfecting fibers [75] and antibacterial textiles have also been developed employing antibacterial NPs [76] [77] [78].

2.2.2 Strategies for Drug Delivery

Figure 2.3 shows the strategies of nano-sized drug delivery systems for antibacterial treatment therapy. NPs might release the drug upon attachment with bacterial cells (a) or in the medium outside the bacterial cells (b) or stimuli responsive release of the drug (c) in which antibacterial agent is released from the NPs when it comes across several specific stimuli i.e change of pH.

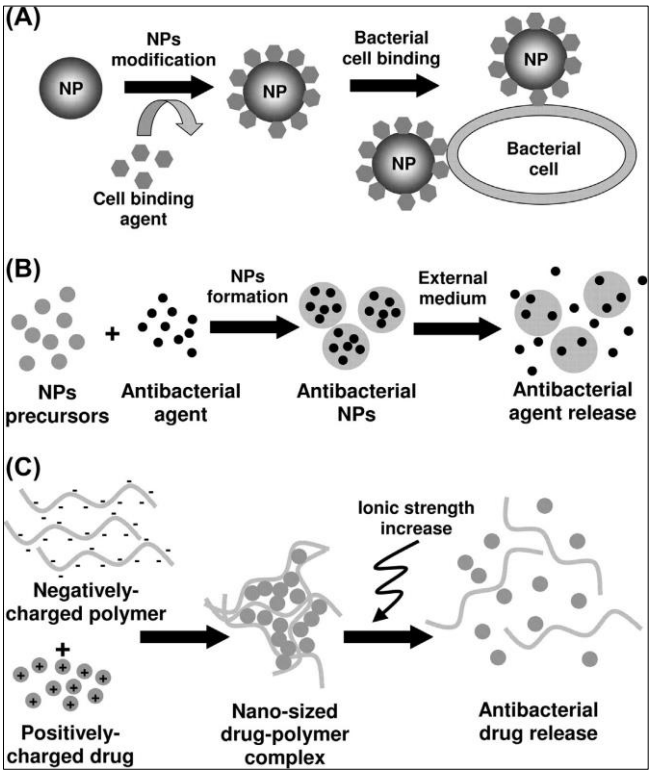

Figure 2.3: Strategies of nano-sized drug delivery systems for antibacterial treatment therapy [8].

2.2.3 Antibiotics Commonly Incorporated into Nanoparticles

Biodegradable nanoparticles have emerged as a promising approach to ship antimicrobial agents into specific cells due to their novel properties. Different classes of antibiotics including Tetracycline, Vancomycin, Antituberculosis drugs, Macrolides, Chloramphenicol, Beta-lactames, Fluroquinolones and Aminoglycosides have been incorporated into the NPs [37].

2.3 Nanoprecipitation Technique

The nanoprecipitation method was developed by Fessi *et al.*, [79] It is one of the easiest methods to produce nanoparticles [80].

Nanoprecipitation finds various definitions in the literature i.e. solvent displacement, interfacial deposition [81], anti-solvent precipitation [82], solvent shifting, solvent replacement, polymeric ouzo effect, solvent exchange and spontaneous emulsion solvent diffusion [83].

2.3.1 Basic Principle

Nanoprecipitation is basically produced from three basic components; polymer, polymer solvent and the non-solvent of the polymer.

Polymer solvent is chosen from organic solvents and requires to be miscible with water and easily removable by evaporation. Most frequently used polymer solvent in nanoprecipitation method is acetone (methanol, ethanol and dimethyl sulfoxide have also been used [84]). Water is generally used as the non-solvent. A stabilizer or surfactant is generally added in the non-solvent to ensure the stability of nanoparticles [85]. It prevents the aggregation of nanoparticle suspensions during long term storage [80].

Amphiphilic polymers as well as completely hydrophobic polymers can be employed to produce nanoparticles through the nanoprecipitation technique [86].

Practically a polymer (in case of drug loaded nanoparticles, drug is also added along with polymer) is dissolved in the water miscible organic solvent and is added drop by drop into a surfactant containing non-solvent of solute (generally water) under constant stirring. The resulting binary solution turns out to be a non-solvent for hydrophobic/amphiphilic polymer molecules and system goes toward phase separation. Nanoparticles form as a result of the 'Marangoni effect' that occurs at the interface of a solvent and non-solvent because of interfacial tension. Organic solvent rapid diffusion into the aqueous phase leads to the precipitation of polymer into nanoparticles [85] [87]. Figure 2.4 is a general representation of the nanoprecipitation technique.

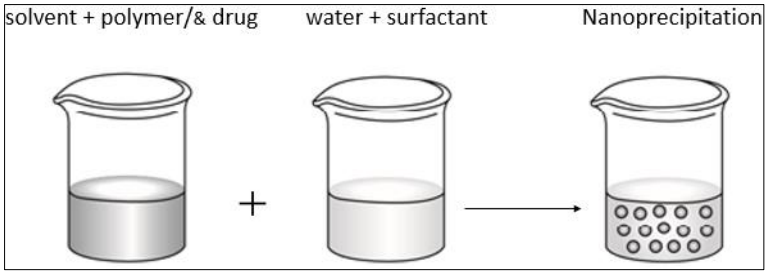

Figure 2.4: Schematic diagram of nanoprecipitation technique [81].

2.3.2 Advantages

As compared to other nanotechnologies, nanoprecipitation needs very simple instrument set up and it is a less complex, more facile, fast, economic, reproducible and easy to scale up method [86] [83] [80]. In nanoprecipitation, particle formation is

spontaneous and does not require sonication, very high temperature or high speed mixing [81].

2.3.3 Applications in Topical Nanomedicine

Conventional nanoprecipitation technique is most suitable to encapsulate hydrophobic drugs [81] and can be used to produce nanoparticles of poorly water soluble drugs through mixing the drug solution with non-solvent. Nanoprecipitation is a widely applicable technique to manufacture defined nanoparticles with high drug loading ability and so it is most commonly used to formulate nanoparticles for skin applications [88].

2.3.4 Previously developed Drug loaded Nanoprecipitations

Anti-inflammatory nanoprecipitation

Dias and colleagues employed nanoprecipitation and dialysis methods to obtain nanoparticles (NPs) of acetylated cashew gum (ACG). NPs synthesized by dialysis showed greater average size compared to those synthesized by nanoprecipitation. NPs were loaded with diclofenac diethylamine. For nanoprecipitation method the particle size was found to be 79.37 nm with a PDI of 0.354 and zeta potential was -20.2 mv [89].

Antibacterial nanoprecipitations

Nafee and colleagues attempted to develop a biodegradable nano-carrier to preserve antibacterial photoactivity of hypericin. They synthesized and used PCL-PEG amphiphilic block copolymers to prepare hypericin-laden NPs of the size of 45nm. The antimicrobial activity was assessed against *S. aureus* and suggested the use of nano encapsulated hypericin particles as antibacterial therapy [90].

Mehdi and colleagues synthesized anethole and carvone loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles to prolong the efficacy of these two essential oils. NPs produced through nanoprecipitation exhibited size of 126 and 158 nm for carvone and anethole respectively. Antimicrobial studies suggested that essential oil loaded PLGA nanoparticles could be useful for biomedical and food applications. Minimum inhibitory concentration (MIC) of carvone loaded nanoparticles against *S. aureus* and *E. coli* was 182 and 374 µg/ml respectively. While MIC of anethole loaded NPs against *S. typhi* was 227 µg/ml [91].

Nanoprecipitations Prepared Incorporating Antibiotics

Ghari *et al.,* prepared azithromycin loaded PLGA nanoparticles using nanoprecipitation technique. Nanoparticles obtained were spherical in shape with an average diameter of 183nm [92].

Mohammadi *et al.,* also formulated azithromycin loaded PLGA nanoparticles using nanoprecipitation technique. Nanoparticles with different drug to polymer ratio were prepared. Size range of 212-252nm and good antibacterial activity was observed [93].

In another study Mohammadi *et al.,* prepared clarithromycin loaded PLGA nanoparticles using different drug to polymer ratios. Size range in this case came out 189-280 nm. NPs exhibited good antibacterial activity against *S. aureus* [94].

According to the best of our knowledge there is no data regarding the production of erythromycin nanoparticles through nanoprecipitation technique or any other technique.

Only a single study was found on nanoparticles of erythromycin. In this study by Bosnjakovic and colleagues erythromycin was fabricated into a nanodevice by conjugating it to polyamidoamine dendrimer. This conjugate showed good potential to be used as a Nano device for targeted and sustained treatment of orthopaedic inflammation. Antibacterial activity of the conjugate against *S. aureus* was found to be similar to free erythromycin [38].

2.4 Commonly Used Polymers in Nanoparticles for Drug Delivery Applications

In the pharmaceutical field polymeric nanoparticles (PNPs) have been extensively studied as drug carriers [92]. Objective of preparing NPs using polymers is to; increase the therapeutic benefits of the drug, efficiently deliver the drug to its target site, minimize the side effects of the drugs and increase its therapeutic benefits [95] [96]. Due to their polymeric composition PNPs when compared to liposomes; exhibit greater stability under storage and in biological fluids [97].

Naturally existing polymers like alginate, gelatin and chitosan are widely used to produce PNPs for drug delivery applications [98]. Synthetic polymers that have been mostly used to produce nanoparticles through nanoprecipitation technique for drug delivery applications, include poly(lactic-co-glycolic) acid (PLGA), poly(lactic acid)

(PLA), polycarpolactone (PCL) and poly(alkyl cyanoacrylate) (PACA) [87]. Among these, PLA and PLGA have been widely used for drug encapsulation studies. Although nanoprecipitation is a very simple and efficient technique but very few studies describe the use of polymers other than mentioned above for the formation of polymeric nanoparticles through nanoprecipitation [86].

2.5 Hydroxypropyl methylcellulose phthalate

Hydroxypropyl methylcellulose phthalate (HPMCP) is a cellulosic polymer and can be defined as a cellulose ester, prepared by esterification of cellulose with phthalic anhydride [99]. Chemical structure is shown in figure 2.5.

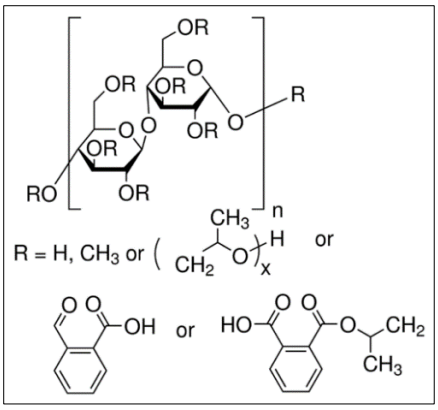

Figure 2.5: Chemical structure of hydroxypropyl methyl cellulose phthalate [100].

HPMCP is basically a negatively charged polymer [101] and exhibits amphiphilic nature [102]. Trade name for hydroxyl propyl methyl cellulose phthalate is HPMCP. HPMCP was introduced as enteric coating polymer in 1971 and is marketed as HPMCP 50, 55 and HP-55-S which are three different form of HPMCP with different degree of esterification/phthalate content [99].

HPMCP solubility depends upon pH. Its insoluble at acidic pH but becomes soluble at mildly acidic to slightly alkaline pH [103]. Dissolution pH depends upon the degree of esterification. HPMCP 50 dissolves at pH 5.0 while HPMCP 55 and 55-s dissolve at 5.5 [103] [104].

2.5.1 Applications in Healthcare

HPMCP has been used as a pH sensitive enteric polymer coating for safe delivery of drugs and proteins to the small intestine protecting them from degradation by gastric acid.

Hydroxylpropyl methylcellulose phthalate is used widely in food and drug delivery applications [105]. In past HPMCP has been modified by thiolation and used for developing vaccines; Singh *et al.,* developed an oral vaccine using thiolated HPMCP to deliver protein antigens specifically to the intestinal cells [106], while Li *et al.,* developed a mucoadhesive nasal vaccine against *Actinobacillus pleuropneumoniae* using thiolated HPMCP [107].

A study by Klayraung *et al.,* proved the ability of HPMCP as a matric material to safely deliver the probiotics to the intestinal cells and to preserve the cell viability of probiotic bacteria [108].

In another study HPMCP was used to produce microcapsules containing diclofenac-resin complexes by a non-aqueous emulsion method [109].

2.5.2 Applications in Preparing Nanoparticles

Due to low water solubility of HPMCP, it has been used to develop organic solvent-based enteric coating technology for many years [110].

HPMCP has been used to develop pH-sensitive insulin nanoparticles aiming for; prevention of insulin from degradation in stomach and intestinal specific delivery of the insulin [111] [112] [113].

In a study by Jin and colleagues Lutein (a natural pigment with excellent anti-oxidation properties) was nano-encapsulated with hydroxypropylmethyl cellulose phthalate (HPMCP) to maintain its bioactivity and avoid thermal/light degradation. Supercritical antisolvent precipitation was applied to prepare lutein/HPMCP nano-capsules. The mean diameter of nanocapsules ranged from 163 nm to 219 nm [114].

In a study by Wang and her colleagues cyclosporine A (CyA) loaded nanoparticles by solvent displacement method were prepared using HPMCP. Particle size of 50–60 nm was observed and bioavailability of CyA loaded HPMCP-HP50 NPs was 82.3% [115].

2.6 Antibacterial Textile

2.6.1 Commonly Used Antibacterial Agent for Antibacterial Coating

Several classes of antibacterial agents have been used for antibacterial functionalization of textiles. Most prevalent antimicrobials used include quaternary ammonium compounds (QAC), metal–organic complexes (such as zinc pyrithione), halogenated phenols (such as triclosan), polybiguanides (such as polyhexamethylene biguanides, PHMB), N-halamine compounds, metals and their corresponding oxides and salts, regenerable peroxides [17] [18] [19] [20] [21].

In addition chitosan and its derivatives has been used as active antibacterial agents to develop antibacterial fabrics in past [116]. Chitosan has also been used in combination with other agents to develop antibacterial fabrics [117].

2.6.2 Previously Developed Antibacterial Fabrics

Han, S and Y. Yang developed antibacterial fabric using curcumin. Curcumin is a natural dye used in food and fabric coloring and possess bactericidal properties [118].

Tessier and colleagues developed a colloidal solution of silver salts stabilized by surfactant. Subsequently the solution was used to coat different fabrics. Efficient antibacterial properties were exhibited by these fabrics due to sustained release of silver ions [119].

Xu *et al.,* developed antibacterial fabric by a simplistic technique. Polyester fabric was first modified by simple dip-coating in aqueous solution of dopamine. Subsequently silver nanoparticles were produced in situ on the surface of modified fabric in aqueous solution of silver nitrate. These fabrics exhibited durable antibacterial properties [120].

Scholz and colleagues coated fabrics (made up of SiO_2 fibers) with precious metals using magnetron sputtering technique. Layers of copper, gold, platinum, silver and platinum/rhodium were deposited on fabrics separately. Antibacterial testing (AATCC 147-1998) revealed that copper was most effective and bacteria and silver was second. Other metals did not exhibit significant antibacterial activity [121].

Wang *et al.,* developed antibacterial polypropylene fibers by depositing nanostructured silver films of different thicknesses through magnetron sputter coating. This treatment imparted antibacterial and electrical conductance properties to the

substrate. An increase in antibacterial activity was observed with increase in thickness of coating [122].

In another study polyester fabric was functionalized with nanostructured silver films through magnetron sputtering. The treatment was shown to impart hydrophobicity, UV protection and efficient antibacterial properties to the treated fabrics [123].

Hegemann and co-workers developed multifunctional fabrics exhibiting good antibacterial activity. The procedure they employed to deposit the antibacterial plasma coatings onto the fabrics was plasma polymerization of acetylene mixed with ammonia and plasma co-sputtering of a silver target. Plasma polymerization deposited accessible amine groups inside a nano-porous hydrocarbon matrix. Plasma co-sputtering of silver generated silver nanoparticles in situ, inserted within the growing plasma polymer. At the coating surface a well-defined size as well as distribution of nanoparticles was achieved and hence the coated fabrics showed good antibacterial properties [124].

Mihailovic *et al.,* developed multifunctional nanocomposite textile. They modified polyester fabric using colloidal TiO_2 nanoparticles and natural polysaccharide alginate. Modified fabric exhibited efficient antibacterial activity [125].

Subash *et al.,* developed antibacterial fabric using Zinc Oxide (ZnO) nanoparticles as a coating. ZnO nanoparticles were produced by wet chemical method using zinc nitrate and sodium hydroxide as precursors while soluble starch as a stabilizing agent. Coated fabrics showed durable and efficient antibacterial activity against *S. aureus* and *E.coli* [126].

Lime *et al.,* developed antibacterial cotton fabric by coating it with a derivative of chitosan. Fiber-reactive chitosan derivative, O-acrylamidomethyl-N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (NMA-HTCC), was applied to cotton fabrics as a textile finish. The cotton treated with NMA-HTCC exhibited efficient bacterial reduction in antibacterial assay [127].

Abdel-Mohsen and colleagues developed antibacterial cotton fabric using a chitosan based copolymer. Copolymer was produced by the reaction of methoxy polyethylene glycol (produced by oxidation of polyethylene glycol with acetic anhydride in dimethyl sulphoxide) aldehyde with chitosan and subsequent reduction through sodium borohydride. Chitosan-N-polyethylene glycol graft copolymer was thus produced and was chemically attached to cotton fabrics using sodium

hypophosphite and citric acid as catalysts. Treated fabrics showed zones of inhibition of 28-30 mm against *S. aureus* and 30-32 mm against *E. coli* in antibacterial testing (AATCC-147) [117].

Ibrahim and his colleagues imparted UV-blocking and antibacterial properties to the polyethylene terephthalate-containing fabrics. Functional groups (carboxyl) were generated onto the surface of polyester component through alkaline hydrolysis of fabrics. Alkali treated fabrics were then treated with basic dyes (c.i. basic red 24 and basic blue 9), metallic salts (zinc acetate and copper acetate) and an antibiotic (Doxymycin). Treated fabrics from each of three groups showed efficient antibacterial and UV-blocking properties [116].

2.7 Layer By Layer Coating Method

Layer by layer (LbL) coating/deposition method involves the alternative deposition of oppositely charged colloids. It was first recommended by R. Iler in 1966. And in early 1990s, LbL assembly was rediscovered by Decher and colleagues to prepare a polyelectrolyte multilayer thin films [128] [129] [130] [131].

LbL assembly technique was initially developed to fabricate composite thin films. Sequential adsorption of oppositely charged polyanions and polycations on solid surfaces leads to the build-up of polyelectrolyte multilayer films. The sequential multilayer deposition can be carried out by immersing the substrate into the cationic and anionic solutions, alternately. After deposition of each layer, the substrate is immersed into the washing solution. Both adsorption steps can be repeated cyclically to form multilayer structures on the surface of a substrate. A wide range of functional molecules can be incorporated within the film, including nanoparticles, dyes, proteins and other supramolecular species [132] [133] [134] [135] [136].

2.7.1 LbL Antibacterial Coatings for Textile

In previous researches various coatings employing LbL technique have been applied to textiles to impart flame retardant, UV blocking, anti-wetting, electrical conductance and antibacterial properties [137] [138] [139] [140] [141].

Fang *et al.,* imparted antibacterial and flame retardant properties to the cotton fabrics using layer by layer method. They employed aqueous solutions of potassium alginate and polyhexamethylene guanidine phosphate (PHMGP) as anionic and cationic deposition solutions respectively. The coating induced antibacterial and flame

retardant properties to the cotton fabrics and antibacterial activity increased with increasing the number of bilayers [141].

While Gomes *et al.,* imparted antibacterial properties to the cotton fabric through LbL technique using sodium alginate and chitosan [142].

In another study Chen *et al.,* flame retarding, electrical conductivity and antibacterial properties were imparted to cotton fabrics through LbL assembly technique using potassium alginate carbon nanotubes and PHMGP. Antibacterial activity was evaluated by Kirby-Bauer test and was shown to increase with increase in number of bilayers. ZOI of 12.1mm was recorded for 5 bilayers coated sample against *E. coli* (at $3x10^6$ cfu/ml) [140].

2.7.2 LbL Antibacterial Coatings for Textile Incorporating Nanoparticles

In various fields of science such as; anti-static coating for plastics, sensors, light emitting diode, fuel cells, polymer capsules, etc., multilayer films containing nanoparticle have been extensively studied for their potential use, but only a few studies have been concluded for textile materials [135] [136] [143] [144] [145] [146] [147] [148] [149].

There are only a few studies about the use of nanoparticles for multilayer film deposition on the textile fibers [150] [151].

Dubasa and colleagues deposited antimicrobial silver nanoparticles on silk and nylon fibers using LbL method. They sequentially dipped the fibers in in dilute solutions of poly (diallyldimethylammonium chloride) (PDADMAC) and poly (methacrylic acid) (PMA) capped silver nanoparticles. Treated fibers showed good antibacterial activity against *S. aureus* [151].

Ugur *et al.,* developed antibacterial cotton fabric by producing nanoparticle based nanocomposite films onto the fabric surface. ZnO nanoparticles were employed as active antibacterial agent. Fabric was pre-treated with 2,3-epoxypropyltrimethylammonium chloride to produce cationic surface charge. The pH of the ZnO nanoparticle suspension was adjusted to 11 and 3 by using NaOH and HCl to produce anionic and cationic ZnO colloidal solutions respectively. Pre-treated fabric was then dipped sequentially in anionic and cationic colloidal solution of ZnO with a washing step in between them. Nano-ZnO films were hence deposited onto the cotton fabric. Antibacterial testing (agar diffusion plate test method ISO 20645) revealed that

treated fabrics exhibited efficient antibacterial activity against *S. aureus*. ZOI of 15mm and 18mm were noted for 10 bilayers and 16 bilayers sample respectively [152].

Chapter 3

MATERIALS AND METHODS

3.1 Materials

Erythromycin (stearate BP 99: pharmaceutical grade) was obtained from Anuh pharma Ltd. Hydroxypropyl methyl cellulose phthalate (HPMCP) (HP 50) was purchased from Shin-Etsu chemical co. Ltd. Tween 80 was purchased from Dae-Jung Chemicals & Metals Co. Ltd. Polyester fabric (Polyethylene terephthalate) was obtained from industry. Safranin dye used was manufactured by Fluka chemicals. Nutrient Broth (CM0001) and Muller-Hinton Agar (CM0337) were purchased from Thermo Scientific. Poly (diallyldimethyl ammonium chloride) (PDAC) (20 wt. % solution) was purchased from Aldrich. *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027) were obtained from SMME, National University of Sciences and Technology.

Ultra-Pure water obtained from Smart2Pure™ Water Purification System (Thermoscientifc) was used to make aqueous solutions and for washing purposes. All the chemicals used in the experiments were of analytical grade and used without any further modification.

3.2 Methods

3.2.1 Nanoprecipitations

Preparation of Nanoprecipitations

Blank nanoprecipitations were prepared and optimized by varying different parameters. Optimized protocol for preparing blank nanoprecipitations is as follows. Aqueous phase was prepared by adding 2ml of surfactant (tween 80) in 12 ml of ultra-pure water and dissolving it by constant stirring. Temperature of water phase was maintained at 40°C. Oil phase was prepared by adding 100mg of HPMCP in 5.5 ml of acetone and sonicating the mixture for 10 minutes. Afterwards oil phase taken into pipette and poured drop by drop into the water phase under constant stirring at 400 rpm.

Resulting mixture was then left on stirring at 40°C for 30 minutes to completely evaporate the acetone.

Erythromycin (EM) loaded nanoprecipitation was prepared in the similar way and 500mg erythromycin was dissolved in oil phase.

Optimization of blank Nanoprecipitations

Nanoprecipitations were optimized by varying different parameters including quantity of surfactant (tween 80), temperature, quantity of acetone, stirring speed and stirring time. Stirring time is the time for which nanoprecipitation is left on stirring after adding the oil phase into water phase.

For optimizing each parameter numerous nanoprecipitations were made by keeping all other factors constant and varying the parameter being optimized. Thus optimum value of each parameter was obtained and final emulsion was made using optimized parameters.

Optimization of blank nanoprecipitations is summarized in the following tables.

Table 3.1: Optimization of temperature of water phase

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
33	1	5	500	15	100
35					
37					
40					
42					
45					

Table 3.2: Optimization of Surfactant (tween 80) quantity

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
40	0.5	5	500	15	100
	1.0				
	1.5				
	2.0				
	2.5				
	3.0				

Table 3.3: Optimization of solvent (acetone) quantity

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
40	2	3.0	500	15	100
		4.0			
		5.0			
		5.5			
		6.0			
		7.0			

Table 3.4: Optimization of stirring speed

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
40	2	5.5	200	15	100
			300		
			400		
			500		
			600		
			900		
			1200		
			1500		

Table 3.5: Optimization of stirring time

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
40	2	5.5	400	10	100
				15	
				20	
				25	
				30	
				35	

While optimizing each parameter, all nanoprecipitations were checked for their stability through naked eye after 15 mins, 30mins, 2hrs and 24hrs of their formation for settling down and clumping of the precipitates. The nanoprecipitations with absent or least settled down and clumped precipitates were considered as the stable ones. For each variable most stable nanoprecipitation among all the prepared nanoprecipitations was selected for further optimization.

Characterization of Nanoprecipitations

1. Optical Microscopy (OM)

Nanoprecipitations (Nps) were checked under optical microscope (Optika B-600 MET) at different magnifications (5x, 10x, 20x, 50x) to visualize the precipitates. A drop of nanoprecipitation was put onto glass slide and seen under microscope. For EM loaded Np, a hydrophilic dye safranin was used to stain the water phase and see the precipitates clearly.

2. Scanning Electron Microscopy (SEM)

For SEM analysis a drop of nanoprecipitations was diluted in 5ml of ultra-pure water. Subsequently a drop of resulting solution was put onto a glass slide and dried in a desiccator. Afterwards it was sputter coated with gold and microscopic images were taken at 20k volts by JSM 6490LA, JEOL microscope, Japan.

3. Dynamic Light Scattering (DLS)

Dynamic light scattering was performed to obtain the zeta potential and size range of nanoparticles (NPs) in the prepared nanoprecipitations (Nps). Samples were diluted with water and analyzed using zeta sizer nano-zsp (malvern).

Antibacterial Assay

Kirby-Bauer Disc Diffusion Method with few modifications was used to determine the antimicrobial Activity of nanoprecipitations. The antibacterial activity of nanoprecipitation was tested against Gram-negative bacterium *Pseudomonas aeruginosa* (ATCC 9027) and Gram-positive *Staphylococcus aureus* (ATCC 6538). Nutrient Broth was prepared as per manufacturer's recommendations and used as stock culture. Muller-Hinton Agar was also prepared as per manufacturer's recommendations. Approximately 25ml of M-H agar was poured into each petri plate and allowed to solidify. Afterwards petri plates were swabbed using fresh 24hr bacterial culture in Nutrient Broth. Sterile filter paper disks (Whattman filter paper No. 1) of 6mm were placed onto the swabbed plates (one disk for each sample). Blank nanoprecipitation was used as a negative control. For positive control 35.7mg/ml solution of erythromycin in acetone was used (as nanoprecipitation also contained 35.7mg/ml of erythromycin). Serial dilutions of the nanoprecipitation up to 10^{-3} were made (Table 3.6) and 50 µl of each sample and control was poured onto separate disks.

Afterwards plates were incubated at 37ºC for 24 hrs. Next day the zones of inhibition (ZOI) around the disks were measured in mm.

Table 3.6: Amount of Erythromycin (mg/ml) in control and Np samples

Negative control	Positive control	Pure Np	10^{-1} dilution of Np	10^{-2} dilution of Np	10^{-3} dilution of Np
0	35.7	35.7	3.57	0.357	0.0357

3.2.2 Layer By Layer Deposition of Nanoprecipitations onto Fabric

For layer by layer method 10mM aqueous solution of APTMS and 5mM aqueous solutions of each EM loaded nanoprecipitation and PDAC were prepared and used as anionic and cationic solutions respectively for LbL coating onto fabric. Fabric swatches (75x25mm) were cut and dipped into APTMS solution for 5hrs (Figure 3.1) to impart positive charge to the fabric.

Figure 3.1: Pre-treatment of fabric with APTMS aqueous solution

Afterwards fabric was dipped into anionic solution for 10 minutes followed by 2 successive washing steps in separate beakers for 5 minutes each. Fabric was then dipped into cationic solution for 10 minutes followed by 2 washing steps again (Figure 3.2).

Figure 3.2: Layer by Layer assembly setup for coating on fabric.

This process leads to the formation of a single bilayer. Whole procedure was repeated to produce 5 BL, 10 BL and 15 BL samples. Samples were then ambient dried and stored in moisture free environment.

Characterization of LbL Coated Fabric

1. Optical microscopy

Coated fabric samples were checked under optical microscope (Optika B-600 MET) at different magnifications (5x, 10x, 20x and 50x) to notice any differences found between uncoated and coated samples and between the samples having different number of bilayers.

2. Optical Profilometry

Optical Profilometry (OP) was performed to calculate the average roughness (nm) of the uncoated and coated samples. Fabric swatches were fixed onto glass slides using paper clips and analysed using Nanovea PS-50.

3. Scanning Electron Microscopy

For SEM analysis fabric swatches were fixed onto glass slides using a double tape. Afterwards it was sputter coated with gold and microscopic images were taken by JSM 6490LA, JEOL microscope, Japan.

4. Atomic Force Microscopy

JSPM-5200, JEOL Scanning Probe Microscope was used to analyze the difference between average roughness of uncoated and 15 BL sample. Fabric sections of 1x1.5 cm were cut and fixed onto glass slides using double tape and analyzed in tapping mode setting. 3D plots were also acquired to better visualize the topographic changes.

Antibacterial Testing of LbL Coated Fabric

Standard antibacterial testing method AATCC 147-2011 with a modification in size of the test sample was employed for antibacterial testing of the LbL coated fabric samples. Sterile Muller Hinton agar was used to make petri plates. 1 ml of 24hr *S. aureus* culture grown in nutrient broth was taken and diluted with 9 ml of sterile distilled water. This diluted culture was used to streak the plates using a 4mm inoculating loop. For streaking each plate loop was dipped into the diluted culture once and 5 parallel streaks of approximately 60 mm in length and 10 mm apart were drawn without changing the slant of the loop. Fabric swatches of 70x10 mm size were placed onto streaked petri plates and gently pressed against the agar surface to ensure proper contact. Plates were then incubated at 37ºC for 24hrs. Each sample was tested in triplicates and average values of ZOI were calculated using standard formula; $W = (T - D) / 2$, used for calculating the zone of inhibition in AATCC 147-2011 method. Here 'W' represents the ZOI (in mm), 'T' is the total diameter (in mm) of clear zone and test sample, and 'D' is the diameter (in mm) of test sample.

Chapter 4

RESULTS AND DISCUSSION

4.1 Nanoprecipitations

4.1.1 Optimization Results

A number of nanoprecipitations (Nps) were prepared as already mentioned, by varying; temperature, surfactant quantity, solvent quantity, stirring speed and stirring time. Each parameter was optimized separately by varying its values while keeping all other factors constant.

Nps were checked for their stability through naked eye after 15 mins, 30mins, 2hrs and 24hrs of their formation for settling down of the precipitates. The nanoprecipitations with absent or least settled down precipitates were considered to be stable ones. After 24hrs most stable one among all the prepared nanoprecipitations was selected for further optimization.

While optimizing the temperature of water phase, 40°C was found to be the most suitable for making nanoprecipitations. Below this temperature Nps although showed good stability for short time period but later precipitates settled down. Above 40°C large clumps of polymer were also formed in Nps, and their size as well as clumping increased with increasing temperature.

For finding optimum volume of surfactant, temperature of the water phase was maintained at 40°C and nanoprecipitations were made varying the amount of surfactant. Nanoprecipitation containing 2ml of tween 80 was found to be well formed and most stable one. After finding the optimum temperature and surfactant concentration, concentration of solvent (acetone) was varied and nanoprecipitations made using 5.5ml of solvent was found to be most stable.

Next stirring speed was optimized. All nanoprecipitations were made by using 2ml of tween 80 for water phase maintaining temperature at 40°C, and 5.5ml of acetone for oil phase. While stirring speed was varied for each nanoprecipitation. Stirring speed of 400rpm was found to be optimum for making Nps. Below 400rpm larger precipitates

were formed that settled down after some time. When speed was increased above 400 rpm apparently stable nanoprecipitations were formed but with passage of time larger precipitates appeared and settled down. Above 400rpm, both amount and size of the settled down precipitates increased with increasing stirring speed.

In the end stirring time was optimized. Nanoprecipitations were made using the previously optimized parameters and varying the stirring time. Stirring time of 30 mins was found optimum for making nanoprecipitations. Below 30 mins nanoprecipitations were not stable in the long term that could be attributed to the fact that acetone might not have evaporated completely that lead to instability later. Table 4.1 shows the optimum parameters for blank nanoprecipitation. Blank nanoprecipitation made by using all the optimized parameters was found to be stable for more than 9 months.

Table 4.1: Parameters of optimized nanoprecipitation

Temperature (ºC)	Tween 80 (ml)	Acetone (ml)	Stirring speed (rpm)	Stirring time (mins)	HPMCP (mg)
40	2	5.5	400	20	100

Drug loaded Np was prepared using the parameters of optimized Np and 500mg of EM was added into the acetone along with the polymer. Drug loaded Np was found to be stable for more than 7 days. Physical appearance of both blank and EM loaded Nps is shown in Figure 4.1.

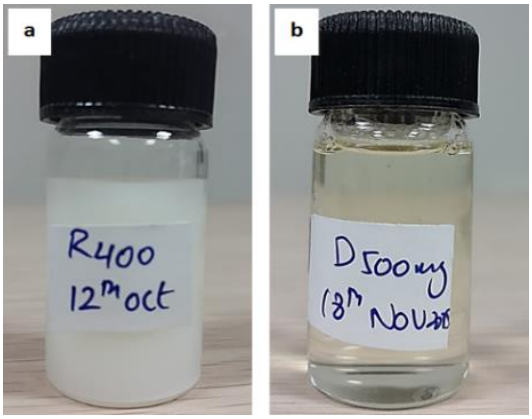

Figure 4.1: Digital photograph showing Nps; (a) Optimized blank Np, (b) EM loaded Np

4.1.2 Characterizations

1. Optical Microscopy

Optimized blank and EM loaded Nps were visualized under optical microscope to observe the dispersion and morphology of the precipitates in the water phase. Precipitates appeared to be well dispersed in the water phase and spherical in shape (figure 4.2).

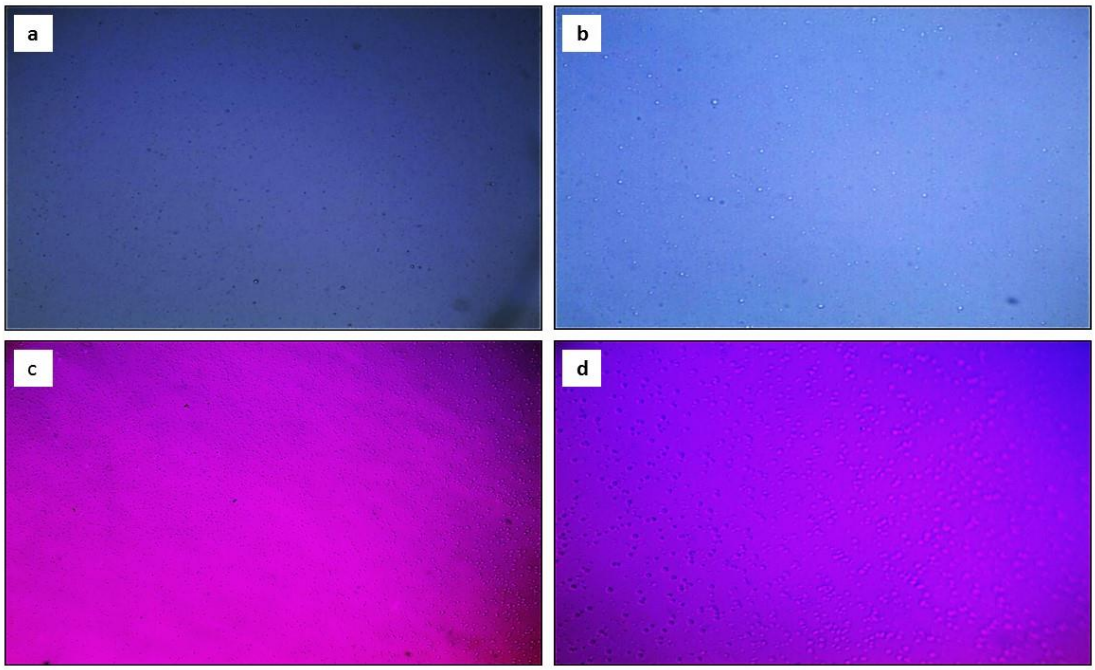

Figure 4.2: Optical Microscopy images of optimized blank and EM loaded Nps; (a) optimized blank Np at 20x, (b) optimized blank Np at 50x, (c) EM loaded Np at 20x, (d) EM loaded Np at 50x

2. Scanning Electron Microscopy

SEM analysis was performed to determine the size and morphology of the particles in both blank and EM loaded Nps. SEM results showed that particles in both blank and EM loaded Nps are spherical in shape and nanometric in size and particle size decreased with the addition of EM (Figure 4.3).

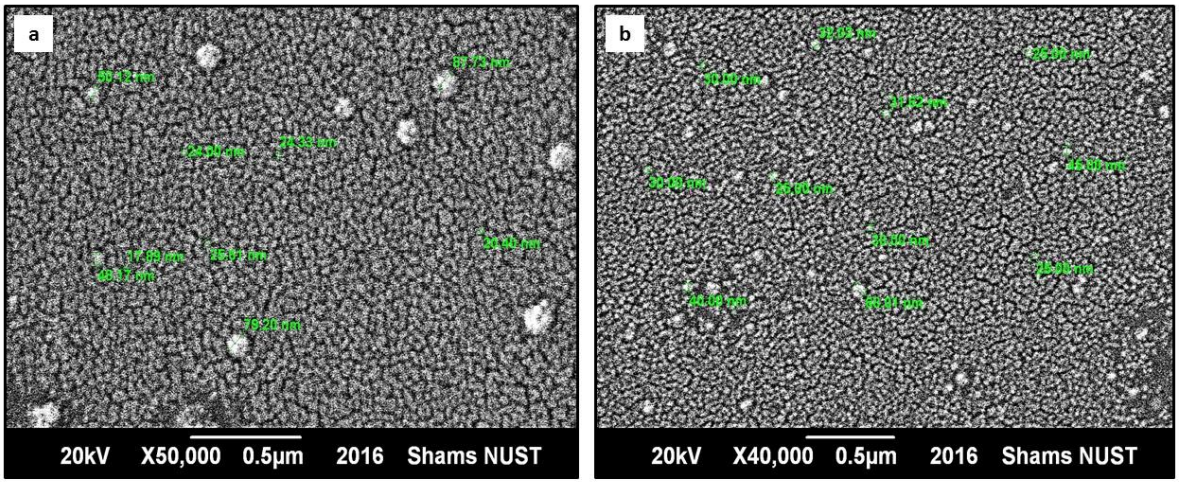

Figure 4.3: SEM images showing nanoparticles in Nps; (a) Optimized blank Np, (b) EM loaded Np

3. Dynamic Light Scattering

Dynamic light scattering technique was used to analyze the charge and size range of nanoparticles in both blank and EM loaded Nps. Results of DLS are summarized in table 4.2

Table 4.2: Size, PDI and Zeta values for optimized blank and EM loaded Nps

Sample	Nanoparticles size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Optimized blank Np	189.4	0.177	-37
EM loaded Np	65	0.169	-17.2

Zeta potential for nanoparticles in blank Np was -37mV while it decreased to -17.2 mV in EM loaded Np. This variation can be explained by the fact that nanoparticles in blank Np were made up of HPMCP only which is a negatively charged molecule. As EM is a positively charged molecule so addition of EM decreased the zeta value. Zeta distribution data for blank and EM loaded nanoprecipitations (Nps) is shown in figure 4.4. It is evident from the figure that -ve value of zeta potential reduced in EM loaded Np.

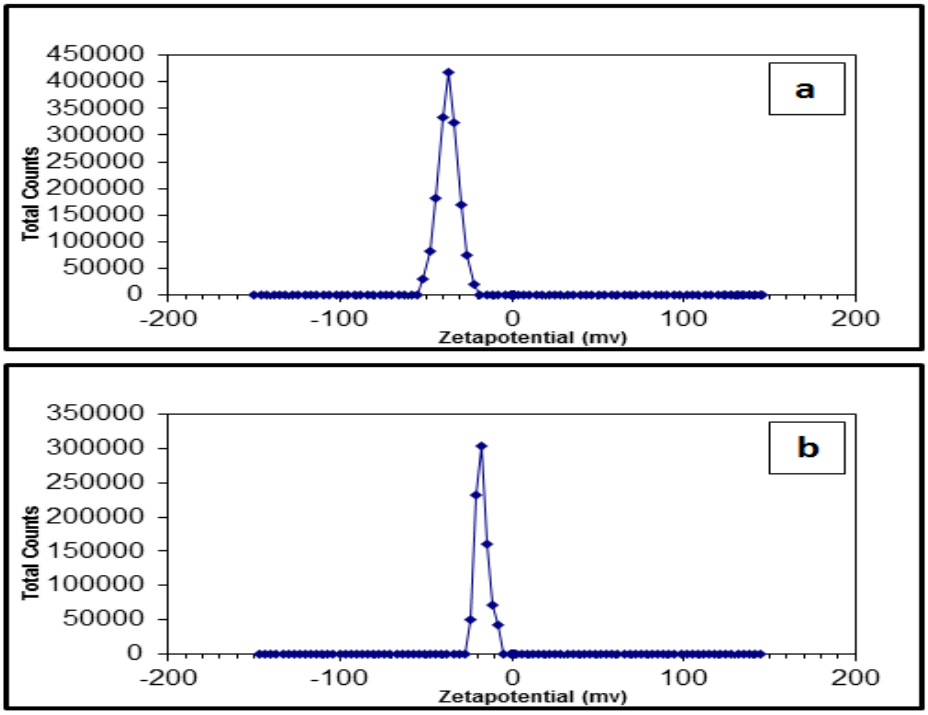

Figure 4.4: Zeta distribution data for Nps; (a) blank Np, (b) EM loaded Np

Zeta potential is an important determinant in the physical stability of Nps. For surfactant stabilized Nps, a minimum zeta potential of ± 20mV is desirable for long term stability [153]. EM loaded Nps (with a zeta potential of -17mV) were found to be stable for one week while blank nanoprecipitations (with a zet potential of -37mV) were found stable for more than 9 months. We can infer that zeta value and consequently stability of EM loaded Nps can be improved by reducing the amount of EM.

Coming towards the size and PDI results of DLS, Blank Np had nanoparticles with average diameter of 189.4 nm and PDI 0.177. While average diameter of nanoparticles in EM loaded Np was 65 nm with PDI of 0.169. This size value is closer to the nanoparticles prepared by Wang *et al*., (50-60nm) [115]. Wang and colleagues prepared cyclosporine-A loaded HPMCP nanoparticles through nanoprecipitation technique [115].

Values of PDI lower than 0.2 indicate good colloidal stability according to Mohnaraj & Chen (2006) [154]. Addition of drug decreased the PDI. This trend of decrease in PDI upon addition of drug was observed earlier as well by Dias *et al.,* [89]. Intensity distribution data (%) for size of nanoparticles in blank Np and EM loaded Np is shown in figure 4.5. It can be seen that size of nanoparticles decreased in EM loaded Np.

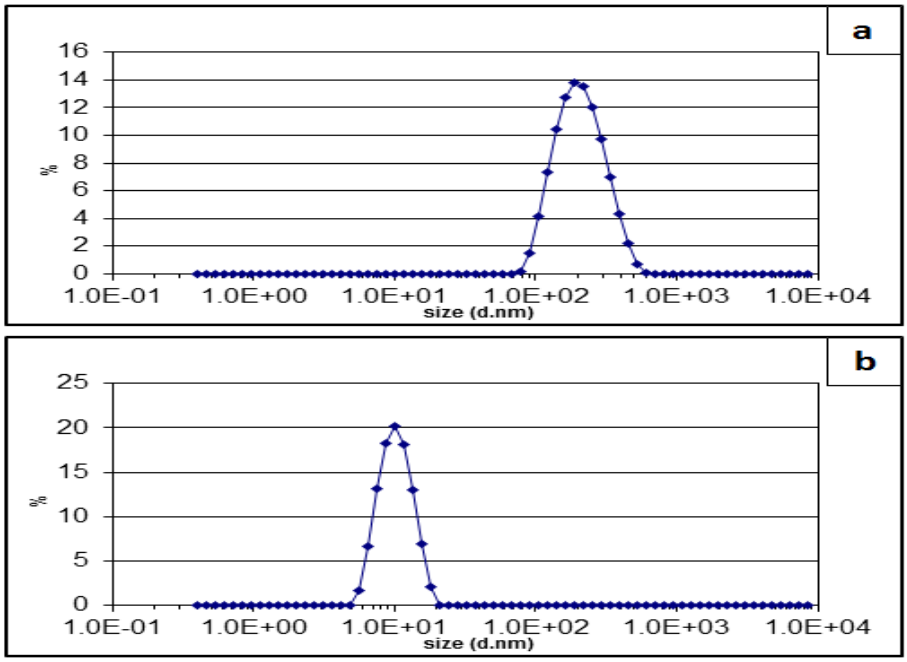

Figure 4.5: Intensity distribution data for size of nanoparticles in Nps; (a) Blank Np, (b) EM loaded Np

4.1.3 Antibacterial Activity

Antibacterial activity of EM loaded nanoprecipitation was evaluated against *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027). All experiments were performed in triplicates. ZOI for different dilutions of nanoprecipitation were noted. Average ZOI with standard deviation were calculated.

Activity against *S. aureus*

Zones of inhibition (mm) for different dilutions of EM loaded nanoprecipitation against *Staphylococcus aureus* (ATCC 6538) are given in Table 4.3.

Table 4.3: ZOI (mm) for antibacterial activity of Nps against *S. aureus*

Control (+ve)	Control (-ve)	Pure Np	Np 10^{-1}	Np 10^{-2}	Np 10^{-3}
39.03 ± 0.45	0	29.53 ± 0.5	21.16 ± 0.75	15.1 ± 1.65	9.13 ± 1.27

ZOI decreased with increasing the dilution factor of Np. Dilutions of up to 10^{-3} were found effective against *S. aureus* (figure 4.6). Amount of EM in control and Np samples is shown in Table 3.6.

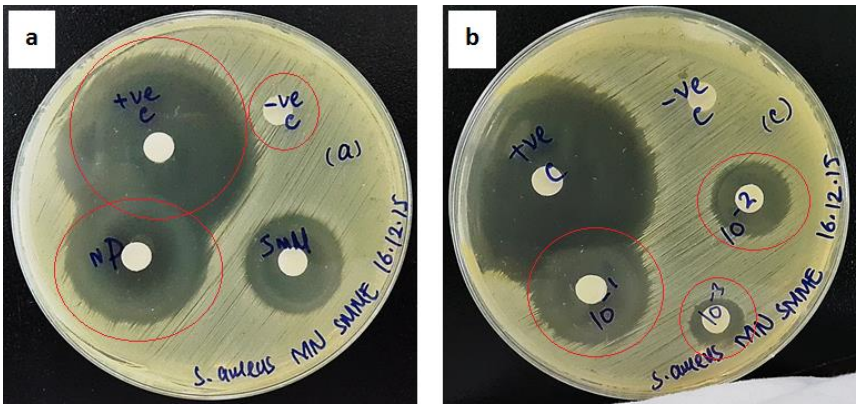

Figure 4.6: ZOI of Nps against *S. aureus* on agar plates; (a) figure showing ZOI for +ve control, -ve control and pure Np, (b) figure showing ZOI for Np 10^{-1}, 10^{-2} and 10^{-3}

Above this dilution no antibacterial activity was observed. Figure 4.7 shows the trend observed for antibacterial activity of different dilutions of Nps against *S. aureus*. Vertical bars on x-axis represent the average ZOI and error bars on them denote standard deviation.

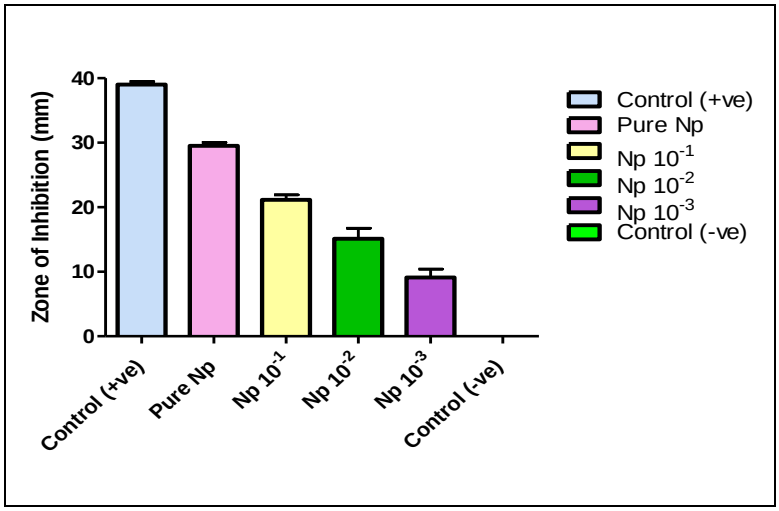

Figure 4.7: Antibacterial activity graph of Nps against *S. aureus*

Antibacterial activity results were statistically analysed via unpaired t-test (two tailed) in 'GraphPad Prism' software. The p value was calculated for two adjacent groups to deduce that weather the change in the dilution factor of the Np had a significant impact on its antibacterial value or not. P value of ≤ 0.05 was considered significant. Groups compared were; +ve control & pure Np, pure Np and 10^{-1} dilution

of Np, 10^{-1} and 10^{-2} dilution of Np, and 10^{-2} and 10^{-3} dilution of Np. Table 4.4 shows the p values obtained for each compared group.

Table 4.4: T-test results of Nps against *S. aureus*

Groups compared	Control (+ve) vs Pure Np	Pure Np vs NP 10^{-1}	Np 10^{-1} vs Np 10^{-2}	Np 10^{-2} vs Np 10^{-3}
p value	< 0.0001	< 0.0001	< 0.0044	< 0.0077

A significant difference of $p<0.0001$ was observed between the antibacterial activity of +ve control and pure Np group. Both contained same amount of EM though but +ve control showed better activity as compared to Np. This can be explained by the fact that addition of polymer and surfactant might have negatively influenced the antibacterial activity of EM and/or EM might not have been completely released from the nanoparticles coated by HPMCP polymer. For other groups too, p value was found significant, indicating a dose dependant relation between the antibacterial activity of Nps, that is; antibacterial activity decreased with increasing the dilution factor/decreasing the amount of drug in the sample. Nps upto 10^{-3} dilution (contained 35.7 µg/ml of EM) were found effective against *S. aureus*.

Activity against *P. aeruginosa*

Zones of inhibition (mm) for control and different dilutions of EM loaded Np against *Pseudomonas aeruginosa* (ATCC 9027) are shown in Table 4.5.

Table 4.5: ZOI (mm) for antibacterial activity of Nps against *P. aeruginosa*

Control (+ve)	Control (-ve)	Pure Np	Np 10^{-1}
36.5 ± 1.65	0	26.2 ± 0.7	15.3 ± 0

In case of *P. aeruginosa* only pure Np and 10^{-1} dilution of Np (contained 3.57mg/ml of EM) showed antibacterial activity and no activity was observed for 10^{-2} [a]nd 10^{-3} dilutions as compared to *S. aureus* (figure 4.8).

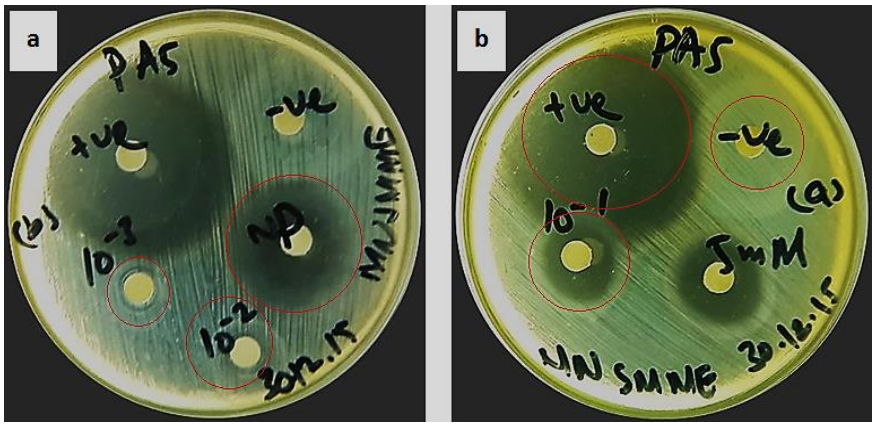

Figure 4.8: ZOI of Nps against *P. aeruginosa* on agar plates; (a) Figure showing ZOI for pure Np, Np 10^{-2} and 10^{-3}, (b) figure showing ZOI for +ve control, -ve control and Np 10^{-1}

Also ZOI for +ve control, pure Np and 10^{-1} dilution were smaller for *P. aeruginosa* as compared to those for *S. aureus*. This can be attributed to the fact that *P. aeruginosa* is a gram-negative bacterium and have thick and less permeable outer membrane that leads towards the decreased penetration of the antibacterial agents [155]. Additionally, *P. aeruginosa* tends to form biofilms and thick protective coatings around them which prevents the penetration of antimicrobial drugs. So this factor might have led to relatively less antibacterial effect of Nps against *P. aeruginosa* as compared to that against *S. aureus*.

Figure 4.9 shows the trend observed for antibacterial activity of different concentrations of Nps against *P. aeruginosa*. Vertical bars on x-axis represent the average ZOI and error bars on them denote standard deviation.

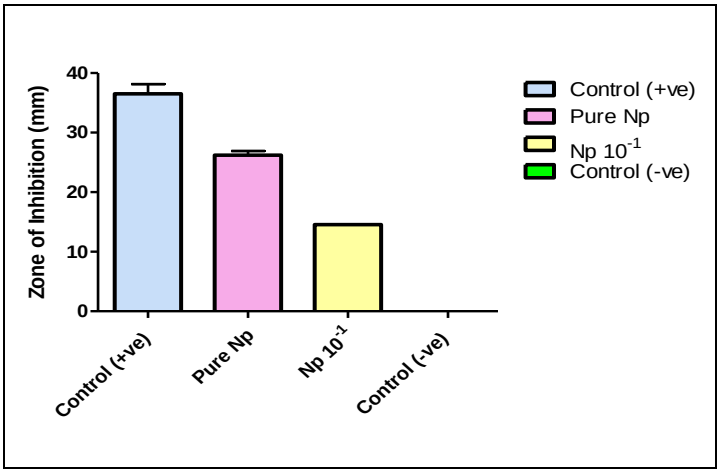

Figure 4.9: Antibacterial activity graph of Nps against *P. aeruginosa*

Antibacterial activity of Nps against *P. aeruginosa* was analysed statistically in similar way as described for *S. aureus* above and trend observed was similar as that for *S. aureus*. Groups compared were; +ve control and pure Np, and pure Np and 10^{-1} dilution of Np (Table 4.6). Similarly to *S. aureus* +ve control showed more efficient antibacterial activity as compared to pure Np (significant p value of <0.0006 was observed) and pure Np showed more activity as compared to 10^{-1} dilution.

Table 4.6: T-test results of Nps against *P. aeruginosa*

Groups compared	Control (+ve) vs Pure Np	Pure Np vs NP 10^{-1}
p value	<0.0006	< 0.0001

From antibacterial results we can conclude that EM loaded Nps showed promising antibacterial effects and can be further developed for topical antibacterial applications. These Nps can also be used as antibacterial coatings on different surfaces, i.e. medical devices and fabrics etc.

The exact mode of action for these antibacterial nanoprecipitations is not known but it is proposed that HPMCP dissolves at pH ≥ 5 and EM is released from the nanoparticles that produces antibacterial effects on the bacterial cells in the surrounding environment.

Normal pH of skin is between the range of 4-6, but it becomes more alkaline upon bacterial colonization and can reach up to 7.4 in case of wounds [156]. As HPMCP dissolves at pH ≥5, the prepared HPMCP nanoparticles have the potential to release the EM in case of skin infections and/or bacterial colonization. EM can be released in a stimuli responsive way and can be used to treat and/or prevent skin infections.

4.2 LbL Coated Fabric

Bilayers were produced based on the principle of electrostatic attraction between polyelectrolytes. Positively charged polymer (PDAC) and negatively charged nanoprecipitations served as oppositely charged species and were deposited on the positively charged fabric as already described in the methodology section.

4.2.1 Characterization

1. Optical Microscopy

Both coated and uncoated fabrics were visualized under optical microscope at magnifications (5x, 10x, 20x and 50x) and differences in the appearance of uncoated and coated samples and also between the samples having different number of bilayers were noted. It was observed that uncoated fabric threads were visualized clearly under the optical microscope while threads of coated fabric samples appeared blurred under optical microscope. Also clarity of the threads decreased with increasing number of bilayers. We can be hypothesize that it happened due to increasing deposition of polyelectrolytes upon threads surface with increasing the number of bilayers. Optical microscopy images at 50x are shown in figure 4.10 for each sample.

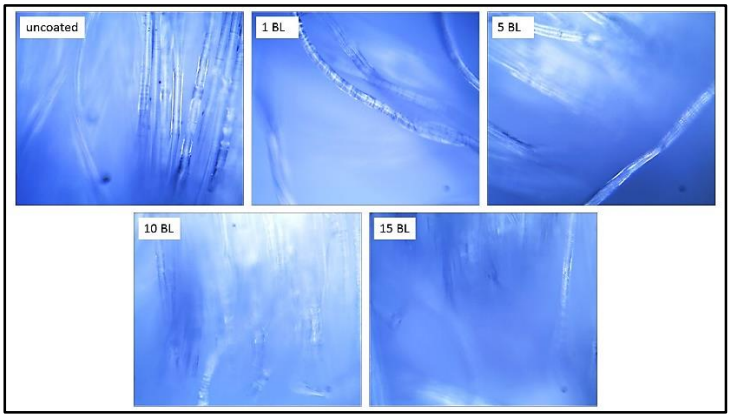

Figure 4.10: OM images of uncoated & LbL coated fabric samples at 50x

2. Optical Profilometry

Optical Profilometry was performed to calculate the average roughness (nm) of the uncoated and coated fabric samples. When smooth surfaces are coated using layer by layer method average roughness is increased with increasing the number of bilayers but an opposite trend was observed in case of our coated fabric. Average roughness decreased with increasing number of bilayers. This can be explained by the fact that fabric surface is very rough and coating made the fabric surface smoother by filling the pores and spaces to some extent. 25-50 mm length of the fabric was scanned. Average roughness (nm) for each sample is shown in Table 4.7.

Table 4.7: OP results for average roughness of LbL coated samples

Uncoated	1 Bilayer	5 Bilayers	10 bilayers	15 bilayers
23927	11871	9454	9294	8760

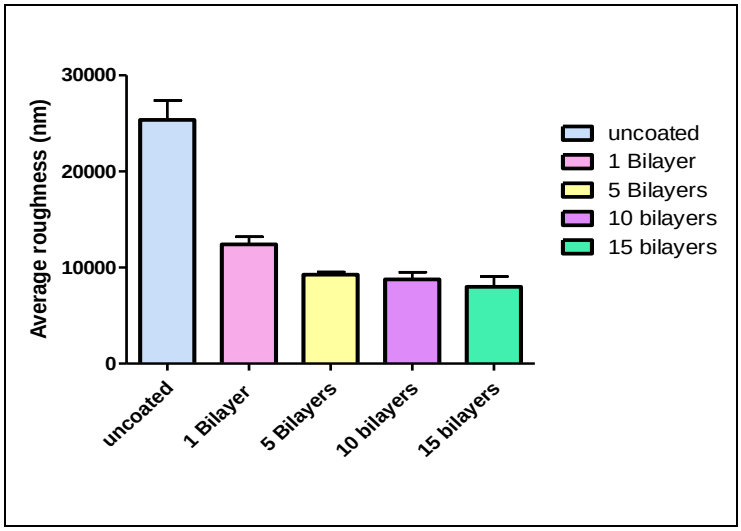

Figure 4.11: Graph showing change in average roughness with increasing number of Bilayers

3. Scanning Electron Microscopy

SEM analysis was performed to visualize the coatings on the fabric surface. It was observed that coated mass increased with increasing the number of bilayers (Figure 4.).

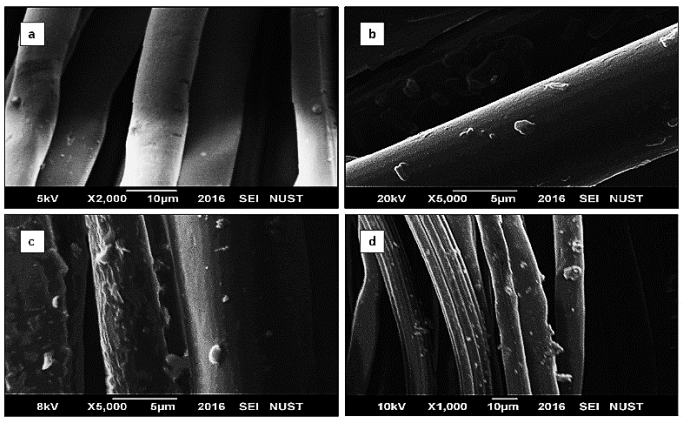

Figure 4.12: SEM images of uncoated and LbL coated fabric samples; (a) uncoated, (b) 5Bl, (c) 10 BL, (c) 15 BL

4. Atomic Force Microscopy

Scanning Probe Microscope was used to analyze the difference between average roughness of uncoated and 15 bilayers sample. 3D plots were also acquired to better visualize the topographic changes.

Fabric samples were analyzed in tapping mode and showed the difference in average roughness between control and 15 BL sample. An average area of 1 µm was scanned.

Coated sample had an average roughness of 14.9 nm as compared to control in which it was found to be 10.4 nm. Figure 4.13 show the average roughness profile of uncoated and 15BL sample.

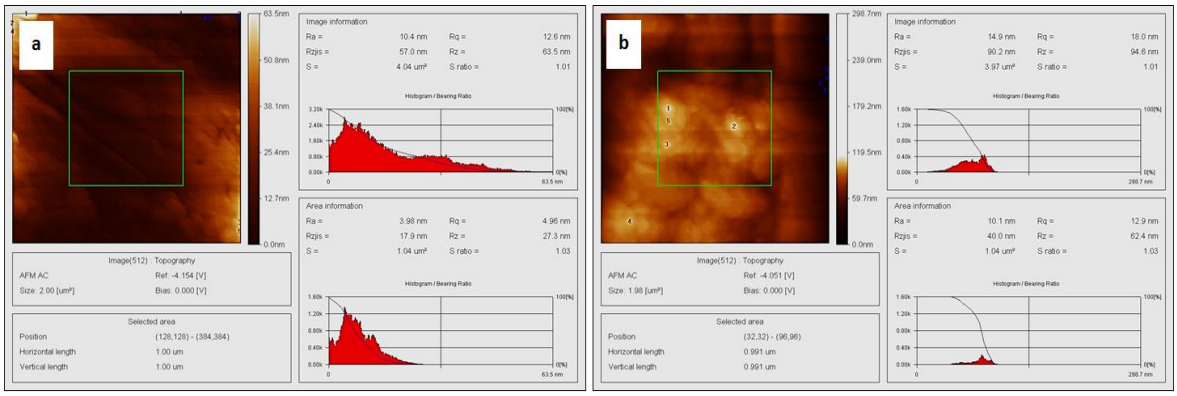

Figure 4.13 AFM results; (a) uncoated sample, (b) 15 BL sample

Contray to OP results average roughness increased in coated sample according to AFM results. It can be explained by the fact that OP calculated the average roughness from a scan length of 25-50 mm while AFM scanned an area of only 1 µm. So OP scanned a large area as compared to AFM and that area contained several wraps and wefts and pores as well. As a result, uncoated fabric had quite uneven surface and hence larger surface roughness as compared to coated samples was observed. Coating supposedly filled the pores and gaps between the threads of fabric so on larger scale length, average roughness decreased in coated samples.

On the other hand, AFM scanned an area of 1µm, that is a very small area and hardly equal to $1/10^{th}$ of the width of a single thread of fabric sample. So on µm scale uncoated sample had relatively smooth surface and coating increased the average roughness due to alternative deposition of PDAC and EM nanoparticles.

3D plots of uncoated and coated samples are shown in figure 4.14. It can be seen that uncoated sample has a clear surface (figure 4.14 a) while deposition can be seen on coated sample (4.14 b).

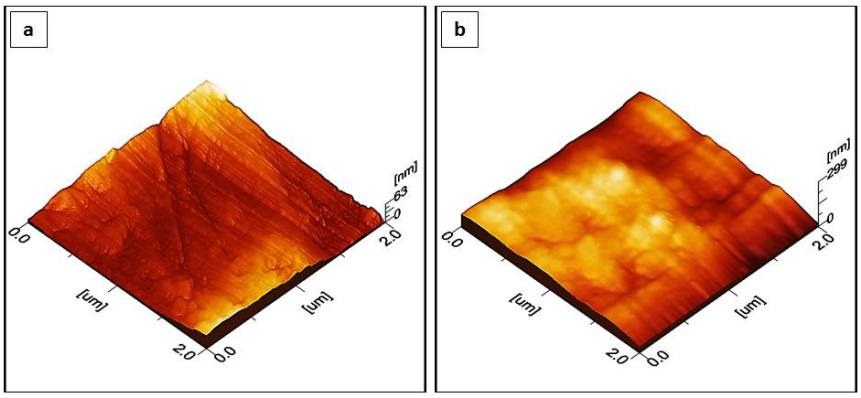

Figure 4.14: AFM 3D plots; (a) uncoated sample (b) 15 BL sample

4.2.2 Antibacterial Activity

Coated fabrics showed good antibacterial activity against *Staphylococcus aureus* (ATCC 6538). ZOI increased with increasing the number of bilayers (figure 4.15). Uncoated fabric showed no antibacterial activity. Average ZOI along with standard deviation are shown in Table 4.8.

Table 4.8: ZOI for LbL coated fabrics against *S. aureus*.

15 BL sample	10 BL sample	5 BL sample	1 BL sample
8.3 ± 0.3 mm	7.26 ±0.55 mm	5.63 ± 0.23 mm	2.93 ± 0.32 mm

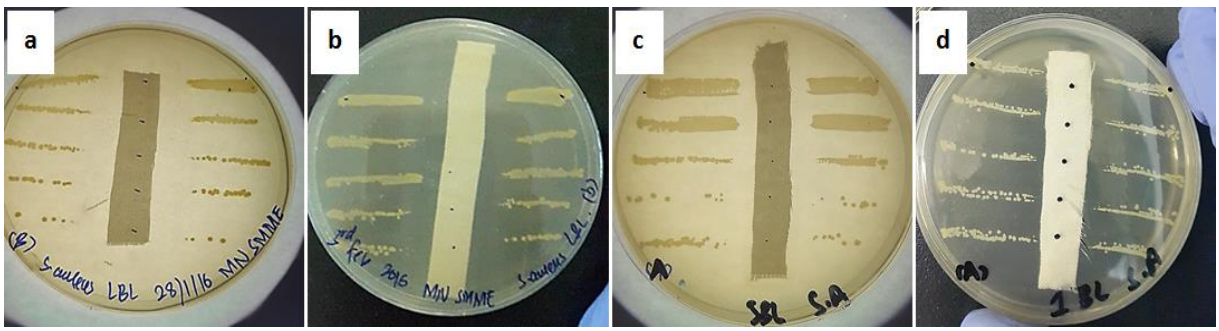

Figure 4.15: Antibacterial activity of different LbL coated fabrics against *S. aureus*; (a) 15 BL (b) 10BL (c) 5 BL (d) 1 BL

Figure 4.16 shows the trend observed for antibacterial activity of different bilayers samples. Vertical bars on x-axis represent the average ZOI and error bars on them denote standard deviation.

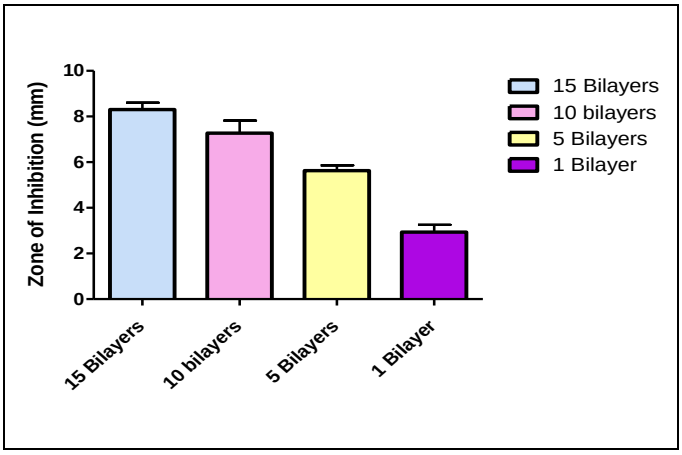

Figure 4.16: Antibacterial activity graph of LbL coated fabrics against *S. aureus*.

Antibacterial activity results were statistically analysed via unpaired t-test (two tailed) in 'GraphPad Prism' software. The p value was calculated for two adjacent groups to deduce the impact of change in the number of bilayers upon antibacterial activity of coated fabrics. P value of ≤ 0.05 was considered significant. Table 4.9 shows the p values obtained for each compared group.

Table 4.9: T-test results for LbL coated fabrics against *S. aureus*

Groups compared	15 BL vs 10 BL	10 BL vs 5 BL	5 BL vs 1 BL
p value	< 0.0462	< 0.0091	< 0.0003

A significant difference was observed between the antibacterial activity of each compared sample. Antibacterial activity increased with increasing the number of bilayers.

From antibacterial results we can deduce that increasing the number of bilayers leads to increased release of erythromycin from HMPCP nanoparticles and hence increased antibacterial activity.

We can conclude that coating of polyester fabric by EM loaded HPMCP nanoparticles successfully induced antibacterial properties to the fabric and carry the potential to be used for prevention and/or treatment purposes of infections of skin and minor wounds.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Antibacterial nanoprecipitations were developed using simple nanoprecipitation technique. Nanoprecipitations were optimized by varying different parameters to find the appropriate factors for the most stable nanoprecipitations. Erythromycin (EM) loaded nanoprecipitations were successfully produced and characterized. SEM and DLS confirmed the nanometric size of the nanoprecipitations. The shape of the nanoparticles was found to be spherical. EM loaded nanoprecipitations showed promising antibacterial activity against *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027).

Erythromycin loaded nanoprecipitations were coated onto polyester fabric using Layer by Layer deposition method. AFM and SEM characterization confirmed the presence of coating on fabric samples. SEM analysis showed that on coated fabrics, number of particles and their size increased with increasing the number of bilayers. AFM also confirmed the coating on fabric sample and showed that surface roughness (nm) increased after coating. LbL coated fabrics exhibited good antibacterial activity against *Staphylococcus aureus* (ATCC 6538), that increased with increasing the number of bilayers. From results we can conclude that prepared antibacterial fabric has the potential to be used as a biomedical textile and can be developed further to prevent and/or treat skin infections, and address the problems related to oral delivery, and increase patient compliance.

5.2 Recommendations

Zeta potential and stability of the nanoprecipitations can be further optimized by varying the drug to polymer ratio.

These easy to produce and cost effective nanoprecipitations could be further developed and/or evaluated for their potential to be used as antibacterial topical application for skin infections and problems as well as gastric side effects related to oral delivery of antibiotic drugs can be prevented. Nanoprecipitations incorporating drugs other than EM can also be prepared and can be tailored according to their application by varying parameters like types of polymers, solvents or non-solvent.

In future these nanoprecipitations can be evaluated for coating onto other fabrics like cotton, wool etc. Also they can be modified and employed for coating onto other surfaces and biomedical textile etc. using Layer by Layer coating method.

Chapter 6

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