DESIGN AND OPTIMIZATION OF NANOFORMULATION LOADED WITH HIV MICROBICIDES

A DSSERTATION IN
Pharmaceutical Sciences
and
Chemistry

Presented to the Faculty of the University
Of Missouri-Kansas City in partial fulfillment of
The requirements for the degree of

DOCTOR OF PHILOSOPHY

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2013
DESIGN AND OPTIMIZATION OF NANOFORMULATION LOADED WITH HIV MICROBICIDES

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ABSTRACT

The objective of this dissertation is to design and optimize nanoformulation for microbicides intended for the prevention of HIV/AIDS transmission. In chapters 1 and 2, the aims and the scope of this work are introduced, as well as the literature review of its technical background.

In chapter 3, the formulation and process variables involved in the production of spray-dried oily-core nanocapsules (NC) are investigated using Box-Behnken Design (BBD) and Fractional Factorial Experimental Design (FFED). Indomethacin (IND) is chosen as a model drug for lipophilic HIV microbicides. Three formulation variables (the amount of polymer, oil, and surfactant) and four process variables (inlet temperature, feed flow rate, atomizing air flow, and aspiration rate) are optimized for NC with smaller mean diameter, higher encapsulation efficiency (EE%) and higher process yield. The spray-dried NC are also characterized for their morphology, density, structure, in vitro drug release, and powder flowability. The optimized production process successfully resulted in NC with mean diameter less than 200 nm, with a drug EE% of 95% and an overall yield of 30.8%. Morphological analysis and density-gradient centrifugation confirmed the existence of an oily core and spherical nanostructure with no
detectable drug crystals. The NC have longer sustained drug release profile than nanosphere (NS) control, with a good fit to the Ritger-Peppas model of drug release ($R^2 > 0.930$).

In chapter 4, we test the hypothesis that actual anti-HIV microbicides such as tenofovir (TNF) or tenofovir disoproxil fumarate (TDF) loaded nanoparticles (NP) prepared with a blend of poly (lactic-co-glycolic acid) (PLGA) and methacrylic acid copolymer (Eudragit® S-100, or S-100) are noncytotoxic and exhibit pH-responsive release of anti-HIV microbicides in the presence of human semen fluid simulant (SFS). After the preparation by emulsification diffusion process, their size, EE%, drug release profile, morphology, and cytotoxicity are characterized by dynamic light scattering, spectrophotometry, transmission electron microscopy, and cellular viability assay/transepithelial electrical resistance measurement, respectively. Cellular uptake is elucidated by fluorescence spectroscopy and confocal microscopy. The NP have an average mean diameter of 250 nm, maximal EE% of 16.1% and 37.2% for TNF and TDF, respectively. There is a 4-fold increase in drug release rate from the 75% S-100 NP in the presence of SFS over 72 h. At a concentration up to 10 mg/ml, the PLGA/S-100 NP are noncytotoxic for 48 h to vaginal endocervical/epithelial cells and Lactobacillus crispatus. The particle uptake (~ 50% in 24h) by these vaginal cell lines mostly occurred through caveolin-mediated pathway.

In chapter 5, we modify the production process, and develop a dual-functional mucoadhesive and pH-responsive microparticles (MS) formulation for TNF. The formulation variables and process parameters are screened and optimized using a $2^{4-1}$ FFED. The MS are characterized for their mean diameter, zeta potential, yield, EE%, Carr's index, drug loading, in vitro release, cytotoxicity, inflammatory responses and mucoadhesion. The optimal MS formulation has an average diameter of 4.73 μm, zeta potential of -26.3mV, 68.9% yield, EE% of 88.7%, Carr's index of 28.3 and drug loading of 2% (w/w). The MS formulation release 91.7% of
its payload in the presence of simulated human semen over 4 hours. At a concentration of 1 mg/ml, the MS are noncytotoxic to vaginal/endocervical epithelial cells and *Lactobacillus crispatus* when compared to control media. There is also no statistically significant level of inflammatory cytokine (IL1-α, IL-1β, IL-6, IL-8, and IP-10) release triggered by these MS. Their percent mucoadhesion is 2-fold higher than that of 1% HEC gel formulation. These MS are found to be 3.8-5.8 fold more potent compared to native drug in HIV-1 inhibition, as a result of CD4-dependent CCR5 tropic cell-free HIV-1 entry essay.

Overall, the present work demonstrates two nanoformulations that could be used. The oily core NC (300 mg of PLA, 0.56 ml of oil, and 239.57 mg of Pluronic F127) which is suitable for lipophilic microbicides, and a microparticle formulation (mean diameter of 4.73 μm with drug loading of 2% w/w) for the more water-soluble TNF. These formulations are promising alternative delivery systems for the purpose of AIDS prevention, offering safe and controlled intravaginal delivery of HIV microbicides.
The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined the dissertation titled “Design and Optimization of Nanoformulation Loaded with HIV Microbicides”, presented by Tao Zhang, candidate for the Doctor of Philosophy Degree, and certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS

BBD: Box-Behnken Design
C-6: Coumarin-6
DOE: Design of experiments
DLS: Dynamic light scattering
EE%: Encapsulation efficiency
EuSNa: Sodium salt of Eudragit® S-100
FFED: Fractional factorial design
IND: Indomethacin
MS: Microparticles
NC: Nanocapsules
NP: Nanoparticles
NS: Nanospheres
PLA: Poly (lactic acid)
PLGA: Poly (lactic-co-glycolic acid)
S-100: Eudragit® S-100
SEM: Scanning electronic microscope
SFS: Semen fluid simulant
TDF: Tenofovir disoprixil fumarate
TNF: Tenofovir
TEM: Transmission electronic microscope
VFS: Vaginal fluid simulant
XRD: Powder X-ray diffraction
ACKNOWLEDGEMENTS

Though the following dissertation is an individual work, I feel like I could never have achieved what I have done without the guidance, support, and encouragement from a lot of people. Firstly I would like to express my sincere gratitude to Dr. Bi-Botti C. Youan, my graduation committee chair. Dr. Youan has been nothing but a true mentor and colleague whose guidance has proven to be invaluable in this project. His unlimited enthusiasm and zeal to the exploration of science have been the major driving force through my graduate career.

I am also grateful to my committee members, Dr. Kun Cheng and Dr. Simon H. Friedman of the division of pharmaceutical sciences, Dr. Zhonghua Peng of the department of chemistry, and Dr. Jacob M. Marszalek of the school of education for their invaluable discussions and comments over the years. Special thanks to Dr. Nathan Oyler, who has been an insightful collaborator as well as a supportive mentor. I would like to acknowledge Dr. James Murowchick in the department of geological sciences for his continuous support in XRD analysis.

Over the years I am fortunate to work with a team of brilliant scientists in my lab. I would like to express thanks to my current and senior lab mates, Di Bei, Nisha Vijay, Jianing Meng, Vivek Agrahari, Fohona Coulibaly, Albert Ngo, Dr. Ibrahima Youm, and Dr. Miezan Ezoulin for their time, support, and friendship.

I would also like to express thanks to other professors, staff members, and fellow students in the Division of Pharmaceutical Sciences for their help and friendship.

My very special thanks to my family whom I owe everything I am today, especially my parents, who have given me nothing but unconditional faith, love and support over the years, and for that I am eternally grateful. Finally, my wife Chi, who has been my enduring source of
strength and helped with the research and writing of this dissertation in numerous ways. To her and my family, I dedicate this dissertation.
CHAPTER

1. INTRODUCTION

1.1 Overview

Acquired immunodeficiency syndrome (AIDS) is an infectious disease caused by Human immunodeficiency virus (HIV). According to the recent report of the global AIDS epidemic, there are 34.0 million people currently living with AIDS, corresponding to 0.8% of the total population of adults aged 15-49 years. With 2.5 million new infections and 1.7 million deaths in 2011, AIDS still remains the deadliest epidemic of our time. Unprotected, heterosexual, vaginal intercourse has become one of the major routes of infection. Although the global percentage of women among people living with HIV has remained stable (50%), women are considered more susceptible to sexually-acquired HIV infection due to physiological, social, and economical factors. Several HIV transmission prevention methods, such as condoms and circumcision, have been implemented, especially in developing countries. However, the results have been unsatisfactory, since it was reported in many regions that men were reluctant to use either method. Besides these facts, a successful HIV vaccine has yet to be developed. Thus it is critical and urgent to design a topical delivery strategy of microbicides that women can use as a pre-exposure prophylaxis (PrEP) method. The idea of PrEP methods started with the oral application of antiretroviral drugs, and later focused on the vaginal/rectal application of anti-HIV substances, known as microbicides. A great variety of HIV microbicides candidates have been studied and tested. In terms of formulation, a lot of the emphasis has been put on the first generation gel formulation, and some positive results have been shown. However, such aqueous gel system suffers from several disadvantages. Besides its limitation of application towards
hydrophobic microbicides, the low retention time requires a high dosing frequency \(^6\). Such “before and after sex” dosing strategy (commonly referred as “coital dependence” \(^7\)) sometimes leads to poor acceptability and adherence. An ideal prevention strategy for women at high-risk of sexually-acquired HIV could be the use of a microbicide formulation that is administered in a coital-independent fashion (e.g. once a day). To achieve this goal, the ideal formulation should a) have high vaginal retention time, and b) be able to release a high dose of microbicides in a short period of time (i.e. semen-triggered release) when sexual intercourse occurs.

With the recent advances in nanotechnology, nanoparticles (NP) provide one possibility of such drug delivery system due to their unique characteristics, such as small size, protection of native drug, ability to reduce irritation at delivery site, and the ability of targeted delivery and controlled release of drugs. The concept of so called “nanomicrobicide” has embraced the great potential of nanomedicine, and efforts have been made to address major health problem of HIV prevention \(^8\). Some examples of new drug delivery system (DDS) designed for the delivery of anti-HIV drugs have been reported \(^9\), but none of them are currently used clinically for the purpose of the prevention of HIV transmission.

The present dissertation aims at exploring different formulation strategies for HIV microbicides utilizing nanotechnology and microencapsulation techniques. The first aim is to design and optimize a spray-dried oily core NC formulation that could serve as a novel DDS for lipophilic anti-HIV microbicides candidates. Design of experiments (DOE) is applied in the formulation and process optimization. The knowledge gained in this study provides a fundamental understanding of various formulation and process variables involved in the NC formation as well as the production process of spray drying. These conclusions are then applied in the second aim of this dissertation, in which a pH-sensitive NP formulation is designed and
prepared for hydrophilic HIV microbicides, tenofovir (TNF). In this work, the physicochemical characteristics of the NP formulation, as well as the in vitro safety study are performed. Finally, the knowledge gained in the previous studies is combined and a modified dual-functional mucoadhesive and pH-sensitive MS formulation is prepared using spray drying. This study also encompasses the formulation optimization, characterization, in vitro safety, in vitro mucoadhesion, immunogenicity testing, and in vitro anti-HIV efficacy study.

1.2 Statement of the Problem

Topical strategy to prevent HIV transmission is particularly challenging compared with other routes of delivery, owing to the complexity of the site of delivery, the female vaginal and endocervical epithelium. The human vagina is a fibromuscular tube approximately 10 cm in length. The intact vaginal mucosa is a multicellular layer of squamous epithelial cells, which gradually changes to columnar ecto- and endocervical epithelium. The squamous epithelium is divided into keratinized epithelium resembling skin which covers the introitus, and non-keratinized epithelium which covers the rest of the vaginal tract. The ectocervix is covered by a mucosal layer which composed of epithelium, basement membrane, lamina propria mucosa, and lamina muscular mucosa. The endocervix consists of a simple columnar epithelium with numerous glands. The epithelium thickness is controlled by several factors such as age and hormone level, and an understanding of the role of epithelium thickness and its effect to drug/particle penetration is essential to successful delivery of topical microbicides. Moreover, the integrity of vaginal and cervical epithelium has been highly associated as a risk factor in vaginal acquisition of HIV. It has been shown that disruption of its integrity due to traumatic sex, bacterial vaginosis, ulcerative diseases, and other sexually transmitted diseases (STDs) heighten
the risk of HIV transmission. Therefore a successful topical microbicides formulation should not pose any damage to the integrity of vaginal or cervical epithelium.

Vaginal fluid, including cervical fluids covers the region of vagina and cervix, providing moist to the environment, and serving as a barrier against the entry of pathogens. It is composed of secretions from cervical vestibular glands, plasma transudate, and endometrial and oviductal fluids. Properties of vaginal and cervical mucus may interact with drug formulation, either facilitate or inhibit the efficacy of microbicides. For example, the presence of vaginal fluids may alter the viscosity of an intravaginal formulation, therefore increase the chance of leakage, and decrease the resident time of such formulation at the target site. Moreover, the dissolution rate, stability, as well as permeability of microbicides may change due to the interactions between the drug substance and the components in the vaginal fluid.

Vaginal flora plays a significant role in maintaining a healthy vaginal environment. The presence of gram-positive and gram-negative species of both cocci and bacilli classes contributes to the balance of vaginal flora. Lactobacilli are one of the major components that are responsible for maintaining the low pH of vagina. Vaginal pH is normally 3.5-5.0 for healthy women, and it is maintained by lactic acid which is produced by Lactobacilli from glucose, which is the metabolite of glycogen. Some of the Lactobacilli strains can also produce H₂O₂, which has proven bactericidal effect and is associated with lower bacterial vaginosis rate. Therefore, a safe topical delivery system must not deplete the number or viability of normal vaginal flora.

Finally, the normal vaginal environment contains all the necessary components to maintain a functional immune response against any genital infection. Immune cells, such as Langerhans cells, T cells, macrophages, and dendritic cells (DC), can be found in the submucosal
layer of vaginal and endocervical epithelium\textsuperscript{18}. In case of a vaginal exposure, especially under the circumstances of damaged epithelium, HIV can rapidly penetrates the mucosa, reaching the dendritic projection of Langerhans cells, followed by transmission and infection to other target cells\textsuperscript{19}. Thus, immunogenicity of the topical formulation of microbicides has been considered another risk factor for facilitating the HIV transmission.

Overall, the present work aims to solve the following problems in the vaginal delivery of HIV microbicides, namely the lack of controlled release, the lack of bioretention, and the lack of assurance in biosafety.

1.3 Objectives

The objectives of the dissertation are:

(1) To design and optimize a NC delivery system that is suitable for the encapsulation of lipophilic microbicides candidate using DOE. To explore the formulation and process variables that could affect the production of NC as well as drug encapsulation. The physicochemical characteristics of NC such as size, EE\%, morphology, density, \textit{in vitro} drug release as well as process yield, powder flowability are evaluated.

(2) To design a pH-sensitive nanoformulation for hydrophilic microbicides such as tenofovir (TNF) for topical prevention of HIV. The proposed formulation should be not only safe, but also has rapid release of microbicides at the presence of human semen. The physicochemical characteristics of NP, as well as \textit{in vitro} cytotoxicity and cellular uptake study are evaluated.

(3) Based on the knowledge gained in the previous two objectives, design and optimize a dual functional pH-sensitive and mucoadhesive MS formulation for topical prevention
of HIV using DOE. The proposed formulation should be safe, not immunogenic, and has semen-triggered release as well as increased bioadhesion. In addition to the tests performed in the previous two objectives, the in vitro mucoadhesion, immunogenicity, and anti-HIV efficacy are also evaluated.
2. CURRENT STATE OF THE ART IN HIV MICROBICIDES DELIVERY

2.1 Vaginal Transmission of HIV

Unprotected, heterosexual, vaginal intercourse has become one of the major routes of infection, especially in developing countries. The incidence of sexually transmitted HIV through unprotected sex is from 0.04% to 0.08% in industrialized countries; however, this rate is 4 to 10 times higher in developing countries. Although the global percentage of women among people living with HIV has remained stable (50%), women are considered more susceptible to sexually-acquired HIV infection due to physiological, social, and economical factors. For example, results show that the prevalence of HIV is generally higher in women compared to men, regardless of their marital status, according to a study done in nine western Africa countries. Women’s greater biological susceptibility is highly associated with the fact that HIV and other STD pathogens are much easier to breach the mucosal barrier of vagina, as compared to penile tissue and anus. The mucosal surface, when intact, serves as a natural barrier for HIV and other STDs. Some features such as low pH in the environment and the H₂O₂ produced by vaginal flora has a virucidal effect. Therefore the chance of vaginal acquisition of HIV when the mucosal barrier is intact is relatively low although conflicting reports exists regarding the ability of HIV to breach intact mucosal epithelial cells. Regardless of the debate, the rate of transmission is remarkably higher when the mucosal barrier is compromised. In such event, cell-free HIV virion can easily gain access to the Langerhans cells (LC), which is a kind of intra-epithelial CD4+/CCR5 dendritic cells (DC) having dendritic projections (dendrites) that might extend to the mucosal surface. This allows the DCs to directly entrap HIV when there’s only minor tissue damage at mucosal surface. Meanwhile, CD4+ T cells and macrophages, the primary
target cells of HIV, usually reside in the lamina propria and columnar epithelium. Within hours of infection, these target cells in the sub-epithelium are infected with the aid of LCs and other sub-epithelial DCs expressing mannose-binding C-type lectins\textsuperscript{18}. The process of vaginal acquisition of HIV is shown in \textbf{Figure 1}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{vaginal_acquisition.png}
\caption{Vaginal acquisition and infection of HIV.}
\end{figure}

Other factors which may disrupt the integrity of the vaginal mucosal can significantly increase the likelihood of HIV transmission, such as dry or traumatic sex\textsuperscript{14a, 24}, bacterial vaginosis\textsuperscript{25}, or inflammatory or ulcerative diseases of the vagina\textsuperscript{26}. These factors act by contributing to the increased amount of target cells to the mucosa, alteration of normal vaginal pH, disruption of H\textsubscript{2}O\textsubscript{2} producing Lactobacillus, or increase the accessibility of virion to the target cells. A growing number of evidence suggests that recurrent or persistent infection of other STDs, such as syphilis, herpes simplex virus type 2 (HSV-2), and gonorrhea increases the
chances of HIV infection. These STDs may have an impact on HIV transmission through the production of: 1) varying degree of mucosal inflammation (Gonorrhea, Trichomonas vaginosis); 2) genital ulcers (HSV 1 and 2), and 3) changes in epithelial cells and/or mucosa neoplasm (Human Papillomavirus). The risk factors are summarized in Figure 2.

Figure 2. Risk factors associated with increased vaginal infection of HIV.

After sexual transmission, viral RNA is not detectable for a period of about 10 days, and it has been suggested that the continued viral expansion in the genital tissues and perpetual seeding of the systemic compartment is necessary to achieve a threshold of sustainable infection at distal sites.
2.2 Topical Microbicides for HIV Prevention

Nucleoside and non-nucleoside reverse transcriptase inhibitors and protease inhibitors have been effectively added to the arsenal of anti-HIV medication for the past decades to significantly reduce the viral loaded of HIV. Known as highly active anti-retroviral therapies (HAART), it has been shown to dramatically reduce the viral load of HIV in clinical setting \(^{29}\). This is also critical to preventing HIV transmission, as the concentration of HIV in the blood of the infected cases can be correlated directly with the sexual transmission of HIV. In the study of Quinn et.al, it is shown that HIV transmission is not observed when the blood concentration of HIV is lower than 1500 copies/ml, and the risk increases dramatically with the increase of blood HIV concentration \(^{30}\) in the host. Therefore it is reasonable to assume that oral administration of anti-viral therapeutics (ART) can be used as a pre-exposure prophylaxis (PrEP) method for HIV prevention. Careful design and consideration must be given in terms of the selection of drug, dose, plasma concentration, and the ability of mucosa penetration \(^{31}\). There have been several human clinical trials utilizing oral PrEP strategy, as shown in Table 1. From the clinical data, there is no absolute conclusion that an oral microbicide formulation is safe and effective for HIV prevention.
Table 1 Summary of clinical trials testing oral PrEP method for HIV prevention

<table>
<thead>
<tr>
<th>Trial ID</th>
<th>Description</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>iPrEx</td>
<td>Phase III trial of once daily oral TDF/FTC in high-risk population</td>
<td>Reduce risk of infection by 43.8%</td>
<td>32</td>
</tr>
<tr>
<td>CDC 4323</td>
<td>Phase II trial of once daily TDF</td>
<td>No serious adverse effect, and PrEP use did not have a significant effect on HIV risk behavior</td>
<td>33</td>
</tr>
<tr>
<td>CDC 4321</td>
<td>Phase II/III trial of oral TDF</td>
<td>Terminated</td>
<td>34</td>
</tr>
<tr>
<td>FEM-PrEP</td>
<td>Phase III trial of once-daily oral TDF/FTC</td>
<td>Failed to draw conclusion of its effectiveness, associated with increased side effect, discontinued.</td>
<td>35</td>
</tr>
<tr>
<td>MTN-001</td>
<td>Randomized cross-over trial for daily oral and topical TNF</td>
<td>Adherence and acceptability of tablet in Africa is relatively low.</td>
<td>5d</td>
</tr>
<tr>
<td>MTN-003</td>
<td>Phase IIb safety and effectiveness study of TNF gel and TDF, TDF/FTC tablet</td>
<td>Closed to follow-up</td>
<td>5c</td>
</tr>
<tr>
<td>Partners PrEP</td>
<td>Randomized trial for oral TDF, TDF/FTC</td>
<td>A relative reduction of 67% HIV infection compared to placebo</td>
<td>36</td>
</tr>
<tr>
<td>CDC 4920</td>
<td>Phase II/III trial for oral TDF, TDF/FTC</td>
<td>Reduction of 62% HIV infection compared to placebo, side-effect observed, long-term safety unknown.</td>
<td>37</td>
</tr>
</tbody>
</table>

Topical microbicides represents themselves as important strategies with clear potential for preventing sexually transmitted HIV (and possibly other STDs). Since the predominant mode
of transmission of HIV globally is through heterosexual intercourse and the number of women suffering from HIV infection is high in specific regions such as Africa, it is critical to develop a safe and effective topical microbicides formulation which women could have control over the risk of sexually transmitted HIV. It has been estimated that a single microbicides with 60% effectiveness could prevent millions of new cases of HIV infection each year worldwide.\(^\text{38}\)

Topical microbicides are products that when applied topically, capable of attacking viral targets and preventing the infection of target cells or viral replication, resulting in decreased likelihood of sexually transmitted HIV.\(^\text{39}\) In the following sections, different types of topical microbicides will be summarized.

Detergents and pH modifiers

Detergents, pH modifiers, and sometimes spermicides are among the very first attempts to disrupt the membrane of HIV and other STD pathogens, therefore providing topical protection strategy to women. There has been a lot of studies focusing on a spermicide, Nonoxynol 9 (N-9), which later was found out to be unsafe and actually increases the risk of HIV infection due to its damage on vaginal epithelium.\(^\text{40}\) Other candidates tested show either increased HIV infection, or failed to protect against HIV, such as Carraguard\(^\text{41}\) and cellulose sulfate.\(^\text{42}\) Recently, PRO2000, a well tolerated gel formulation, has successfully completed a phase IIb study with a modest 30% reduction of HIV acquisition in women.\(^\text{43}\) Because HIV is inactive below pH of 4.5, a number of acidifying agents are under development as microbicide candidates. Tested products vary from something as simple as lime juice\(^\text{44}\) to buffered gel formulation (Buffergel).\(^\text{43, 45}\) However, these reports are not conclusive enough since they are either in vitro data, or the number of sample is not large enough to draw a general conclusion. Besides, human semen has a pH value around
7.6, and a higher buffer capacity compared to vaginal fluid. Therefore a small rise in the pH may allow infection.

Reverse transcriptase (RT) inhibitors

Given their successful application in the HAART therapy, RT inhibitors have been widely tested as topical microbicide candidates. Tenofovir (TNF), Dapivirine (TMC 120), and UC-781 are some of the examples that are currently under clinical trial. A summary of clinical trials for RT inhibitors is listed in **Table 2**

The advantage of using RT inhibitors as a topical prevention strategy is the fact they have succeeded in demonstrating abilities to prevent maternal to infant transmission, and have been applied systematically as pre-or post-exposure prophylaxis. Their already proven systematic safety may also expedite the development process. However, as shown in some of the clinical studies, the effectiveness of topical microbicides is highly dependent on a high adherence rate. The acceptability and adherence of topical formulation (gel, ring, etc.) has always been an issue in the development process, which deserves more attention and discussion (see later).

Among RT inhibitors, TNF is one of the most studied compounds on the battlefield of HIV prevention. Tenofovir (TNF, \([(2R)-1-(6\text{-amino-9H-purin-9-yl})\text{propan-2-yl}]\text{oxy}\)methylphosphonic acid) is a nucleotide analog HIV reverse transcriptase inhibitor whose prodrug (Tenofovir disoproxil fumarate, TDF) is now marketed in an oral dosage form (Viread®, Gilead Science). It is also available as a combination with emtricitabine in a product with the brand name Truvada® (Gilead Science). Atripla®, a combination of Gilead Science’s Truvada with Bristol-Myers-Squibb’s Efavirenz, was also approved by FDA in 2006.
Table 2 Summary of clinical trials testing RT inhibitors for topical PrEP method for HIV prevention

<table>
<thead>
<tr>
<th>Trail ID</th>
<th>Description</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATH HS-522</td>
<td>Safety evaluation for two TNF applicators</td>
<td>Both applicators were acceptable</td>
<td>49</td>
</tr>
<tr>
<td>MTN-003</td>
<td>Safety and effectiveness of TNF gel compared to TDF and TDF/FTC tablets</td>
<td>Only phase I safety data available, TNF gel appears to be well tolerated.</td>
<td>5d, e</td>
</tr>
<tr>
<td>RMP02-</td>
<td>Safety and effectiveness of rectal TNF gel compared with oral tablet</td>
<td>No serious adverse effects reported, rectal TNF gel results in higher tissue detection than oral tablets</td>
<td>50</td>
</tr>
<tr>
<td>CAPRISA 004</td>
<td>Safety and effectiveness of TNF gel</td>
<td>An overall 39% reduction in HIV infection was observed. Highly associated with gel adherence.</td>
<td>5b</td>
</tr>
<tr>
<td>IPM 012</td>
<td>Phase I safety and PK study of TMC 120 gel</td>
<td>Gels are well tolerated and able to deliver effective concentration to the lower genital tract.</td>
<td>51</td>
</tr>
<tr>
<td>MTN-020</td>
<td>Phase III trial of TMC 120 vaginal ring</td>
<td>Recruiting</td>
<td>52</td>
</tr>
<tr>
<td>U19-</td>
<td>Safety and acceptability of rectal UC-781 gel</td>
<td>PK-PD assessment of topical microbicides and comparisons of microbicides efficacy available</td>
<td>53</td>
</tr>
</tbody>
</table>

HIV entry/fusion inhibitors
Besides early-stage detergents (N-9, Carraguard) which targeting at disrupting HIV viron, detailed understanding of HIV entry and process and mechanism has lead to a new generation of entry/fusion inhibitors. The interactions between HIV envelop protein gp120 and proteoglycans on the cellular surface initiate the infection process, followed by recognition and binding of gp120 to its major target, CD4 receptor. In humans, the conformational change after gp120-CD4 binding triggers the binding of gp120 to chemokine coreceptor CCR5 or CXCR4, which finally expose the hydrophobic peptide of HIV envelop protein gp41 to insert itself to the host cell. Then the last step of HIV entry involves conformational change of gp41 to form a six-helix bundle structure which brings the transmembrane region of gp41 in close proximity to the host cell membrane, thus facilitating the entry of viral capsid. There are a number of different strategies that targeting different stages of HIV entry process, focusing on either blocking gp120-CD4/CCR5 or CXCR4 (entry inhibitor) or blocking gp41 mediated membrane fusion (fusion inhibitor). Table 3 summarizes some of the entry/fusion inhibitors in these two categories. Since most HIV entry/fusion inhibitors target HIV envelop protein, the variability of effectiveness to this category of drug could differ by several orders of magnitude, which is much larger than RT inhibitors. Moreover, the HIV viral resistance to entry/fusion inhibitors can occur rapidly, as it has been shown in different reports. In a formulation standpoint, most entry/fusion inhibitors are peptides or antibodies. Therefore the stability of these agents, particularly when applied topically, may also have a critical impact on their effectiveness.
Table 3 Summary of HIV entry/fusion inhibitors

<table>
<thead>
<tr>
<th>Name</th>
<th>Mechanism</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD4 (solution CD4)</td>
<td>CD4 mimics</td>
<td>Discontinued in phase II (resistance)</td>
</tr>
<tr>
<td>BMS-378806/BMS-488043</td>
<td>Competitive binding to CD4</td>
<td></td>
</tr>
<tr>
<td>TNX-355 (Ibalizumab)</td>
<td>CD4 antibody</td>
<td>Phase II</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>Non-competitive CCR5 antagonist</td>
<td>Approved 2007</td>
</tr>
<tr>
<td>Chemokine CCL5 (RANTES)</td>
<td>Block binding of CCR5, inducing CCR5 internalization</td>
<td>Planning clinical trials</td>
</tr>
<tr>
<td>PRO-140</td>
<td>CCR5 antibody</td>
<td>Phase II</td>
</tr>
<tr>
<td>ALX40-4C</td>
<td>Polypeptide binds to CXCR4</td>
<td>Did not have significant effect in human test</td>
</tr>
<tr>
<td>AMD3100</td>
<td>Small molecule bind to CXCR4</td>
<td>Clinical trial halted due to side effect</td>
</tr>
<tr>
<td>Enfuvirtide (T-20)</td>
<td>Inhibit gp41 conformational change</td>
<td>Approved 2003</td>
</tr>
<tr>
<td>Sifuvirtide (SFT)</td>
<td>Inhibit gp41 conformational change</td>
<td>Phase IIb clinical trial</td>
</tr>
</tbody>
</table>

Source: www.clinicaltrials.gov

2.3 Clinical Consideration for Topical Micorbicide for HIV Prevention
Overview

There has not been a specific guidance document issued by the Food and Drug Administration (FDA) regarding the development of anti-HIV microbicides. However, as most Investigational New Drug (IND) development usually includes safety and efficacy studies using appropriate models, it is needless to say that the development of topical microbicides must follow the same rule. Specifically, some points needs to be modified to precisely reflect vaginal or rectal environment where these products apply. Some development guidelines even test algorithm have been proposed, and one must also take into consideration other factors such as viral resistance, combinational assay with other anti-HIV drugs, PK/PD of topical microbicides formulation, and storage stability in certain region (Sub-Saharan Africa, for example).

Formulation and delivery

Although choosing the ideal biological target and identifying the best microbicides for this target is clearly central to the success of developing topical prevention strategy, it is also necessary to acknowledge the importance of designing an appropriate formulation that is capable of safely and effectively delivering the microbicides to the site of action. An ideal formulation should be able to achieve absolute mucosal safety, long bioretention time, ensure the efficacy of microbicides when delivered, and has sufficient tissue penetration or controlled release, depending on the mechanism of action. Moreover, a successful formulation must also be well tolerated and well accepted, covert use, and economically affordable.

Semi-solid gels are the most common form for vaginal delivery. In clinical trials, a gel formulation based on hydroxyethylcellulose (HEC) has been widely used as the topical formulation for a variety of microbicides. The advantage of gel formulation lies in its
safety, and the development and preparation is relatively easier compared to other formulations, which is probably why it is the most commonly applied formulation in topical microbicides clinical trials. However, gel formulation suffers from leakage and a general “messy” feeling, and these facts not only create issue for its acceptability, but also lower its anti-HIV efficacy. Other factors affecting the acceptability of the gel formulation include the ease of incorporation into typical sexual practices, and the different types of sexual partnership. The short duration of the bioretention of the formulation requires the user to apply microbicides formulation hours before sex (coital dependence), which leads to significant patient compliance issues and decreased protection against HIV infection as recently observed in the clinical trial of tenofovir gel. To counter the poor bioretention and lack of controlled drug release, a temperature and pH-sensitive hydrogel formulation is developed as a topical microbicides DDS. A random terpolymer of N-isopropyl acrylamide, butyl methacrylate, and acrylic acid is synthesized, and the formulation is designed to coat vaginal tissue with a stable gel layer and to release entrapped model agents in a burst release profile in a response to the presence semen. The results indicate a prolonged retention, and semen-triggered release compared to traditional gel, demonstrating potential of solving adherence issue by incorporation of bioretention with controlled release.

Intravaginal rings (IVR) offer an alternative to gel-based formulation, as their solid nature and the capability of long-lasting release may provide a solution for leakage and coital dependence. IVR are torus-shaped polymeric devices either loaded with API within the polymer matrix of the ring or within a reservoir core at the center of the ring. The rings needs to be carefully compressed and placed in the upper third of vagina to avoid involuntary expulsion. Dapivirine (TMC 120) is currently under clinical trial evaluation using a silicon based IVR.
formulation, and the preliminary results support its safety and capability of zero-order release in a period of 30 days 64.

Vaginal contraceptive film (VCF), a rapid-dissolving polymeric film formulation for spermicide, has shown its acceptability among consumers through its ease of storage and application 65. The final product form of VCF is a solid polymer film, therefore it is most likely odorless and it will not be as “messy” as gels. Once inserted into the vagina, VCF is rapidly dissolved, allowing immediate release of microbicides. Due to these superior attributes over gel formulation, VCF has gained research interests in development of contraceptive as well as microbicides formulations 66.

Safety and acceptability

Safety concerns have emerged regarding topical microbicides formulation, especially when detergent based microbicides such as N-9 fails to protect HIV infection 40a. As described in the previous section, the ability of maintaining an undamaged vaginal mucosa is critical for any topical microbicide formulation. Although “reduced” irritation of N-9 has been reported, it is not guaranteed that long-term use of such product would be safe 67. Other microbicides, such as low pH buffering agents (Buffergel), have shown mild or moderate adverse effects (mostly irritation) among two thirds of the participants 68, indicating a possible damage to the mucosa over a long-term use. Some topical agents may disturb the normal vaginal ecology, thus counterbalance their protective effect over HIV infection 18. For example, there is a report showing PSC-RANTES (a synthetic CCR5 antagonist) is toxic to some of the Lactobacillus strains 69. Acidify agents, such as lemon or lime juice, have also been tested and proven to inhibit the viability of beneficial Lactobacillus species that are associated with vaginal health 44b. These undesired disruption to
normal vaginal flora must be avoided, otherwise the damaged intrinsic defense system will lead to increased risk of HIV infection.

Any artificial agent applied to topical mucosa on a long-term basis has the risk of being immunogenic. In the case of topical microbicides, this is unquestionably dangerous, and must be well characterized in the preclinical setting. Immune responses triggered by irritation may damage the vaginal epithelium through inflammatory cytokines such as IL-1, IL-6, IL-8 and IP-10. Moreover, the immune response leads to localization of T-cells and macrophages, both are targets for HIV infection. Infections with STDs that cause inflammation are a significant risk factor for HIV infection, and induction of local inflammation is a possible reason for the increased risk of HIV infection in the N-9 clinical trial. In vivo intravaginal lavage using rabbit model may show a good correlation between the rabbit vaginal irritation scores and IL-1β levels, but developing new models that is appropriate to reflect the variability between species is definitely needed.

A successful topical microbicides formulation must be well accepted to its end users to maximize its biological effectiveness. As shown in the CAPRISA 004 trial, the success rate of HIV prevention is highly dependent on the adherence, which is mostly decided by the acceptability of the formulation. In the low adherence group the overall protection is only 28%, while, it is almost 60% in the high adherence group. While the necessity of using topical microbicides has been well recognized among the high-risk population, there are a lot of factors that contribute to the acceptability of a topical microbicides formulation. Factors such as leakage, ease of application, odor, texture, ability to affect sexual pleasure (both positive and negative), whether it is coital-dependent, and the ability to use covertly will have great impact on whether a woman wants to use such product. There are a number of studies focusing on the
acceptability of different formulation in different population \(^{73}\), and conducting acceptability research in the early stage of development is highly recommended to be able to obtain critical data which may lead to a successful formulation strategy \(^{39}\).

2.4 Nanotechnology and its Application in Topical Microbicides Research

Nanoparticles (NP) are particles having dimensions vary from 100-1000 nm, and they may or may not contain drug substances. Applying NP as a drug delivery system (DDS) poses a number of advantages compared with traditional formulation, such as 1) No risk of blocking blood vessels during systemic circulation after intravenous administration because of their nano-size range \(^{74}\); 2) Prolonged circulation time in the body by avoiding the reticuloendothelial system (RES) \(^{75}\); 3) Unique accumulation behavior at the tumor site (passive tumor targeting of long circulation) \(^{76}\); 4) Controlled release of drugs by biodegradable polymer degradation and diffusion mechanisms after they accumulate at the target site \(^{77}\); 5) Improve the efficacy of carried drugs, owing to small size, prolonged circulation time, and sustained drug release profile \(^{78}\); 6) More stability during storage \(^{79}\); 7) Reduce the multi-drug resistance by internalizing drug and reducing its efflux from cells mediated by the P-glycoprotein \(^{80}\). Various NP based DDS, including liposomes \(^{81}\)/cubosomes \(^{82}\)/niosomes \(^{83}\), polymeric micelles \(^{84}\), nanocapsules (NC) \(^{85}\), quantum dots \(^{86}\), gold \(^{87}\)/silver NP \(^{88}\), solid lipid NP \(^{89}\), and dendrimers \(^{90}\), have been designed for different therapeutic agents (small molecules, proteins, peptides, siRNA, etc.) via different delivery routes (intravenous, oral, topical, subcutaneous, etc.). The physicochemical characteristics of NP, such as their size, surface charge, shape, morphology, texture, and surface modification may be controlled through engineering and production process, and these characteristics may greatly influence the function and the biological responses of NP \(^{91}\). The
shape and the size/size distribution of NP are very important characteristics which may have influence on the distribution, penetration, cellular uptake, and drug release profile of the NP. Therefore a rational design of particle shape and size is required in the early stage of development, and a narrow size distribution is usually more desirable for its better controlled in vivo distribution and release of drug substances. Characterization tools such as dynamic light scattering (DLS) and electron microscopy (SEM or TEM) are commonly used to evaluate the size and morphology of NP. Zeta potential measurements are usually applied to measure the surface charge. Drug loading, encapsulation efficiency, and drug release are characterized using dissolution techniques and chromatographic methods.

Since DDS in the nanoscale can offer advantages such as enhanced delivery efficiency, specific targeting, controlled release and ability to bypass biological barriers, they have gained interests in formulation development for the HIV therapeutics. For example, NP formulations are designed to deliver therapeutic agents specifically to the lymphatic system and macrophages through careful control of their particle size. The large surface area per unit volume of NP display enhanced solubility compared to larger particles, therefore nanocrystals have been prepared to increase the water solubility of anti-viral drugs, which would otherwise considered not viable due to their poor water solubility. Other solubility-enhancing strategy may also include preparation of nano-micelle or NC. NP can be easily modified with targeting moieties or functionalized materials to increase their biological functions. For example, NP formulation modified with polyethylene glycol (PEG) can significantly increase the half life of anti-viral drug in animal studies. NP coated with lectin or bryostatin-2 can target the cells of RES, or activate the latent HIV virus while ensuring successful delivery of anti-viral drugs to
CD4+ T cells\textsuperscript{100}. **Table 4** shows a number of antiviral drugs that uses NP as their delivery platform.

**Table 4 Summary of nanoparticles delivery system for anti-HIV drugs**

<table>
<thead>
<tr>
<th>Antiviral drug</th>
<th>NP type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saquinavir (SQV)</td>
<td>Cationic solid lipid NP</td>
<td>101</td>
</tr>
<tr>
<td>SQV</td>
<td>Cyclodextrin and poly(alkylecyanoacrylate) NP</td>
<td>102</td>
</tr>
<tr>
<td>SQV</td>
<td>PEO-PCL NP</td>
<td>103</td>
</tr>
<tr>
<td>Stavudine (D4T)/Delavirdine (DLV)/SQV</td>
<td>Solid lipid NP, polybutylecyanoacrylate NP</td>
<td>104</td>
</tr>
<tr>
<td>Indinavir</td>
<td>CD4 targeted lipid NP</td>
<td>105</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>Tat-conjugated NP</td>
<td>106</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>PLA and PLA-PEG NP</td>
<td>107</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>Thermosensitive gel incorporating polymeric NP</td>
<td>108</td>
</tr>
<tr>
<td>Lamivudine/Zidovudine</td>
<td>Polymeric and solid lipid NP</td>
<td>109</td>
</tr>
<tr>
<td>Dapivirine</td>
<td>PCL NP</td>
<td>110</td>
</tr>
<tr>
<td>Ampenavir</td>
<td>Transferrin conjugated quantum dots</td>
<td>111</td>
</tr>
</tbody>
</table>

More advanced formulations are also highly sought-after in the field of topical microbicides. A nanoparticle drug delivery system for a CCR5 chemokine inhibitor, PSC-RANTES, has been investigated\textsuperscript{112}. In the *in vitro* study, the release profile demonstrates an effective anti-HIV concentration of PSC-RANTES for 30 days. Tang et.al reported a self-
assembled NP formulation delivering peptide-based vaccine to induce effective T cell responses against established cervical cancer \(^\text{113}\). In the recent studies, intravaginal delivery of siRNA has been achieved by PLGA or pegylated lipoplex system for intravaginal gene knockdown \(^\text{114}\) or herpes simplex virus type 2 (HSV-2) microbicides \(^\text{115}\). Owing to its size and surface chemistry, NP formulation has the potential of delivering “once-a-day” microbicides to address the adherence issue \(^\text{116}\). Mucus-penetrating NP may provide an uniformly distributed layer of microbicides throughout the vaginal tract, avoiding the drug lost due to leakage of gel formulation. Lai et al. develop a mucus-penetrating NP capable of diffusing through human cervical-vaginal mucus \textit{in vitro} \(^\text{117}\). Several parameters that affect the biodistribution of NP in vaginal tract have been discussed, such as size, surface charge, and PEGylation \(^\text{118}\). Due to the complexity of vaginal environment and the challenges involved in developing a successful topical microbicides, the number of references supporting a successful attempt in this field is limited. However, this technology is being applied in several cases and data generated will provide the evidence needed to support the use of such delivery systems in the field.

2.5 Design of Experiments (DOE) and its Application in Formulation Design

Overview

In general, design of experiments (DOE) refers to the design of any information-gathering exercises where variation is present \(^\text{119}\). Specifically, DOE is a systematic, rigorous approach to problem-solving that applies statistical principles and techniques at the data collection and analysis so as to ensure the generation of valid, defensible, and supportable conclusions. In addition, all of this is carried out under the constraint of a minimal expenditure of experimental runs, time, and money \(^\text{120}\). Traditionally, the general method of scientific research
is referred as “one-factor-at-a-time” (OFAT), where the design of experiments involving the testing of factors, or causes, one at a time instead of all simultaneously. The principles of DOE start to establish as James Lind, Charles Peirce, and Abraham Wald proposing principles such as control, statistical inference, randomization, polynomial regression, and sequential analysis. Modern concept of DOE was developed by Ronald A. Fisher, who described a method studying the effect of nature fluctuation such as temperature, soil condition, and rain fall. This was also the first publication that coined the term “null hypothesis”. Although the first application of DOE was in the field of agriculture, it has been successfully gained interested in military and engineering (see later Besse Day study). Some of the modern principles of DOE are somewhat similar to the traditional point of view, such as: 1) Randomization. It has been widely accepted that the random assignment of individual group or condition in an experiment by various methods (tables of randomized numbers or randomization devices) provides a calculable thus reducible risk of error. 2) Replication. Measurements are usually subject to uncertainty and variation, and replication may help to identify the sources and the true effects of variation. 3) Blocking. Blocking is the arrangement of grouping experimental runs that shears similar conditions together. Blocking reduces known but irrelevant sources of variation between conditions, thus allow more precise estimation of the real sources of the variation. 4) Factorial experiments. Instead of OFAT, modern DOE favors factorial experiments, which consists of all possible combination of different levels of factors involved in study. For an experimental design involves n factors and x levels for each factor, it is usually denoted as x^n factorial design, and the total number of experimental runs is x^n. The concept of DOE is later developed and modified by a lot of scientists such as Besse Day, who applied DOE in naval experimentation on fleet testing in WWII, and George Box, who is one of the leading scientists in development of responsive
surface methodology (RSM). Perhaps the most well-known scientist among them is Genichi Taguchi, whose quality improvement methods are adopted by Japan and later US auto industries such as Toyota and GE. His methods are the foundation of modern quality improvement system such as “Total quality” and “Six Sigma”. To analyze the result of a DOE, especially in a factorial design, an Analysis of Variance (ANOVA) is usually applied. ANOVA is a statistical hypothesis test that makes conclusion on whether a test result is statistically significant (that it is unlikely to occur by chance), assuming the truth of the null hypothesis. A statistically significant result usually indicated by a p-value below a threshold (significance level) would justify the rejection of the null hypothesis. Typically, the null hypothesis in an ANOVA states that all variance between treatment groups are random, which implies there is no effect of treatments. Thus rejecting the null hypothesis concludes that the treatments have different impact on the results. The calculations of ANOVA can be characterized as computing a number of means and variances, dividing two variances and comparing the ratio to a handbook value to determine statistical significance. The sample variance is calculated as:

\[ s^2 = \frac{1}{n-1} \sum (y_i - \bar{y})^2 \]  

(1)

Where the divisor (n-1) is called the degree of freedom (DF), the summation is called sum of squares (SS), and the result (s^2) is called mean sum of squares (MS). If we consider the source of SS as coming from the model fit and residue (error), they can be calculated individually as:

\[ SSM = \sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2 \]  

(2)

\[ SSR = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 \]  

(3)
Where \( \hat{y}_i \) is the value predicted by the model, \( \bar{y} \) is the mean value of \( y \) values, and \( y_i \) is the experimental value. An F ratio is therefore calculated as the ratio of MS of the model (MSM) to the MS of the residue (MSR) according to their DF. The calculation can be found in Table 5. The probability (p-value) of a value of F greater than or equal to the observed value under null hypothesis can be calculated as the area under F distribution curve that is beyond the calculated F ratio. The null hypothesis is rejected if this probability is less than or equal to the significance level (\( \alpha \)).

Table 5. Calculation of the F ratio.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>SSM</td>
<td>P</td>
<td>MSM=SSM/P</td>
<td>MSM/MSR</td>
</tr>
<tr>
<td>Residual</td>
<td>SSR</td>
<td>N-P-1</td>
<td>MSR=SSR/N-P-1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>SSM+SSR</td>
<td>N-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DOE in formulation design and optimization

The principle and application of DOE has gained numerous interests by the FDA, and is reflected in the ICH guideline Q8 R2, in which “Quality by Design” (QbD) is proposed. According to FDA, QbD is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management. Specifically, DOE is listed as one of the “systematic approach” throughout the life circle of the product. A quick glance at the chronological chart (Figure 3) of last decade indicates that there has been a surge in the number
of publications regarding the formulation development utilizing DOE, especially since the publication of FDA guideline in 2006. The optimized formulations comprise of tablets \(^{131}\), capsules \(^{132}\), liquids \(^{133}\), semi-solids \(^{134}\), MP \(^{135}\), NP \(^{136}\), and other formulations (inhalations, aerosols, films, etc.) \(^{137}\). As seen in these examples, optimizing the formulation and process variables in formulation development using DOE will help in achieving the desired goals with phenomenal ease. DOE can prove to be useful, even if it fails to provide an optimum formulation that meets all requirements, because it unveils the internal correlation between the critical quality attributes (CQAs) to these variables \(^{138}\).

![Figure 3 Bar diagram portraying the chronological development in the number of research publications on use of DOE in formulation development. Source: pubmed.gov](image)

There are a lot of experimental designs applied to the process of screening and optimization. A full factorial design is an experimental design in which independent variables take on all possible combinations of their respective levels. By doing so it allows a thorough investigation of the effects of all independent variables and their interactions on the dependent
variables. However, the purpose of screening is to identify few important main effect from the many less important others\(^{139}\). For this purpose, fractional factorial design (FFD) is implemented. FFDs are experimental designs consisting of a subset of the full factorial design. A FFD with two levels are usually denoted by \(2^{k-p}\), where \(k\) is the number of factors screened, and \(p\) is the size of the fraction of the full factorial design\(^{140}\). For example, a \(2^{4-1}\) design stands for a FFD which is half of a two-level-four-factor full factorial design. The total number of runs (8 runs) is only half of its full factorial counterpart (16 runs). The trade-off of FFD is its inability to distinguish main effect with high-order interaction between independent variables. For example, in a \(2^{4-1}\) design, main effects are unconfounded with any of the two-factor interactions, but two-factor interactions are confounded with each other, rending this design only suitable to screen the main effects of independent variables. After the screening process, a response surface methodology (RSM) is usually applied to optimize the experimental conditions. RSM explores the relationship between a set of independent variables and the dependent variables by fitting the data using a polynomial equation\(^{141}\). One may choose different designs such as central composite design (CCD) and Box-Behnken Design (BBD).
3. DESIGN AND OPTIMIZATION OF SPRAY DRIED OILY CORE NANOCAPSULE FOR ENCAPSULATION OF LIPOPHILIC ANTI-HIV MICROBICIDES

3.1 Rationale

There are two major obstacles in drug delivery including poor water solubility/stability and limited drug targeting. For example, PHI-113, PHI-346, and PHI-443 are novel anti-HIV microbicides under development, and they have very poor water solubility (< 0.001 mg/ml). Poor solubility may lead to problems such as poor dissolution kinetics, lowered bioavailability, thus leads to ineffective therapy and the need for higher dosing. Various researches have been focused on this aspect, while lipid nanocapsules are intensively studied. Nanocapsules (NC) are heterogeneous vesicular systems in which the drug is confined to a cavity surrounded by a single polymeric membrane. Therefore, NC are considered as “reservoir” systems and the core may be aqueous or composed of a lipophilic solvent, usually oil. NC have several advantages such as: 1) No risk of blocking blood vessels during systemic circulation after intravenous administration because of their nano-size range; 2) Prolonged circulation time in the body by avoiding the reticuloendothelial system (RES); 3) Unique accumulation behavior at the tumor site (passive tumor targeting of long circulation); 4) Controlled release of drugs by biodegradable polymer degradation and diffusion mechanisms after they accumulate at the target site; 5) Improve the efficacy of drugs, owing to small size, prolonged circulation time, and sustained drug release profile; 6) More stability during storage; 7) Reduce the multi-drug resistance by internalizing drug and reducing its efflux from cells mediated by the P-glycoprotein. Therefore NC has the possibility serving as a promising vector for lipophilic microbicides.
The concept of NC as drug delivery system was first introduced by Couvreur et.al. Previous work on NC has been done by different groups. Emulsion-diffusion technique was first reported by Quintanar-guerrero et.al. in 1998, which enabled the preparation of NC in a simple, efficient, reproducible and versatile manner, and the mechanism as well as the challenges in formulation and process of this method were further elucidated.

In order to explore the possibility of a time-saving and scalable preparation of the NC, spray-drying NC have been extensively studied. Spray drying, a rapid process for generating powder, in which a feed solution containing the pharmaceutical is atomized into droplets that rapidly due to their high surface area and intimate contact with the drying air. The concept of spray drying has been extensively applied in the drying of various pharmaceutical excipients. This technology exhibits several advantages: drying in a rapid one step process, comparable low price with easy scale-up, suitable with heat sensitive molecules such as enzymes and proteins, and the possibility of modulating powder characteristics such as size by process analytical technology (PAT). For the operator of the spray drying process, several major process variables which may have direct influence on product quality should be considered and controlled, such as: the inlet temperature of the drying air, the drying air flow rate, the supply rate of the feed liquid, and the pressure/volume of the atomizing air as well as the drying air flow. Therefore, the process variables in the spray drying technique should be carefully controlled in order to avoid unwanted consequences such as particle arrogation, low yield and high moisture content. Previous literatures have focused on the effect of process variables during spray drying and/or spray congealing process.

Recently several publications have shown the ability of spray drying technique to improve the physicochemical stability of the NC formulation, meanwhile remain intact core-shell
structure, which is likely to corrupt in conventional freeze drying method \(^{153}\). A series of methods for spray drying polymeric NC and nanospheres (NP) have been proposed and studied, with appropriate spray drying adjuvant \(^{154}\). Although NC around 200 nm have been successfully prepared by spray drying \(^{155}\), there is a knowledge gap on the systematic and statistical analysis of the effect of formulation and process variables, specifically during spray drying of oily core NC. The lack of information regarding the effect of different formulation variables on the final formulation characterization hinders further application of this technique. Hence, there’s an urgent need in filling the knowledge gap between the roles of different formulation variables and final formulation characteristics in spray drying NC, so to predict their effect. Thus, this study is designed to bridge this knowledge gap.

In this study, an easy and effective method of preparing spray-dried NC without solvent rotoevaporation is developed. The influence of three different formulation variables and four process variables is studied using Box-Behnken design (BBD) and Fractional factorial design (FFED) to optimize spray dried NC. Indomethacin is chosen as a model drug because of its relative poor water solubility. The present study is finally the first application of DOE on spray dried IND containing oily core NC, which could eventually be used as a powerful tool in formulation strategy.

3.2 Materials and Methods

Reagents

Poly (D,L-lactide) Resomer® R208 was supplied by Boehringer Ingelheim Inc. (Ingelheim, Germany). Labrafac CC (caprylic/capric triglyceride) was kindly supplied by Gattefosse Corporation (Saint-Priest, France) as a gift. Poloxamer 407 (Pluronic F127) was a gift from BASF (Baden Aniline and Soda Factory) Corporation (Ludwigshafen, Germany).
Indomethacin (IND), Coumarin-6, Percoll® and sodium chloride were purchased from Sigma Aldrich (St. Louis, MO, USA). α-Lactose and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemical used in this study were of analytical grade and used without further purification.

Box-Behnken experimental design

BBD is an experimental design for response surface methodology, which could be thought of a combination of a two level factorial design with an incomplete block design. There are 3 blocks in a three factor Box-Behnken design. In each block, 2 factors are varied through the 4 possible combinations of high and low level, while a third factor is set at middle level. Compared to other response surface design such as central composite circumscribed (CCC) design and central composite inscribed (CCI) design, which both require 20 runs and 5 level of the factor, Box-Behnken design requires less number of runs (12 plus 3 center points) and less level of the variables (3 levels). Moreover, since Box-Behnken design does not take factors at extreme high/low level into account, it’s useful when the experimenter wants to avoid unreasonable extreme results.

Our group has studied the effect of different formulation variables on fabricating freeze dried oily core NC using same emulsion-diffusion technique proposed. It was found that NC ranging from 115.0 nm to 582.6 nm can be produced using different amount of PLA (40 to 360 mg) and Labrafac CC (0.1 to 0.9 ml), and polyvinyl alcohol was used as surfactant (unpublished data). A preliminary study was performed comparing F127 and PVA as surfactant. It was found spray dried NC using F127 could provide smaller particle size (360 nm compared to 470 nm). In this study, three formulation variables, namely, polymer amount, oil amount and surfactant
amount were selected at their low, medium and high levels with replicated center points. The independent and dependent variables and their coded factors are listed in Table 6.

Table 6. Variables and their Levels in Box-Behnken Design

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Levels</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁=amount of PLA 208 (mg)</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100(0.04)*</td>
<td>200(0.08)</td>
<td>300(0.12)</td>
<td></td>
</tr>
<tr>
<td>X₂= volume of oil (ml)</td>
<td>0.4(0.15)</td>
<td>0.6(0.22)</td>
<td>0.8(0.30)</td>
<td></td>
</tr>
<tr>
<td>X₃= amount of Pluronic F127 (mg)</td>
<td>120(0.05)</td>
<td>240(0.10)</td>
<td>360(0.15)</td>
<td></td>
</tr>
<tr>
<td>Coded values</td>
<td>-1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Dependent Variables**

Y₁= particle size (nm)

Y₂= encapsulation efficiency (%)

*: the number in brackets stands for formulation variable concentration in final nanosuspension (wt/wt %). The total mass of liquid phase was calculated by adding the total mass of ethyl acetate (10ml, 0.897 g/cm³) and water (240ml, 1.0 g/cm³). The specific density of Labrafac cc was from 0.930 to 0.960 g/cm³ according to manufacturer’s data sheet and an average value of 0.945 g/cm³ was used in calculation.

Similar formulation viable setting was also reported in previous studies ¹⁴⁷a. The polynomial equation obtained by Box-Behnken design software (JMP 8, SAS Institute) was as follows:

\[
y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (4)
\]
Where $Y_i$ was the dependent variable; $X_1$ through $X_3$ were independent variables; $b_0$ was the intercept with y axis; and $b_1$ through $b_{33}$ were regression coefficients. Two dependent variables, $Y_1$ (particle size) and $Y_2$ (encapsulation efficiency, EE%) were chosen in optimization process. It was reported that nanoparticles in the size range of 20 to 200 nm could have several advantages such as better stability (hours to months), narrow size distribution, and ability to transport through biological barrier $^{92,157}$. However, the drug release kinetics as well as drug loading could be improved by increasing the size of the nanoparticles $^{158}$. It was found in our preliminary study that spray dried NC sized between 100 to 500 nm, therefore our optimization goal was set to maximize EE% while minimize size within this range.

Preparation of IND loaded oily core nanocapsules suspension

IND loaded oily core nanocapsules were prepared by an adopted emulsion-diffusion technique previously described by Quintanar-Guerrero et al $^{146}$. First, mutually saturated deionized water and ethyl acetate were prepared. PLA was dissolved in 10 ml of water saturated ethyl acetate, and meanwhile Pluronic F127 was dissolved in 40 ml of ethyl acetate saturated water. Then, different amount of oil containing 10 mg of IND was added to the above water saturated ethyl acetate. The resulting organic phase was then poured into the water phase and emulsified with a homogenizer (IKA ULTRA-TURRAX T-25, Staufen, Germany) at 9,500 rpm for 10 min in which the oil-in-water emulsion was formed. Then, by adding deionized water (200 ml) to the emulsion under gentle stirring, ethyl acetate was extracted from the emulsion droplet, thus facilitating the precipitation and formation of NC.

Spray-drying of the IND loaded oily core nanocapsules
NC powder was obtained by spraying the emulsion in previous step (adding 3% w/v α-Lactose (7.5 g in 250 ml nanosuspension) as adjuvant) through the nozzle of a Bushi Mini Spray Dryer Model B290 (Buchi Laboratoriums - Technik AG, Flawil, Switzerland). The process variables were set as follows: inlet temperature 145 °C, feed flow rate 10ml/min, aspiration rate 100%, atomizing air flow 30 mmHg (357 L/h). The solid NC that had precipitated into the bottom collector (57–63°C outlet temperature) were collected and kept at room temperature for future testing and evaluation.

Particle size determination

The particle size and size distribution of IND loaded NC was measured by Dynamic light scattering (DLS, Brookhaven Instruments Corporation, Austin, TX, USA). DLS is a non-invasive, well-established technique for measuring the size of particles at nanometer scale. The spray dried powder was redispersed in deionized water and sized at temperature of 25°C. The measurements were taken 3 times. The polydispersity index (PI) is given by the following equation:

$$PI = \frac{K_2}{K_1^2}$$

(5)

Where $K_1$ is an effective mean diffusion coefficient while $K_2$ describes the relative width of the size distribution if normalized by $K_1^2$.

According to National Institute Standard, polydispersity index (PI) < 0.05 was considered monodispersed.

Encapsulation efficiency
The encapsulation efficiency, (EE%), was measured at wavelength of 318 nm by UV spectrometer (Spectronic Genesys 10 Bio, Thermo Electron Corporation, WI, USA). The standard curve was prepared using drug concentration ranging from 2.5~30 µg/ml, and had a regression equation of \( y = 0.1697x + 0.0201 \), with \( R^2 = 0.9989 \). In each of the 15 samples, EE% was measured by separating the aqueous phase with the colloidal one after centrifuge at 14,000 rpm for 30 min (VWR micro 18R, VWR Inc., West Chester, USA). The encapsulation efficiency of the drug loading was calculated using equation below,

\[
\text{Encapsulation efficiency (\%)} = \left( \frac{A_T - A_F}{A_T} \right) \times 100
\]

In which \( A_T \) is the total drug amount, and \( A_F \) is the nonencapsulated drug amount.

Scanning electron microscopy

Scanning electron microscopy (SEM) technique was used to assess the morphology of NC. Selected samples were analyzed by SEM for their surface morphology. The spray dried samples were sonicated for 15 min to disperse the particle and sugar. Samples were then pipetted onto a Nucleapore filter membrane and vacuum deposited. The membrane was mounted on 1/2" SEM stubs with double-sticky carbon tape and secured with Ag paint around the perimeter of the membrane to the stub. The samples were sputter coated (Emitech EMS575SX) with ~20 nm of Pt. The SEM picture was taken on a Hitachi S4700 Cold-cathode Field Emission Scanning Electron Microscope (Hitachi High-tech cooperation, Tokyo, Japan).

Transmission electron microscopy

Cryo-ultramicrotomy TEM was performed in order to provide visual evidence of the oily core structure. The spray dried samples were mixed with a small amount of 2.3M sucrose to form
a paste. A small amount of paste was placed on a cryo pin then placed in liquid nitrogen until frozen, and carefully transferred to the cryo box and locked in place. The samples were sectioned at a temperature between -109 °C and -115°C with the sample thickness set between 65nm and 75nm using diatome cryo-immuno diamond knife. Cryo sections were picked up and placed onto a 200 mesh copper grid with a carbon support film. After water was wicked off, the dried grids were examined in the JEOL 1400 TEM (JEOL Inc., Peabody, USA).

Density gradient centrifugation

Density gradient centrifugation was performed according to previous method. 200 µl of NC and control NP emulsion were centrifuged in 3.8 ml of density gradient media using Beckman L8-M Ultracentrifuge (Beckman-Coulter Inc., Brea, USA) with SW55Ti rotor under 21,319 g for 1 hour. A colloidal silica gradient was prepared using 54% (v/v) Percoll® media in 0.15M sodium chloride (with initial density 1.074g/cm³), and different gradients were generated in situ after centrifuge. The refractive index of the Percoll® gradients where NC and NP settled was measured under 20°C using a refractometer (Auto Abbe 10500 Refractometer, Reichert Analytical Instruments, Depew, USA). The refractometer was calibrated using pure water according to instrument manual. The standard correlation of Percoll® gradient density (d, g/cm³) in 0.15 M sodium chloride and refractive index at 20°C (nD²⁰) was provided by GE Healthcare (GE Healthcare Instructions 28-9038-34 AB) as d=6.5505×nD²⁰−7.7365, R²=0.9995. The density of NC and NP was therefore calculated using this equation.

Statistical analysis
Polynomial equations of the response values of Size ($Y_1$) and EE ($Y_2$) were derived from the total result of the 15 runs in the 3-factor 3-level Box-Behenken design. Analysis of variance (ANOVA) was performed to ensure the model fit. Formulation variables that significantly affect size and EE% were identified through Pareto chart. A theoretical optimum condition was obtained by setting the maximum desirability of minimum size and maximum EE%.

After obtaining the polynomial equation, a checkpoint analysis was performed to further confirm the model validation. Three random points (0, -0.5, 0.5); (0.5, 0, -0.5); (-0.5, 0.5, 0) as well as the theoretical optimum point (1, -0.1812, -0.0036) were selected. These checkpoints were prepared in triplicates and check for size and EE% to ensure reproducibility.

Powder X-ray diffraction analysis

Powder XRD analyses of spray dried NC were conducted using a MiniFlex automated X-ray diffractometer (Rigaku, The Woodland, Texas, USA) at room temperature. Ni-filtered Cu Ka radiation was used at 30 kV and 15 mA. The diffraction angle covered from $2\Theta = 5^\circ$ to $2\Theta = 40^\circ$ a step size of 0.05°/step and a count time of 3 sec/step (effectively 1°/min). The samples were under test for 35 minutes for the scan. The diffraction patterns were processed using Jade 8+ (Materials Data, Inc., Livermore, CA).

In vitro release study

The in vitro release study was conducted in a water bath (BS-06 Lab. Companion, Jeio Tech Co., LTD, Seoul, Korea) under 37 °C, shaking speed 60 rpm. 50 milligram of spray dried powder was redispersed in 1ml of 0.1M PBS (pH=7.4), and enclosed in a dialysis bag (MW cut-off 12,400, Sigma-Aldrich, St. Louis, MO, USA), which was immersed in 40 ml of PBS buffer at
pH=7.4. At predetermined time interval, 1 ml of buffer solution outside the dialysis bag was removed and replaced with 1ml of fresh buffer. The released amount of IND was measured by UV spectrometer at 318 nm. The standard curve was prepared using the same method in previous section. All samples were done in triplicates.

$2^{4-1}$ Fractional factorial design for optimizing process variables

In this study, four process variables for spray drying, namely, inlet temperature, feed flow rate, atomizing air flow and aspiration rate were selected at their low (coded -1) and high (coded +1) levels with triplicate centerpoints. The two-factor interactions investigated ($X_1X_2$, $X_1X_3$, and $X_1X_4$) are chosen based on preliminary results. The centerpoints provide a check for curvature and a measure of process stability and inherent variability $^{126b}$. Therefore, it’s recommended to add approximately 3 to 5 centerpoints to a full or fractional factorial design $^{156}$. The independent and dependent variables and their coded factors are listed in Table 7. The equation obtained by statistical software (JMP 8, SAS Institute) was as follows:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4$$  \hspace{1cm} (7)

Where $Y_i$ was the dependent variable; $X_1$ through $X_4$ were independent variables; $b_0$ was the intercept with y axis; and $b_1$ through $b_{14}$ were regression coefficients. In FFED, every two-factor interaction is aliased with another two-factor interaction, and those relationships are $X_1X_2=X_3X_4$, $X_1X_3=X_2X_4$, and $X_2X_3=X_1X_4$ $^{161}$. In this study, the independent variables were chosen based on preliminary experiments.
Table 7. Variables and their Levels in $2^{4-1}$ Design.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_{A1}$=inlet temperature ($^\circ$C)</td>
<td>Low: 140</td>
</tr>
<tr>
<td>$X_{A2}$=feed flow rate (L/h)</td>
<td>0.18</td>
</tr>
<tr>
<td>$X_{A3}$=atomizing air flow (L/h)</td>
<td>450</td>
</tr>
<tr>
<td>$X_{A4}$=aspiration rate (%) $^a$</td>
<td>80</td>
</tr>
<tr>
<td>Coded values ($X_1$, $X_2$, $X_3$, $X_4$)</td>
<td>-1</td>
</tr>
</tbody>
</table>

Dependent Variables

$Y_1$= Particle size (nm)

$Y_2$= Yield (%)

$^a$ Aspiration rate represented the percentage of the maximum drying gas flow (35,000 L/h) of the Buchi B 290 spray dryer.

Assessment of process yield and powder flowability

The process yield ($Y_2$, %) was calculated by the following equation:

$$Yield \, (%) = \frac{W_r}{W_i} \times 100$$  (8)

Where $W_r$ was the weight of spray dried powder collected and $W_i$ was the weight of total solids in the dispersion before spray drying.
The powder flowability was characterized via Carr’s index and Hausner ratio through published method\textsuperscript{162}. Briefly, the bulk density was measured by placing approximately one gram of powder under gravity into a calibrated measuring cylinder and record the volume occupied. The tapped density was further measured following established method by tapping the measuring cylinder on a wooden platform with an approximate amplitude of 20 mm until no further change in powder volume was observed\textsuperscript{163}. Carr’s index and Hausner ratio were calculated through:

\[
\text{Carr’s Index (\%)} = \frac{(\text{Tapped Density} - \text{Bulk Density})}{\text{Tapped Density}} \times 100
\]

\[
\text{Hausner Ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}
\]

3.3 Result and Discussion

Statistical analysis and optimization of formulation variables

Table 8 describes the responses obtained with the Box Behnken design for the particle size ($Y_1$), and the encapsulation efficiency ($Y_2$), with additional information on polydispersity index (PI).

These data show that NC are indeed formed with the particle size ranging from 208 to 504 nm. NC with similar size range was reported in references\textsuperscript{146, 155a}. It is noted that nanosuspension prior to spray drying had a size range from 280 to 568 nm. The particle size and size distribution is rarely the same as the original droplet. Higher available input energy levels in the spray drying may decrease the mean size of the droplet\textsuperscript{164}. The range of PI of the sample is from 0.005 to 0.352. Except from sample 2, 6, 7, 13, other 11 samples showe relatively large polydispersity, which may be due to formation of particles with different size as shown in SEM image (Figure 4).
Table 8. Polynomial equations of the response values of Size ($Y_1$) and EE% ($Y_2$); $X_1$ was for amount of PLA, $X_2$ was for amount of oil, $X_3$ was for amount of F 127.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$Y_1$ (nm)</th>
<th>PI</th>
<th>$Y_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>216.2</td>
<td>0.246</td>
<td>79.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>308.7</td>
<td>0.019</td>
<td>93.3</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>374.5</td>
<td>0.352</td>
<td>94.5</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>386.4</td>
<td>0.206</td>
<td>65.3</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>0</td>
<td>1</td>
<td>208.6</td>
<td>0.275</td>
<td>64.7</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>463.8</td>
<td>0.005</td>
<td>94.3</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>380.6</td>
<td>0.019</td>
<td>90.2</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>-1</td>
<td>1</td>
<td>328.3</td>
<td>0.276</td>
<td>79.2</td>
</tr>
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<td>9</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>258.6</td>
<td>0.206</td>
<td>88.3</td>
</tr>
<tr>
<td>10</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>464.8</td>
<td>0.308</td>
<td>85.7</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>338.4</td>
<td>0.210</td>
<td>81.5</td>
</tr>
<tr>
<td>12</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>429.9</td>
<td>0.336</td>
<td>69.4</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>504.4</td>
<td>0.005</td>
<td>65.5</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>273.5</td>
<td>0.299</td>
<td>78.6</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>366.1</td>
<td>0.224</td>
<td>94.6</td>
</tr>
</tbody>
</table>
Figure 4. Scanning electron microscopy image of IND-NC (Run No.6 in Table 7). Scale bar set at 500 nm.

The nonencapsulated poorly water soluble drug may have also precipitated out leading to crystal formation. Based on the analysis of the data of 15 runs of the experiments, the polynomial equations for both response values are:

\[ Y_1 = 276.03 + 8.50X_1 + 42.74X_2 - 62.66X_3 - 24.85X_1X_2 + 66.15X_1X_3 + 4.37X_2X_3 + 0.59X_1^2 + 67.82X_2^2 + 76.87X_3^2 \]  \hspace{1cm} (11)

\[ Y_2 = 79.73 + 8.08X_1 - 8.09X_2 - 4.54X_3 + 3.98X_1X_2 + 5.22X_1X_3 + 3.71X_2X_3 + 7.18X_1^2 - 1.60X_2^2 - 2.05X_3^2 \]  \hspace{1cm} (12)

Where the coded independent factors are \( X_1 \) for amount of PLA 208 (mg), \( X_2 \) for volume of Labrafac CC (ml), \( X_3 \) for amount of F127 (mg). \( Y_1 \) is for particle size (nm), and \( Y_2 \) is for EE (%).

The result from ANOVA analysis is given in Table 9. For model validation of drug loaded NC system, the P value obtained from ANOVA should be no more than 0.05, and
correlation coefficient ($R^2$) should be greater than 0.9\textsuperscript{165}. For $Y_1$ and $Y_2$, the P values are less than 0.05, indicating that the model adequately fit the data. The correlation coefficients ($R^2$) for equations (11) and (12) are 0.906 and 0.950, respectively, indicating that the sum of square of the model accounts for over 90% of the total sum of square. In both equations, the coefficient of the interaction terms ($X_1 X_2$, $X_1 X_3$, $X_2 X_3$, $X_1^2$, $X_2^2$, $X_3^2$) show how the response values change when two variables simultaneously change. The positive signs of the coefficient indicate positive effect on size or EE% while the negative signs show a negative effect on those responses. In the study of $Y_1$, size increases with $X_2$ and $X_1$ amount, while the amount of $X_3$ has a negative effect on the NC size.

Table 9. ANOVA analysis for both responses. SS: sum of square, MS: mean sum of square, DF: degree of freedom, F ratio=Model MS/Residual MS, P value: the area under F distribution curve to the right of tabulated critical F value.

<table>
<thead>
<tr>
<th>Response</th>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_1$ (Size)</td>
<td>Model</td>
<td>103060.3</td>
<td>9</td>
<td>11451.1</td>
<td>5.4</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>10641.3</td>
<td>5</td>
<td>2128.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>113701.6</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Y_2$ (EE%)</td>
<td>Model</td>
<td>1668.4</td>
<td>9</td>
<td>185.4</td>
<td>11.1</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>83.1</td>
<td>5</td>
<td>16.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1751.5</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In order to find out the significant variables in both equations, two Pareto charts are constructed in Figure 5 and 6, which indicate the main effect of the independent variables and interactions that will exert significant influence on Y\(_1\) and Y\(_2\). The values on the x-axis of Pareto charts of Figure 5 and 6 are the so-called standardized effects, which are in fact the t ratio values. These t ratios are obtained based on the estimate of factor effect \(E_x\), which are the coefficients in equation (11) and (12) and

\[
t = \frac{|E_x|}{SE_e}
\]  

(13)

Where SE\(_e\) is standard error of an effect. The obtained t value is compared to a tabulated critical t value (\(t_{\text{critical}}=2.571\), as shown in the vertical line in Pareto chart). Critical t value is associated with the residual degree of freedom (residual df=number of runs-number of terms-1, which is df=15-9-1=5), and determined at significance level \(\alpha=0.05\). In Fig. 5 and 6, the absolute t value of the factors whose length of the chart pass the vertical line (\(t_{\text{critical}} \) at P>0.05) are significant on response values.

![Table](image)

Figure 5. Pareto chart shows the standardized effect of formulation independent variables and their interaction on particle size. X axis shows the t ratio of the variables. Bars extending past the line indicate values reaching statistical significance (\(\alpha=0.05\)) \(X_1\) : PLA amount, \(X_2\) : Oil amount, \(X_3\) : F127 amount.
Figure 6. Pareto chart shows the standardized effect of formulation independent variables and their interaction on EE. X axis shows the t ratio of the variables. Bars extending past the line indicate values reaching statistical significance (α=0.05) X_1 : PLA amount, X_2 : Oil amount, X_3 : F127 amount.

For a nanocapsule system which constitutes a discontinuous phase and a continuous phase, an important parameter that describes droplet deformation is the Weber number, $W_e$; which gives the ratio of the external stress $G\eta$ (where $G$ is the shear stress, and $\eta$ is the viscosity) over the interfacial tension $\gamma$.

$$W_e = \frac{G\eta R}{2\gamma} \quad (14)$$

Where $R$ is the radius of the droplet. Rewriting equation (14) gives:

$$R = \frac{2We\gamma}{G\eta} \quad (15)$$

The deformation of the droplet increases with increase in the Weber number, which means in order to get small droplet, one need high stress. Figure 7 shows responses of size and EE while changing the level of different formulation variables. In Figure 7A, the size of the NC slightly increases with more polymer in the organic phase, however, this effect is insignificant, which corresponds to previous report that increasing polymer amount has no significant effect on particle size \textsuperscript{147a}. The effect of oil amount on particle size is shown in Figure 7B. The increase of
oil amount increases viscosity value \( \eta \), which increases Weber number and results in high deformation, so size starts to decrease. But if the oil amount continued to increase, an increase in particle size is observed. This may be explained by change in the interfacial tension. Defay et al. provided a thermodynamic model for monolayer adsorption at the interface between two immiscible liquids, which could be extended to a linear mixing model based on the linear combination of mole fractions for individual components \(^{169}\):

\[
\gamma = \sum \gamma_i \chi_i
\]  

(16)

Where \( \gamma_i \) was the interfacial tension of each component and \( \chi_i \) was their mole fraction in the system.

Figure 7. Prediction and desirability plot showing the effect of polymer amount (X1), oil amount (X2) and F127 amount (X3) on the particle size and EE of formulation. The coding of the variables (PLA=1, Oil=-0.1812, PLA=-0.00355) represents the optimized formulation (300mg of PLA, 0.52 ml of oil, and 239.62 mg of F127).
Since the o/w interfacial tension of oil is greater than that of ethyl acetate (30 mN/m compared to 6.8 mN/m\(^\text{170}\), increase mole fraction of oil will increase overall interfacial tension, and if this effect overwhelms the effect of viscosity increase, an overall decrease in Weber number as well as deformation can be observed, which can lead to size increase. This phenomenon was also reported by Heurtault et.al.\(^\text{171}\). Figure 7C reveals the effect of surfactant on polymer size. Surfactants play a major role in the formation of nano-emulsions, by lowering the interfacial tension $\gamma$, therefore giving greater Weber number and greater deformation. However, the ability of surfactant to reduce the interfacial tension depends on the surfactant concentration as well as the surface excess (number of moles adsorbed per unit area of the interface)\(^\text{168}\), which was given by:

$$-d\gamma = RTd\ln C$$

(17)

Where R is the gas constant, $T$ is the absolute temperature and $\Gamma$ is the surface excess. This could explain the fact that size of NC increased with increase of F127 concentration after certain point (as shown in Figure 7C), because $\Gamma$ increases with surfactant concentration and eventually reaches saturation. After this point, more oil is solublized into micelles, which also increases with F127 concentration, appears to surpass the effect of reducing interfacial tension. This will enhance Ostwald ripening, which leads to condensation of all droplets into forming bigger droplets\(^\text{172}\).

Variables influencing the encapsulation efficiency (EE%) can be derived from equation (12). Higher EE% is achieved by higher amount of polymer and less amount of oil, as shown in Figure 7D,E, which is in accordance with previous research, where encapsulation increases with the feed drug concentration\(^\text{173}\). Because IND is a hydrophobic drug, it may diffuse through the capsule during the diffusion of ethyl acetate. Higher amount of polymer may slow down the
diffusion, thus increases the amount of drug entrapped. Also, by increasing the oil amount, the diameter of NC also increases, thus reducing the total surface of the oily core. Previously a correlation was observed between maximal encapsulation of hydrophobic drug and the total surface of oily core,¹⁷⁴ which suggested lower oil amount would have higher EE%.

**Figure 8 A and B** show the three dimensional response surface of the interactions between formulation variables that have a significant impact. The effects of PLA and F 127 amount and their interaction on size are shown in **Figure 8A** (P=0.03).

![Figure 8 A](image)

**Figure 8 A.** The response plot showing effects of PLA amount ($X_1$) and F 127 amount ($X_3$) on response, size ($Y_1$).

At low polymer amount, size significantly decreases with increased amount of F 127. However, at high polymer amount, increase in F 127 amount beyond 240 mg in the aqueous phase leads to an increase in particle size. This may be attributed to the higher level of micelles formation described, which can lead to the formation of bigger particles. The effect of factors (PLA and oil
amount) on EE% is shown in Figure 8B. At high F127 amount, EE% decreases significantly with less PLA amount. This may be due to the less capability of preventing drug leakage during the preparation. It is also noted that under low polymer amount, EE% significantly decreases with higher amount of F127. Since in this case higher amount of F127 is related to smaller size of NC, it is contradicted with previous discussion that more hydrophobic drug will be encapsulated into NC if total surface of oily core is increased. Same contradiction was also observed in previous reports.

![Figure 8B](image)

Figure 8 B. The response plot showing effects of PLA amount ($X_1$) and F 127 amount ($X_3$) on response, EE% ($Y_2$).

This remains to be clarified by further experiment as to whether hydrophobic drug is encapsulated into the oily core or stayed at the surface of the oily core. The relationship between independent variables and response values are further investigated by constructing prediction and
desirability plot. The concept of desirability function in optimization was described by Derringer and Suich. Our goal is to minimize the size of NC meanwhile having the maximized EE% since the size observed is above 200 nm. The desirability functions are shown below:

\[
\begin{align*}
    d_r^{\text{max}} &= \begin{cases} 
    0 & f_r(x) < A \\
    \frac{f_r(x) - A}{B - A} & A \leq f_r(x) \leq B \\
    1 & f_r(x) > B 
    \end{cases} \\
    d_r^{\text{min}} &= \begin{cases} 
    0 & f_r(x) > B \\
    \frac{f_r(x) - B}{A - B} & A \leq f_r(x) \leq B \\
    1 & f_r(x) < A 
    \end{cases} \\
    D &= \left( \prod_{r=1}^{R} d_r \right)^{\frac{1}{R}}
\end{align*}
\]  

Where \(d_r^{\text{max}}\) and \(d_r^{\text{min}}\) are desirability function of EE (maximize) and size (minimize), respectively. D is the total desirability of size and EE. In eqn.18, A=60 and B=95 (%), while in eqn.19, A= 200 and B=550 (nm). Since there’s only two dependent variables, R=2 in eqn.20. The calculated \(d_r\) and D values are shown in Table 10, and a maximized D value is generated by JMP 8.0. In Figure 7 predicted optimized condition is translated as follow: 300 mg of PLA, 0.56 ml of oil, and 239.57 mg of F127. The predicted size is 284.1nm and EE% is 95.7% with a desirability (d=0.86) which is relatively close to the ideal value of 1.

Based on polynomial equation (11) and (12), a check point analysis is performed to evaluate \(Y_1\) and \(Y_2\), as shown in Table 11. Results indicate that the measured values are approximately the same as predicted, as the differences between measured and predicted values are found to be insignificant (P>0.05) using Student’s t-test. Therefore, it can be concluded that these equations fit the data satisfactorily, and are valid for predicting size and EE%.
Table 10. Calculated partial and overall desirability values based on eqn.18-20.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Size (nm)</th>
<th>EE (%)</th>
<th>d size (d min)</th>
<th>d EE (d max)</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>216.2</td>
<td>79.0</td>
<td>0.95</td>
<td>0.54</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>308.7</td>
<td>93.3</td>
<td>0.69</td>
<td>0.95</td>
<td>0.81</td>
</tr>
<tr>
<td>3</td>
<td>374.5</td>
<td>94.5</td>
<td>0.50</td>
<td>0.99</td>
<td>0.70</td>
</tr>
<tr>
<td>4</td>
<td>386.4</td>
<td>65.3</td>
<td>0.47</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>5</td>
<td>208.6</td>
<td>64.7</td>
<td>0.98</td>
<td>0.13</td>
<td>0.36</td>
</tr>
<tr>
<td>6</td>
<td>463.8</td>
<td>94.3</td>
<td>0.25</td>
<td>0.98</td>
<td>0.49</td>
</tr>
<tr>
<td>7</td>
<td>380.6</td>
<td>90.2</td>
<td>0.48</td>
<td>0.86</td>
<td>0.65</td>
</tr>
<tr>
<td>8</td>
<td>328.3</td>
<td>79.2</td>
<td>0.63</td>
<td>0.55</td>
<td>0.59</td>
</tr>
<tr>
<td>9</td>
<td>258.6</td>
<td>88.3</td>
<td>0.83</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>10</td>
<td>464.8</td>
<td>85.7</td>
<td>0.24</td>
<td>0.73</td>
<td>0.42</td>
</tr>
<tr>
<td>11</td>
<td>338.4</td>
<td>81.5</td>
<td>0.60</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>12</td>
<td>429.9</td>
<td>69.4</td>
<td>0.34</td>
<td>0.27</td>
<td>0.30</td>
</tr>
<tr>
<td>13</td>
<td>504.4</td>
<td>65.5</td>
<td>0.13</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>14</td>
<td>273.5</td>
<td>78.6</td>
<td>0.79</td>
<td>0.53</td>
<td>0.65</td>
</tr>
<tr>
<td>15</td>
<td>366.1</td>
<td>94.6</td>
<td>0.53</td>
<td>0.99</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 11. Checkpoint experiments comparing measured and predicted response values (n=3)

<table>
<thead>
<tr>
<th>Run No.</th>
<th>X₁</th>
<th>X₂</th>
<th>X₃</th>
<th>Y₁</th>
<th>Cal. Y₁</th>
<th>% Error</th>
<th>P value</th>
<th>Y₂</th>
<th>Cal.Y₂</th>
<th>% Error</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0</td>
<td>-0.5</td>
<td>0.5</td>
<td>254.5±2.9</td>
<td>258.4</td>
<td>-1.5</td>
<td>0.25</td>
<td>77.4±1.3</td>
<td>79.7</td>
<td>-2.9</td>
<td>0.15</td>
</tr>
<tr>
<td>C2</td>
<td>0.5</td>
<td>0</td>
<td>-0.5</td>
<td>318.5±2.1</td>
<td>314.4</td>
<td>1.3</td>
<td>0.12</td>
<td>87.5±1.6</td>
<td>86.0</td>
<td>1.7</td>
<td>0.40</td>
</tr>
<tr>
<td>C3</td>
<td>-0.5</td>
<td>0.5</td>
<td>0</td>
<td>320.3±1.8</td>
<td>316.5</td>
<td>1.2</td>
<td>0.10</td>
<td>74.4±0.8</td>
<td>72.0</td>
<td>3.3</td>
<td>0.25</td>
</tr>
<tr>
<td>C4</td>
<td>1</td>
<td>-0.1812</td>
<td>-0.0036</td>
<td>281.0±2.4</td>
<td>284.1</td>
<td>-1.1</td>
<td>0.19</td>
<td>93.4±1.0</td>
<td>95.7</td>
<td>-2.4</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Physicochemical characterization of the oily core nanocapsules

**Figure 5** shows the SEM images of IND-loaded NC. The particles are observed to have a smooth spherical surface and the size observed is correlated well with the data measured by DLS. In transmission electron microscope (TEM), the electron beam’s interaction with the specimen under TEM is like the light beam with a microscope slide in a projector. Areas of the specimen which block the beam cause fewer electrons to be transmitted to the camera, causing a dark field of view, which can be due to denser or thicker material, and lighter areas tend to be either less denser or thinner or both. **Figure 9 A** and **B** show the TEM images of sectioned NC and NP, exhibiting electron density difference, which may contribute to the oily core nature of NC. Same morphology pattern of oily core NC has also been reported by previously published literature.  

![Figure 9 A Cryo-ultramicrotomy TEM image of IND-NC (Run No. 6 in Table 7)](image)
The result from the density gradient provides further evidences on NC formation by the proposed method. NP have a significantly higher density and can be easily distinguished from that of NC. Various research groups have used the same method to characterize the NC density. Refractive index is one of the basic physical properties of pharmaceutical solids. Studies have already used refractive index measurement to assess the density of density-gradient medium. The measured refractive indexes of NC/NP layer are $n_D^{20} = 1.3437$ and $n_D^{20} = 1.3606$, respectively. The calculated density is 1.065 g/cm³ for NC and 1.176 g/cm³ for NP, which is consistent with previous findings. Together, the analysis of electron microscope and density gradient confirm the formation of NC.

Figure 10 show the diffraction patterns of the IND-NC and the individual components by powder XRD. Characteristic diffraction peaks for indomethacin are observed at $2\theta = 11.7^\circ$, $16.7^\circ$, ...
19.7°, 21.9°, and 26.7°, which is consistent with previous IND crystallography\textsuperscript{180}. The characteristics peaks of α-lactose are 12.5° and 16.4°, and those of Pluronic F127 are 18.8° and 23.1°. Similar crystallographic characteristics of Pluronic F127\textsuperscript{181} and α-lactose\textsuperscript{182} are observed compared to previous papers. The PLA crystallography observed has diffraction in the 2θ range from 36° to 38°, suggesting crystallite formation\textsuperscript{183}. The XRD patterns also clearly showed the characteristic peaks of IND are absent in IND-NC with different polymer to oil ratio, suggesting the drug is completely amorphous within the NC.

Figure 10 Powder X-ray diffraction (Cu K\textsubscript{α} radiation) pattern for IND-NC and individual component in the formulation: IND\textsubscript{-NC} 3 (High polymer to oil ratio 300:567 w/w); IND-NC 10 (Low polymer to oil ratio 100:567 w/w); IND\textsubscript{-NC} 11 (Medium polymer to oil ratio 200:567 w/w). Absence of characteristic peaks of IND in NC was shown (peaks 1 through 5).

\textit{In vitro} drug release kinetics
The effect of different formulation variables on IND release kinetics are shown in Figure 11 A through C. A sustained release profile of IND is observed over a period of 72 hr. Similarity factor is calculated using following equations:

\[
\begin{align*}
    f_1 &= \left( \frac{\sum_{i=1}^{n} |R_i - T_i|}{\sum_{i=1}^{n} R_i} \right) \times 100 \\
    f_2 &= 50 \times \log \left\{ 1 + \frac{1}{n} \sum_{i=1}^{n} \left( R_i - T_i \right)^2 \right\}^{-0.5} \times 100
\end{align*}
\] (21) (22)

Where R_t and T_t are release amount of reference formulation and test formulation at time t. \( f_1 \) (difference factor) should <15 and \( f_2 \) (similarity factor) should > 50 to ensure sameness and equivalence of two release profiles \(^{184}\).
Figure 11 Release profile of different formulation conditions (n=3). (a): High vs. low polymer amount (300mg, run No.2 vs.100mg, run No.9); (b): High vs. low oil amount (0.8ml, run No.4 vs.0.4ml, run No.8); (c): High vs. low surfactant amount (360mg, run No.3 vs.120mg, run No.15); (d): IND-NC (optimized formulation) vs. IND-NP.

Although similarity factor results indicate that none of these differences is statistically significant (data not shown), one can obtain from the release profile that higher polymer to oil ratio as well as higher surfactant amount will result in better sustained release. The release rate of IND from optimized NC formulation is significantly decreased compared to IND nanosphere control according to similarity factor analysis ($f_1=51.86$, $f_2=29.50$), and release profiles are compared in Figure 11D.

The Ritger-Peppas equation is used to elucidate the release mechanism:\(^{185}\):

$$\frac{M_t}{M_\infty} = k_p t^n$$

(23)

Where $M_t/M_\infty$ is the fraction of drug released, $t$ is the release time, and $k_p$ is kinetic constant incorporating different factors of the release device, $n$ is the release exponent explaining the mechanism of the release.

Rewriting this equation gives:

$$\log \frac{M_t}{M_\infty} = n \log t + \log k_p$$

(24)

By determining the release exponent $n$, one can tell the physical mechanism controlling drug release from a particular device, which can be classified as Fickian diffusion ($n=0.5$), Case II transport ($n=1$), Anomalous transport ($0.5<n<1$) and Super case II transport ($n>1$)\(^{186}\). Figure 12 shows the model fit of the release profile of optimized NC formulation to equation (24). Table 12 describes estimated parameters obtained from fitting release profile to Ritger-Peppas

59
model. When polymer amount is high, drug release is more dependent on polymer chain relaxation, and appears to be Case II and Super case II kinetics. When 0.5<n<1, drug release is the combinative effect of Fickian diffusion and polymer chain relaxation. Similar release pattern and mechanism of biodegradable polymers are also reported \(^{187}\).

Figure 12 Model fit of release profile of optimized IND-NC formulation to equation 24.
Table 12. Estimated parameters obtained from fitting release profile to Ritger-Peppas model.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio of tested</th>
<th>Correlated equation</th>
<th>n value</th>
<th>$R^2$</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Low polymer</td>
<td>100mg vs. 300mg</td>
<td>$y=0.919x+0.339$</td>
<td>0.919</td>
<td>0.945</td>
<td>Anomalous</td>
</tr>
<tr>
<td>High polymer</td>
<td></td>
<td>$y=1.032x+0.192$</td>
<td>1.032</td>
<td>0.935</td>
<td>Super case II</td>
</tr>
<tr>
<td>B. Low oil</td>
<td>0.4ml vs. 0.8ml</td>
<td>$y=0.544x+0.989$</td>
<td>0.544</td>
<td>0.980</td>
<td>Anomalous</td>
</tr>
<tr>
<td>High oil</td>
<td></td>
<td>$y=0.574x+0.965$</td>
<td>0.574</td>
<td>0.965</td>
<td>Anomalous</td>
</tr>
<tr>
<td>C. Low surfactant</td>
<td>120mg vs. 360mg</td>
<td>$y=0.476x+0.782$</td>
<td>0.476</td>
<td>0.948</td>
<td>Fickian</td>
</tr>
<tr>
<td>High surfactant</td>
<td></td>
<td>$y=0.383x+1.308$</td>
<td>0.383</td>
<td>0.979</td>
<td>Fickian</td>
</tr>
<tr>
<td>Nanosphere</td>
<td></td>
<td>$y=0.353x+1.386$</td>
<td>0.353</td>
<td>0.975</td>
<td>Fickian</td>
</tr>
<tr>
<td>Optimized NC</td>
<td></td>
<td>$Y=0.562x+0.983$</td>
<td>0.562</td>
<td>0.984</td>
<td>Anomalous</td>
</tr>
</tbody>
</table>

Statistical analysis and optimization of spray drying process variables
Table 13 describes the responses obtained with the $2^{4-1}$ design for the particle size ($Y_1$), and the yield ($Y_2$), with additional information on polydispersity index (PI). This data show that NC are indeed formed with the particle size ranging from 129.5 to 444.8 nm.

Table 13. Response values of Size ($Y_1$) and Yield ($Y_2$).

<table>
<thead>
<tr>
<th>Run No.</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$X_4$</th>
<th>$Y_1$ (nm)</th>
<th>PI</th>
<th>$Y_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>150.3</td>
<td>0.199</td>
<td>19.7</td>
</tr>
<tr>
<td>2</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>264.0</td>
<td>0.291</td>
<td>18.3</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>201.9</td>
<td>0.251</td>
<td>19.7</td>
</tr>
<tr>
<td>4</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>153.4</td>
<td>0.162</td>
<td>28.5</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>129.5</td>
<td>0.121</td>
<td>31.1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>149.6</td>
<td>0.148</td>
<td>22.7</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>151.7</td>
<td>0.211</td>
<td>23.5</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>153.8</td>
<td>0.200</td>
<td>21.4</td>
</tr>
<tr>
<td>9</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>167.4</td>
<td>0.231</td>
<td>31.1</td>
</tr>
<tr>
<td>10</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>208.6</td>
<td>0.157</td>
<td>14.1</td>
</tr>
<tr>
<td>11</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>444.8</td>
<td>0.304</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Note: $X_1$ through $X_4$ represented coded values for process parameters. $X_1$ was for inlet temperature, $X_2$ was for feed flow rate, $X_3$ was for atomizing air flow, and $X_4$ was for aspiration rate.

Figure 13 shows a typical size distribution of the spray dried NC. The particle size ranging from 81.3 to 195.8 nm accounts for over 95% of the population. The size of the prepared NC emulsion before spray drying varies from 149.6 to 439.2 nm.
Figure 13 Multimodal size distribution curve of sample No.5 (effective mean diameter 129.5 nm, PI=0.121). The particles ranging from 81.3 to 195.8 nm accounts for over 95% of the population.

The particle size and size distribution is rarely the same as the original droplet. Higher available input energy levels may decrease the mean size of the droplet.\textsuperscript{164} Spray dried NC with similar size range were also reported in reference\textsuperscript{155a}. The yield of NC is from 14.1\% to 31.1\%. The range of PI of the sample is from 0.121 to 0.304. These 11 samples exhibit relatively large polydispersity, which may be due to aggregation of particles after preparation. Based on the analysis of the data, the equations for both response values are as follows:

\[ Y_1 = 197.73 - 31.21X_1 + 22.61X_2 + 8.79X_3 - 27.79X_4 - 54.54X_1X_2 - 32.16X_1X_3 + 52.71X_1X_4 \] (25)

\[ Y_2 = 23.24 + 0.9X_1 - 0.15X_2 + 5.55X_3 + 0.9X_4 - 0.15X_1X_2 - 0.15X_1X_3 - 1.9X_1X_4 \] (26)

Where the coded independent factors (X\textsubscript{i}), which have the value of -1, 0, or +1 are: X\textsubscript{1}= (X\textsubscript{A1}-145)/5, X\textsubscript{2}= (X\textsubscript{A2}-0.27)/0.09, X\textsubscript{3}= (X\textsubscript{A3}-525)/75 and X\textsubscript{4}= (X\textsubscript{A4}-90)/10. The actual independent factors (X\textsubscript{Ai}) are: X\textsubscript{A1} for inlet temperature, X\textsubscript{A2} for feed flow rate, X\textsubscript{A3} for atomizing air flow and X\textsubscript{A4} for aspiration rate. The key measured response values are: Y\textsubscript{1} for particle size (nm), and Y\textsubscript{2} for yield (\%).
The results from ANOVA and Lack-of-fit analysis are given in Table 14 and 15. For model validation, the P value obtained from ANOVA should be no more than 0.05, correlation coefficient (R²) should be greater than 0.9, and the P value obtained from Lack-of-fit should be no less than 0.05. For Y₂, the P values in ANOVA analysis are less than 0.05, indicating the model adequately fit the data. The correlation coefficient (R²) is 0.985, indicating that the sum of square of the model accounts for over 90% of the total sum of square. The Lack-of-fit test shows that the P value for Y₂ was >0.05, which indicates that at 95% confidence level, there is no lack of fit in the model. Therefore we accept the model prediction for Y₂. On the other hand, since the P value of Y₁ in ANOVA is greater than 0.05, we reject the model prediction for Y₁.

Table 14. ANOVA analysis for both responses.

<table>
<thead>
<tr>
<th>Response</th>
<th>Source</th>
<th>R²</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y₁ (Size)</td>
<td>Model</td>
<td>0.892</td>
<td>72978.27</td>
<td>7</td>
<td>10425.50</td>
<td>3.57</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td></td>
<td>8747.67</td>
<td>3</td>
<td>2919.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>0.892</td>
<td>10</td>
<td>81725.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₂ (Yield)</td>
<td>Model</td>
<td>0.985</td>
<td>288.80</td>
<td>7</td>
<td>41.26</td>
<td>28.88</td>
<td>0.0094</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td></td>
<td>4.28</td>
<td>3</td>
<td>1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>0.985</td>
<td>10</td>
<td>293.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Note: SS: sum of square, MS: mean sum of square, DF: degree of freedom, F ratio=Model MS/Residual MS, P value: the area under F distribution curve to the right of tabulated critical F value.

In both equations, the coefficient of the interaction terms ($X_1X_2$, $X_1X_3$, and $X_1X_4$) indicates how the response values changed when two variables simultaneously change. The positive signs of the coefficient indicate a positive effect on size or yield while the negative signs show a negative effect on those responses. In the study of $Y_2$, yield increases with $X_1$ and $X_3$ and $X_4$ settings, while the increase of $X_2$ has a negative effect on the NC yield.

Table 15. Lack-of-fit analysis for both responses.

<table>
<thead>
<tr>
<th>Response</th>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_1$ (Size)</td>
<td>Lack-of-fit</td>
<td>8738.85</td>
<td>1</td>
<td>8738.85</td>
<td>1981.60</td>
<td>0.0005</td>
</tr>
<tr>
<td>Pure error</td>
<td>8.82</td>
<td>2</td>
<td>4.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8747.67</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Y_2$ (Yield)</td>
<td>Lack-of-fit</td>
<td>2.04</td>
<td>1</td>
<td>2.04</td>
<td>1.81</td>
<td>0.31</td>
</tr>
<tr>
<td>Pure error</td>
<td>2.25</td>
<td>2</td>
<td>1.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.29</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Note: SS: sum of square, MS: mean sum of square, DF: degree of freedom, F ratio=Lack-of-fit MS/Pure error MS, P value: the area under F distribution curve to the right of tabulated critical F value.

In order to visualize the significant variables in equation (26), a Pareto chart is constructed in Figure 14, which indicates the main effect of the independent variables and interactions on $Y_2$. The values on the x-axis of the Pareto chart in Figure 14 are the so-called standardized effects, which are in fact the t values. Those values are obtained based on the estimate of factor effect $E_x$, which is the coefficient in equations (25) and (26). Then t values are calculated based on the following equation (13). The obtained t values are compared to a tabulated critical t value ($t_{critical}=3.18$, as shown in the vertical line in Pareto chart). The critical t value is associated with the residual degree of freedom (residual df = number of runs – number of terms – 1), and usually determined at significance level $\alpha=0.05$.

Figure 14 Pareto chart show the standardized effect of process parameters and their interaction on yield in equation (26). X axis shows the t ratio of the variables. Bars extending past the line indicate values reaching statistical significance ($\alpha=0.05$)

In Figure 14, the absolute t values are represented by the length of the bar and the critical t value is represented by the vertical line. Factors whose bar passes the vertical line ($t_{critical}$ at $P>0.05$) have a significant impact on response values. From Figure 14, it appears that among the
four process parameters, only atomizing air flow has a significantly positive impact on the process yield. In order to further study the influence of process parameters on yield, statistically irrelevant interaction terms ($X_1X_2$, $X_1X_3$) are omitted from the initial model to generate a reduced model. The prediction equation of reduced model for $Y_2$ is:

$$Y_2 = 23.24 + 0.9X_1 - 0.15X_2 + 5.55X_3 + 0.9X_4 - 1.9X_1X_4$$  \(27\)

The ANOVA and Lack-of-fit analysis are shown in Table 16. The $R^2$ value of the reduced model is 0.984. According to the same acceptability criteria, the results of $R^2$ value, ANOVA and Lack-of-fit indicate that omitting interaction terms does not impair the model validation. A comparison of initial and reduced models using ANOVA is shown in Table 17.

<table>
<thead>
<tr>
<th>Response</th>
<th>Source</th>
<th>$R^2$</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_2$ (Yield)</td>
<td>Model</td>
<td>0.984</td>
<td>288.44</td>
<td>5</td>
<td>57.69</td>
<td>62.09</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td></td>
<td>4.65</td>
<td>5</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.984</td>
<td>293.09</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-fit</td>
<td>2.40</td>
<td>3</td>
<td>0.80</td>
<td>0.71</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure error</td>
<td>2.25</td>
<td>2</td>
<td>1.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.65</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Note: SS: sum of square, MS: mean sum of square, DF: degree of freedom, F ratio=Lack-of-fit MS/Pure error MS, P value: the area under F distribution curve to the right of tabulated critical F value.

A Pareto chart of process parameters in reduced model is shown in Figure 15 (t_{critical}=2.57, as shown in the vertical line). It is clear from Fig. 15 that increasing inlet temperature, aspiration rate and atomizing air flow can significantly increase NC yield. These observations can be explained using the following basic operating principles of spray drying as described below.

![Pareto chart](image)

Figure 15 Pareto chart shows the standardized effect of process parameters and their interaction on yield in equation (8). X axis shows the t ratio of the variables. Bars extending past the line indicate values reaching statistical significance (α=0.05)
Table 17. Results of ANOVA of initial (Eqn 25) and reduced (Eqn 27) models for $Y_2$.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eqn (25)</td>
<td>7</td>
<td>288.80</td>
<td>41.26</td>
<td>28.88</td>
</tr>
<tr>
<td>Eqn (27)</td>
<td>5</td>
<td>288.44</td>
<td>57.68</td>
<td>62.09</td>
</tr>
<tr>
<td>Residuals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eqn (25)</td>
<td>3</td>
<td>4.28(C_1)</td>
<td>1.43(D_1)</td>
<td>p-value=0.9745</td>
</tr>
<tr>
<td>Eqn (27)</td>
<td>5</td>
<td>4.65(C_2)</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

Note: $F_{cal} = [(C_2 - C_1)/N_{TO}]/D_1=0.13$, where $N_{TO}$ was the number of terms omitted.

The investigated inlet temperature ($X_{A1}$) varies from 140 to 150 °C. Inlet temperature determines the temperature of the drying air at the contact of the feeding solution. The inlet temperature is measured before entry to the drying chamber and it has influence on the amount of solvent that can be removed per unit time. Although the inlet temperature is close to the melting point of IND (158°C), it is reasonably speculated that the drug and/or oil component is not significantly degraded for the following reasons: I) IND has been shown to be relatively stable (with only 1.56% degradation) under high temperature and after melt-quench cooling process; II) Spray drying is a recognized method for the processing of heat-sensitive materials such as proteins and enzymes. This may be explained by the fact that the actual droplet
temperature during the drying process is far lower than inlet temperature, and the exposure time is only 5-30 seconds \(^{190}\). In this study, the outlet temperature during the spray drying of the NC varies from 48 to 52°C. Moreover, it has been reported that usually the spray dried particles reach a maximum temperature which is 15-20°C below the outlet temperature \(^{149a}\). It suggests that these substances are actually briefly exposed to an even lower temperature than the one observed experimentally. The overall thermal efficiency (\(\eta_{\text{overall}}\)) is defined as the fraction of total heat supplied to the dryer used in the evaporation process and can be approximately expressed as:

\[
\eta_{\text{overall}} = \left( \frac{T_1 - T_2}{T_1 - T_0} \right) \times 100
\]

Where \(T_1\) is inlet temperature, \(T_2\) is outlet temperature, and \(T_0\) is the atmospheric temperature \(^{164}\).

It shows an increase in thermal efficiency, which results from increasing inlet temperature for fixed outlet and ambient temperature. Although the outlet temperature cannot be predetermined theoretically, it is experimentally observed during the spraying process that it is stabilized between 48 to 52°C. Overall the inlet temperature has a positive effect on the process yield, because it raises the thermal efficiency leading to effective drying of the particles. Therefore fewer particles are stuck to the drying chamber and cyclone, and yield increases.

The feed flow rate (\(X_{A2}\)) is set from 0.18 L/h to 0.36 L/h in this study. It is found in the preliminary study that higher feed rate (> 0.36 L/h) will cause insufficient drying of the solvent leading to low yield. The phenomenon remains to be fully explained. However, according to equation (29), if increased liquid feeding rate (\(V_{lf}\)) counteracts the effect of atomizing air flow rate (\(V_{aa}\)), the decreasing air/fluid mass ratio (\(n_{a/f}\)) will result in insufficient drying. In this case, water may not be fully evaporated given the relative low outlet temperature and high water content in the nanosuspension. Although it is not found to be a significant factor in the range
studied, the importance of controlling feed flow rate has been elaborated through a previous study, as shown in the equation below in which $\rho_a$ and $\rho_f$ are the density of atomizing air and feed fluid intended to be atomized:

$$n_{a/f} = \frac{V_{aa} \times \rho_a}{V_{lf} \times \rho_f}$$ \hspace{1cm} (29)

The coupling of the liquid feeding rate ($V_{lf}$) with the atomizing air flow rate ($V_{aa}$) and their influence on spray drying is defined by the air/fluid mass ratio ($n_{a/f}$) \textsuperscript{191}. The so-called air/fluid mass ratio represents the energy available for atomization, and decreasing $n_{a/f}$ will result in insufficient drying of particles \textsuperscript{192}. In this study, the difference between low and high settings of the feed flow rate appears to have a statistically insignificant effect. Therefore using the above equation (27), the energy available for atomization appeared here to be mainly dependent on atomizing air flow rate which positively impacts the process yield (Figure 14 and Figure 15). For example, indeed samples 5 and 11 (from Table 13) do have relatively high yield (31.1\% and 25.5\%), but this fact may be mainly explained by the high value of $V_{aa}$ (in equation 29) providing more effective drying energy. On the contrary, sample 10 with low $V_{aa}$ value has a low yield of 14.1\%.

The atomizing air flow ($X_{A3}$) in this study is set from 450 L/h to 600 L/h. It refers to the gas flow in the nozzle. As shown in equation (29), this provides more energy available for atomization, causing the formation of a very large surface area owing to small droplet size that is exposed to the drying gas, which increases the drying efficiency \textsuperscript{151}. The droplet drying time $\tau_d$ can be described using the following equations (30) and (31) \textsuperscript{193}:

$$\tau_d = \frac{d_0^2}{K}$$ \hspace{1cm} (30)
Where $\tau_d$ is the droplet drying time, $d_0$ is the initial diameter of the droplet, and $\kappa$ is the evaporation rate:

$$\kappa = 8D_g \frac{\rho_g}{\rho_l} (Y_s(T_e) - Y_x)$$  \hspace{1cm} (31)

Where $D_g$ is the diffusion coefficient of the gas phase, $\rho_g$ and $\rho_l$ are density of the gas and liquid phases, respectively; $Y_s$ and $Y_x$ are the mass fraction of the solvent at droplet surface and in atmosphere, respectively; and $T_e$ is the equilibrium temperature of the droplet. In a case where evaporation rate is fixed, higher atomizing air flow will help decrease initial diameter of the droplet and therefore significantly reduce drying time. Together with the effect of increasing air/liquid mass ratio as shown in equation (29), higher atomizing air flow rate will lead to effective drying of the particle and higher yield.

The aspiration air flow setting ($X_{A4}$) represents the drying gas flow rate in the spray dryer, which is the volume of drying air supplied per unit time. $X_{A4}$ is set to 80% (low level) and 100% (high level) of the maximum drying air flow (35,000 L/h) of the Buchi B 290 spray dryer according to manufacturer specifications. The higher the aspiration setting, the shorter the particle transfer time ($t_p$) will be from the nozzle to the collector. When $t_p$ is low, the interaction time between the particle and the drying gas will be relatively shorter. However, higher $X_{A4}$ setting can increase the process yield by providing higher drying air velocity. Therefore the increased centrifugal forces in the cyclone will increase cyclone efficiency, and $X_{A4}$ has a positive effect as shown in equation (27).

The interaction between aspiration air flow and inlet temperature shows a significant negative effect on the process yield. As shown in Figure 16, yield increases when inlet temperature and aspiration rate increase. But after certain point, yield starts to decrease significantly, exhibiting an overall of negative interaction effect in Figure 15.
Figure 16. The response surface plot showing effects of inlet temperature and aspiration air rate on process yield.

This may be explained by the antagonistic effect of both parameters. Increasing inlet temperature contributes to reducing droplet drying time $\tau_d$, but increasing aspiration air flow rate can shorten $t_p$. If $t_p$ is short enough to overwhelm the effect of faster drying (reduced $\tau_d$), then the moisture content in the particle will be high enough which lead to insufficient drying and forming sticky particles. These particles can be retained on the surface of drying chamber and decrease recovery of spray dried NC in the collector.

The relationship between the process variables and NC yield is further investigated by constructing a prediction and desirability plot as shown in Figure 17. The solid line indicates the
prediction of process yield (%) as a function of the selected process variables and the dotted lines around the solid lines indicate standard deviation.

Figure 17. Prediction and desirability plot showing the effect of process parameters on the yield.

The concept of desirability function in optimization is described by Derringer and Suich with a desirability on the scale of 0 to 1, where 0 is not acceptable and 1 is the perfectly desirable response. Basically the desirability was calculated as a function of the differences between fitted response value and the target value. Our goal was to maximize yield. The predicted yield is 30.8% with a desirability (d=0.90) which is close to the ideal value of 1. In Figure 17, the predicted optimized condition can be translated as follows: inlet temperature 140 °C, atomizing air low 600 L/h, feed flow rate 0.18 L/h and aspiration air flow set at 100%.

Based on the reduced equation (27), a check point analysis is performed to evaluate $Y_2$. The triplicate optimization points have an average yield of 32.5±2.4% (n=3), with a P value of 0.415 ($\alpha=0.05$) compared to calculated value.
Powder flowability and process yield

The powder flowability of spray dried NCs powder is summarized in Table 18. The bulk density and tapped density are measurements of the degree of packing or conversely the amount of space between the powder in the powder bed \(^{162}\). A Hausner ratio value less than 1.20 is indicative of good flow, whereas a value greater than 1.5 indicates poor flow. The Carr’s index value less than 25% indicates a fluid powder (good flow) whereas a value greater than 25% indicates a cohesive powder with poor flow \(^{196}\). The results in Table 18 exhibit poor powder flow, which may be due to insufficient drying related to the great amount of water added into the diffusion process. In spray drying theory, increase in solid content of the feed solution (concentration of particles in the feeding solution) usually increases the bulk density of the powder and powder flow \(^{164}\). The solid content in the feed solution tested in this study is relatively low (approximately 3.2% w/w), indicating a very high heat requirement for drying. Moreover, the powder flowability also improves with increasing particle size and greater particle sphericity \(^{164}\), as the relatively small size of NC can also contribute to poor powder flowability due to increased Van der Waals interaction. In practice, poor powder flowability is correlated with high moisture content in spray dried powder shown in previous reports \(^{197}\). With the relatively low solid content in feed solution used, there is high possibility that the moisture content is high in the spray dried NC thus suggesting a high chance of wet particle sticking to the drying chamber and the cyclone wall. This may explain the low yield in this process. Also, small particles such as those produced in this study may be much more easily carried out by Brownian motion in the drying gases, therefore bypassing the collector through the cyclone outlet and further explaining the low yield observed.
Table 1. Summary of powder flowability.

<table>
<thead>
<tr>
<th>Runs</th>
<th>Bulk density (g/ml)</th>
<th>Tapped density (g/ml)</th>
<th>Hausner ratio</th>
<th>Carr’s index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.29</td>
<td>0.50</td>
<td>1.70</td>
<td>41.18</td>
</tr>
<tr>
<td>2</td>
<td>0.27</td>
<td>0.46</td>
<td>1.73</td>
<td>42.11</td>
</tr>
<tr>
<td>3</td>
<td>0.31</td>
<td>0.56</td>
<td>1.78</td>
<td>43.75</td>
</tr>
<tr>
<td>4</td>
<td>0.27</td>
<td>0.47</td>
<td>1.73</td>
<td>42.11</td>
</tr>
<tr>
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<td>0.49</td>
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<td>0.30</td>
<td>0.56</td>
<td>1.87</td>
<td>46.43</td>
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</table>

Influence of process parameters on particle size

Although the major use of FFED is in screening experiments, it is found in this study the FFED cannot provide an adequate model to predict particle size. Since FFED neglects the polynomial effect of the individual factor and considers that two-factor interactions are confounded with each other \(^{161}\), the inadequate fit of the model for particle size prediction may suggest the existence of a higher-order relationship in this case. It is found in the study, although the model does not fit the data well, smaller size can be obtained when the drying and
atomization energy is high (high temperature, low feed rate, and high atomizing air flow according to equation 29), especially in the case of samples 5 and 9 with smallest size of 129 nm obtained with run 5 (Table 13). It is well established in spray drying technique that an increase in the energy available for atomization will reduce particle size. Experimental results from other investigators also confirm this theory. For instance, Tajber et.al. reported that the higher atomizing air flow was associated with smaller spray dried microparticle, and this trend was also commonly observed. Mackaplow et.al. studied the effect of disk speed on rotary spray congealing of microspheres. Disk rotation speed (similar to atomization air flow rate in spray drying) had the most significant effect on microsphere size, with higher speed yielding smaller congealed particles. Although feeding speed was high, particle size might be reduced due to higher centrifugal forces in spray congealing. The impact of nozzle temperature was also studied in spray congealing process. Passerini et.al. studied two different atomizing nozzles (wide pneumatic and air pressure type) in spray congealing process. Their data showed atomizing air pressure and nozzle temperature also had a positive effect on smaller particle size, which concur with spray drying theory. Moreover, particle size usually increases as the feed concentration increases. The effect of temperature on the particle size is more material dependent. But these conclusions were not made based upon nanoparticles. Although FFED fails to provide an adequate model to predict the size, our objective is partially achieved, which is to produce spray dried NC under 220 nm suitable for sterile filtration and i.v. injection. Alternative experimental design approaches such as Central Composite Design or Box-Behnken Design needs be studied to predict the effect of process parameters on size.
3.4 Conclusion

In this study, Box-Behnken design enables to elucidate and optimize the size and encapsulation efficiency of IND into spray dried oily core NC prepared by emulsion-diffusion technique. Optimum combination of formulation variables is calculated to be 300 mg of PLA, 0.56 ml of oil, and 239.57 mg of Pluronic F127, which corresponds to size of 284.1 nm and EE% of 95.7%. This is suitable for many routes of drug delivery. Checkpoint analysis proves the validity of the model and optimum formulation. Morphological and XRD analysis confirm the existence of oily core structure, and the drug is encapsulated in amorphous form. *In vitro* release study shows that IND-NC has a better sustained release over 72 hrs compared to control drug-PLA nanosphere, mechanistic study indicates that drug release from NC is the combinative effect of diffusion and degradation. Spray dried oily core NC can serve as a template of novel nanocarrier for lipophilic drugs, and Box-Behnken design shed light on future study of formulation optimization. Fractional factorial design enables to investigate and optimize the yield of indomethacin (IND) loaded oily core Nanocapsules (NCs) prepared by emulsion-diffusion technique and spray drying, which lead to a yield of 30.8%. Statistical analysis proves the validity of the model and optimization process. The powder flowability is low, which may be due to low feed solid concentration and small particle size. Further experiments should explore the effect of formulation and process variables in order not only to increase the yield of the process but also to optimize the preparation of NC with lower PI. Experiments should be also designed in order to elucidate critical issues such as safety, performance, stability, and manufacturability.
4. pH-RESPONSIVE NANOPARTICLES RELEASING TENOFOVIR FOR THE PREVENTION OF HIV TRANSMISSION

4.1 Rationale

Unprotected, heterosexual, vaginal intercourse has become one of the major routes of infection. Although the global percentage of women among people living with HIV has remained stable (50%), women are considered more susceptible to sexually-acquired HIV infection due to physiological, social, and economical factors. Several HIV transmission prevention methods, such as condoms and circumcision, have been implemented, especially in developing countries. However, the results have been unsatisfactory, since it was reported in many regions that men were reluctant to use either method. Besides these facts, a successful HIV vaccine has yet to be developed. It is critical to design a topical delivery strategy of microbicides that women can use as a pre-exposure prophylaxis (PrEP) method. Some examples of new drug delivery system (DDS) designed for the delivery of anti-HIV drugs have been reported, but none of them are currently used clinically for the purpose of the prevention of HIV transmission.

Extensive research activities have been dedicated to the field of HIV microbicides development, such as detergents/pH buffers, entry/fusion inhibitors, and HIV reverse transcriptase inhibitors. Tenofovir (TNF, [(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methylphosphonic acid) is a nucleotide analog HIV reverse transcriptase inhibitor whose prodrug (Tenofovir disoproxil fumarate, TDF) is now marketed in an oral dosage form (Viread®, Gilead Science), and its 1% vaginal gel formulation has recently been proven effective in clinical trial. Other vaginal delivery strategies of tenofovir along with dapivirine and emtricitabine have also been studied. However, factors affecting the acceptability of the gel formulation include the ease of incorporation into typical sexual practices, and type of sexual partnership.
The vaginal route has been a site of local delivery as well as systemic delivery. Several dosage forms have been investigated as vaginal delivery systems, such as vaginal rings, films, and gels. Nanoparticles (NP) also potentially provide one possibility of such drug delivery system due to their unique characteristics, such as small size, protection of native drug, ability to reduce irritation at delivery site, and the ability of targeted delivery and controlled release of drugs. The concept of so called “nanomicrobicides” has embraced the advanced potential of nanomedicine, and efforts have been made to address major health problem of HIV prevention. Several insightful studies of NP vaginal formulation have indicated that it is a promising strategy toward the delivery of peptides and even SiRNA.

Since the HIV virus can be present in human semen during the intercourse, it is promising to design a semen-triggered topical delivery system. The ambient human vagina pH varies from 4-5, whereas human semen has a higher pH (typically 7.5) as well as higher buffer capacity. Therefore, the local acidic pH will be altered during intercourse, which has been utilized in semen triggered delivery and pH sensitive hydrogel. However, vaginal retention of such a delivery system is also important. Otherwise, the short duration of the drug requires the user to apply microbicides formulation hours before sex (coital dependence), which leads to significant patient compliance issues as recently observed in the clinical trial of tenofovir gel. Based on the above consideration, the focus has been given to the preparation of a semen-triggered delivery system having a sustained release characteristic for vaginal delivery. It is hypothesized that a semen-triggered polymeric nanoparticulated delivery system, using poly(lactic-co-glycolic acid) (PLGA) and the methacrylic acid copolymer Eudragit® S-100 (S-100), would be effective and safe in the prevention of HIV transmission by vaginal route. PLGA is a FDA-approved and widely accepted biodegradable copolymer used in NP formulation,
which can also provide the sustained release of an encapsulated drug. Eudragit S-100 is a methacrylic acid - methyl methacrylate copolymer (1:2) synthesized from methacrylic acid and methacrylic acid methyl ester, which is soluble in an alkaline environment. Therefore, it has been widely used in intestine or colon delivery systems where pH is above 7.

The present investigation is aimed at testing the hypothesis that Tenofovir or tenofovir disoproxil fumarate loaded nanoparticles prepared with a blend of poly(lactic-co-glycolic acid) (PLGA) and methacrylic acid copolymer (Eudragit S-100, or S-100) are noncytotoxic and exhibit significant pH-responsive release of anti-HIV microbicides in presence of human semen. Basically, the TNF and TDF loaded, pH-sensitive NP was prepared, and physicochemical characteristics, as well as biological responses, were investigated. The results of this study demonstrates that a PLGA/S-100 NP formulation may provide semen-triggered delivery and sustained release of an encapsulated microbicides for the prevention of HIV/AIDS transmission.

4.2 Materials and Methods

Materials

Tenofovir (TNF) was purchased from Zhongshuo Pharmaceutical Co. Ltd. (Beijing, China). Tenofovir disoproxil fumarate (TDF) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Rockville, Maryland. Poly(D,L-lactide-co-glycolide) Resomer® RG756 with L/G ratio of 75:25 (MW 76,000-116,000) was purchased from Boehringer Ingelheim Inc. (Ingelheim, Germany). Eudragit® S-100 (Methacrylic acid-methyl methacrylate copolymer 1:2) was purchased from Evonik Industries (Darmstadt, Germany). Poloxamer 407 (Pluronic® F127) was a gift from the BASF Corporation (Rhom, Germany). Coumarin-6, propidium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased
from Sigma Aldrich (St. Louis, MO, USA). CytoTox-ONE™ and CellTiter 96™ Aqueous kits were purchased from Promega (Madison, WI, USA). All other chemicals used in this study were of analytical grade and used without further purification.

Nanoparticle preparation

TNF and TDF loaded PLGA/S-100 nanoparticles were prepared at room temperature using a previously described emulsification solvent diffusion method \(^{206}\). Briefly, TNF (5 mg) and the polymer (100 mg, PLGA/S-100 ratio 25:75, 50:50 and 75:25) were co-dissolved in 4 ml DMSO. This mixture served as the organic phase for the NP preparation. The organic phase was added drop wise to 25 ml of aqueous phase, containing 0.6% (w/v) Pluronic F127, under homogenization at 13,500 rpm for 10 min (IKA ULTRA-TURRAX T-25, Staufen, Germany). The suspension was further ultracentrifuged at 15,000 rpm for 1 hr (Beckman L8-70 M Ultracentrifuge, Brea, CA, USA) to collect NP, and then washed three times with distilled water to remove the surfactant. The supernatant was used for the determination of drug encapsulation efficiency (EE%). Finally, the NP was first frozen in liquid nitrogen then lyophilized for 12 hours using a lab-scale freeze dryer (Labconco Corporation, Kansas City, MO, USA) under -46 °C, and stored at 4 °C until use. Blank NP and Coumarin-6 (C-6) loaded PLGA/S-100 NP were prepared using the same method.

Particle size and zeta potential measurement

The particle size and size distribution of the various NP solutions were measured at 25 °C by dynamic light scattering method (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). The particle size of the different samples (nanosuspension before
lyophilization and suspension of lyophilized NP powder) were evaluated and represented as Z-average diameter. The zeta potential of the PLGA/S-100 NP suspension was measured using the zeta potential analysis mode of the instrument. Nanosphere™ size standard (59±2.5 nm) and zeta potential standard (-68±6.8 mV) were used to calibrate the instrument prior to the analysis.

Morphology

The images of the NP formulation were taken by transmission electron microscopy (TEM). Particles were diluted in 2.5% uranyl acetate (UA), sonicated, and then 8 µL of the solution was put on a carbon coated grid and allowed to equilibrate for 5min; excess solution was wicked off. Then 5% UA was put on the grid to increase contrast. The grids were viewed under a JEOL JEM 1400 Transmission Electron Microscope (JEOL Inc., Peabody, USA), and photographed digitally with a Gatan axis-mount 2k×2k digital camera.

Encapsulation efficiency

The encapsulation efficiency (EE%) was measured at a wavelength of 260 nm by UV spectrometer (Spectronic Genesys 10 Bio, Thermo Electron Corporation, WI, USA). The standard curves of TNF and TDF were prepared using drug concentration ranging from 2~100 µg/ml. The amount of encapsulated drug was calculated using mass balance by subtracting the amount of the free drug present in the supernatant from the total drug amount initially added in the preparation medium.

In vitro release of tenofovir from nanoparticles
To estimate the amount of drug released from the PLGA/S-100 NP and assess the rate of pH-responsiveness, an in vitro release study was conducted over 72 hr using a vaginal fluid simulant (VFS, pH 4.2±0.1) and a semen fluid simulant (SFS, pH 7.6±0.1). The components used in the VFS and SFS formulations are adopted from previous articles \(^{46,207}\). Briefly, the VFS was prepared using sodium chloride, potassium hydroxide, calcium hydroxide, albumin bovine fraction V, lactic acid, glycerol, urea, acetic acid, glucose and water. The components of SFS were: sodium phosphate monobasic/dibasic, sodium citrate, potassium chloride, potassium hydroxide, fructose, glucose, lactic acid, urea, and bovine serum albumin.

The samples tested included drug loaded NP with various weight ratios of S-100 to PLGA in the polymetric matrix. The release profile of TNF and TDF was also compared. Each experiment was run in triplicate, along with a blank control formulation. For this release study, 1 ml of the resuspended pellet after ultracentrifugation (corresponding to 250 µg of encapsulated drug) was put into a Spectra/Por cellulose ester membrane dialysis bag (Spectra/Por Float-A-Lyzer G2, MWCO 3.5-5 KD, Spectrum Laboratories Inc. Rancho Dominguze, CA, USA), and maintained in 40 ml of release medium using a shaking water bath (BS-06 Lab. Companion, Jeio Tech Co., LTD, Seoul, Korea) at 37 °C with an agitation speed of 60 rpm. In order to test the pH-responsiveness, VFS alone, and 1:4 ratio of VFS/SFS mixture (final pH 7.57), were used as release medium. It has been reported by Owen et.al. that the volume of normal human vaginal fluid is 0.75 ml, and the volume of human ejaculate is 3.4 ml (Need to insert reference 22 and 26 here). At predetermined time intervals, 1 ml of buffer solution outside the dialysis bag was removed and replaced with 1 ml of fresh buffer. The amount of drug released was measured by a UV spectrometer at 260 nm as described previously. The standard curve was prepared using the
same method as in the previous section. The standard curve was $y=0.0446x+0.0052$ ($R^2=0.9993$) for TNF and $y=0.0209x+0.0215$ ($R^2=0.9998$) for TDF.

Cell culture

Human vaginal epithelial cell line (VK2/E6E7, ATCC Number CRL-2616), Human Endocervical epithelial cell line (End1/E6E7, ATCC Number CRL-2615), and Lactobacillus crispatus (ATCC Number 33197) were obtained from the American Type Culture Collection (Manassas, VA, USA). Unless otherwise stated, culture medium and reagents were purchased from Invitrogen (Carlsbad, CA, USA). VK2/E6E7 and End1/E6E7 cells were grown and routinely maintained at 37 °C in 75-cm$^2$ culture flasks, in keratinocyte-serum free medium supplemented with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, additional calcium chloride 44.1 mg/L, and in atmosphere of 5% CO$_2$. Lactobacillus crispatus was grown in an ATCC medium 416 Lactobacilli MRS broth (BD, Franklin Lakes, NJ, USA) at 37°C.

Cytotoxicity studies on vaginal/endocervical cell lines

Cell viability was determined by a DTX 800 multimode microplate reader (Beckman Coulter, Brea, CA, USA). Cells were transferred to 96-well plates to ensure $1\times10^4$ cells per well, and were allow to grown until they reached 80% confluence. Then the medium was changed with 100 µl medium of blank PLGA/S-100 NP with different PLGA to S-100 ratios. The concentration of NP in the cell culture medium varied from 25 µg/ml to 10,000 µg/ml. The average size of 100% S-100 NP used in cytotoxicity study was 535.8 nm. The plates were incubated for 24 and 48 hr. The medium was used as a negative control and 1% Triton X as a positive control. Twenty microliters of $[3-(4,5$-dimethylthiazol-2-yl)-5-(3-
carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution (MTS, CellTiter 96™ Aqueous, Promega, Madison, WI, USA) was added to each well and incubated for 1 hr at 37°C. Cell viability was determined using equation (32):

\[
\text{Viability (\%)} = \frac{\text{ABS}_{\text{test}}}{\text{ABS}_{\text{control}}} \times 100
\]  

(32)

where \(\text{ABS}_{\text{test}}\) and \(\text{ABS}_{\text{control}}\) represented the amount of formazan detected in viable cells.

The cellular membrane integrity was determined by the release of lactate dehydrogenase (LDH). Briefly, cells were seeded in 96-well plates and incubated with PLGA/S-100 NP using the same condition as stated above. One row of the 96-well plate without cells was used to determine the background fluorescence that might be present. At different time intervals, the plates were equilibrated at 22 °C and 100 µl of CytoTox-ONE™ reagent (Promega, Madison, WI, USA) was added to each well. The plate was incubated at 22 °C for 10 min, and then 50 µl of stop solution was added to each well. The fluorescence was detected using the above microplate reader at excitation wavelength of 560 nm and emission wavelength of 590 nm. The percent cytotoxicity for a given treatment can be expressed using equation (33):

\[
\text{Cytotoxicity (\%)} = 100 \times \frac{\text{Experimental} - \text{Background}}{\text{Positive} - \text{Background}}
\]  

(33)

where Experimental, Background, and Positive represent the absorbance of NP-treated wells. The background control wells contained cells not treated with NP, and positive control wells contained cells treated with 1% Triton X, respectively.

Lactobacillus viability assay

To assess the effect of PLGA/S-100 NP on *L. crispatus* growth, the viability assay was performed using a MTS assay. The soluble tetrazolium salt method has previously been tested on
various Gram-positive and Gram-negative bacteria including \textit{Lactobacillus}, showing linear relationships between the absorbance and viable microbial cell density.\textsuperscript{208} Briefly, the bacteria density was adjusted to an OD\textsubscript{670} of 0.06, which corresponds to a 0.5 McFarland Standard or 10\textsuperscript{8} CFU/ml.\textsuperscript{209} \textit{L. crispatus} was seeded in 96-well plates at a volume of 100 \(\mu\)l and incubated with 100 \(\mu\)l of a series of NP dilutions at 37\textdegree{}C for 24 and 48 hr. Bacterial wells treated with commercially available Penicillin-Streptomycin solution (Invitrogen, Carlsbad, CA, USA) at 10 \(\mu\)g/ml were used as positive controls. After incubation, a 20 \(\mu\)l MTS reagent was added to each well and the bacterial viability was determined by measurement of the OD\textsubscript{490} using the microplate reader. The percent viability was expressed using equation (32).

Transepithelial electrical resistance (TEER) measurement

To determine the effect of PLGA/S-100 NP on the epithelial integrity, the transepithelial electrical resistance (TEER) was measured. Polycarbonate transwell inserts (Corning Costar Transwell dual-chamber system, Fisher Scientific) were coated with 0.05\% type IV collagen (Sigma) prepared in 0.2\% acetic acid and 7.5\% ethanol overnight at 37\textdegree{}C, 5\% CO\textsubscript{2}. End1/E6E7 cells (4x10\textsuperscript{5}) were seeded onto the coated transwell inserts and grown in a 12-well dual chamber system for seven days to achieve polarization. At that time, PLGA/S-100 NP (75\% S-100 at 1,000 or 10,000 \(\mu\)g/ml) was added to the apical surface of the monolayer, and TEER was measured at 30 min, 1, 2, 4, and 24 hr using an EVOM voltmeter (World Precision Instruments, Sarasota, FL, USA). As controls, wells treated with medium, cells treated with Triton X, or no cells were used. The TEER was expressed as (\(\Omega\)xcm\textsuperscript{2}) value of the treated wells subtracting that of the cell free wells.
Cellular uptake studies

Cellular uptake studies were performed as previously described\textsuperscript{210}. The cells (1x10\textsuperscript{4}) were seeded in the 96-well plates. After 80% confluence was reached, the cells were incubated with C-6 NP suspension (250, 500, and 1,000 µg/ml in medium) for 4, 24, and 48 hr. Cells treated with medium only were used as background, and control was kept by adding C-6 NP suspension to the cell-free wells. At different time intervals, suspension was removed and the cells were washed three times using a phosphate buffer saline (PBS, pH=7.4) solution. After adding 1% Triton X for cells lysis, the fluorescence of the plate was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA). The excitation and emission wavelengths were 430 and 485 nm, respectively. The cellular uptake efficiency was calculated by the ratio of the fluorescence intensity in the cell coated wells to those in control wells.

For the confocal microscopy experiment, cells were grown on a 16-well CultureWell\textsuperscript{TM} chambered coverglass (Grace Bio-Labs, Inc., Bend, OR, USA). After 80% confluence was reached, medium was substituted with 100µl C-6 NP suspension (250 µg/ml). After incubation for 4hrs, the suspension was removed and 10 µl of 70% ethanol was used to fix the cells at 37 °C for 20 min. Ethanol solution was then removed and the cells were washed three times using PBS. Ten µl of propidium iodide (PI, 5 mg/ml) was added to stain the nucleus for 30 min. After the PI was washed three times using PBS, the coverglass was separated and observed by a confocal laser scanning microscope using a 40x water lens with a KrAr laser (Zeiss LSM 410) and Fluoview FV300 software.

The elucidation of the mechanism of the NP endocytosis was done through uptake inhibition assay previously described\textsuperscript{211}. Cytoskeleton recognition was prevented by incubation
the cells with cytochalasin D (10 µg/ml) for 30 min followed by C-6 NP application. Macropinocytosis was promoted through pre-incubation with phorbol myristate acetate (PMA, 1 µM) for 30 min followed by C-6 NP. Clathrin-dependent endocytosis was inhibited by co-treatment C-6 NP with chlorpromazine (10 µg/ml) after the pre-incubation at the same concentration. Finally, the caveolins-mediated endocytosis was disturbed by pre-incubate the cells with genistein (200 µg/ml) for 30 min followed by co-treatment with C-6 NP at the same concentration. Cells treated with C-6 NP alone were used as control. After 24 hours, cells were analyzed as described in the previous section.

Data analysis

After triplicate experiments, data were expressed as mean ± standard deviations. Compare to controls, the statistical significant difference of a given mean was determined using a t-test. A P value <0.05 was considered statistically significant.

4.3 Results

Physicochemical characterization of TNF or TDF loaded PLGA/S-100 NP

The size, EE%, PDI, and zeta potential of TNF/TDF loaded PLGA/S-100 NP are listed in Table 1. Figure 18 A shows a typical size distribution of the NP sample. As shown in Figure 18 B, the morphology of drug-loaded NP appeared to be spherical in shape and smooth in surface. There is variance in size distribution, as shown in a PDI value greater than 0.005, but the majority of the particles have a diameter below 300 nm. The encapsulation efficiency of TNF is less than 20%. The EE% of TDF is significantly higher than that of TNF (P value = 0.008, 0.004, 0.005 for NP with S-100/PLGA ratio of 25/75, 50/50 and 75/25, respectively). The EE% decreases with increasing S-100 percentage for both TNF and TDF.
Table 19. Physicochemical characterization of TNF and TDF loaded pH-responsive nanoparticles\(^a\)

<table>
<thead>
<tr>
<th>Drug (5 mg)</th>
<th>S100/PLGA ratio</th>
<th>Particle size before freeze drying (nm)</th>
<th>Particle size after freeze drying (nm)</th>
<th>EE (%)</th>
<th>Drug loading (% w/w)</th>
<th>PDI</th>
<th>Z-potential (mV)</th>
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<tr>
<td>TNF 25/75</td>
<td>240.0±16.9</td>
<td>315.6±24.1</td>
<td>15.4±1.2</td>
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<tr>
<td>TNF 50/50</td>
<td>225.2±16.5</td>
<td>336.2±18.9</td>
<td>16.1±1.0</td>
<td>0.81±0.05</td>
<td>0.224±0.032</td>
<td>-2.09±0.12</td>
<td></td>
</tr>
<tr>
<td>TNF 75/25</td>
<td>298.0±14.9</td>
<td>501.7±44.5</td>
<td>9.5±1.9</td>
<td>0.48±0.10</td>
<td>0.269±0.094</td>
<td>-3.26±0.25</td>
<td></td>
</tr>
<tr>
<td>TDF 25/75</td>
<td>226.1±13.5</td>
<td>310.6±29.1</td>
<td>37.2±3.9*</td>
<td>1.86±0.19*</td>
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<td>-3.37±0.50</td>
<td></td>
</tr>
<tr>
<td>TDF 50/50</td>
<td>251.1±27.0</td>
<td>301.5±47.6</td>
<td>26.9±1.4**</td>
<td>1.34±0.07**</td>
<td>0.190±0.046</td>
<td>-3.87±0.28</td>
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</tr>
<tr>
<td>TDF 75/25</td>
<td>336.8±41.4</td>
<td>433.6±36.2</td>
<td>24.0±1.6***</td>
<td>1.20±0.08***</td>
<td>0.278±0.068</td>
<td>-2.38±0.33</td>
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</tr>
</tbody>
</table>

\(^a\) Each data point shown is the average of three samples (n=3)

*, **, ***  Statistical significant difference (\(P<0.05\)) between means of TNF and TDF groups.
Figure 18. (A) Size distribution by volume of tenofovir disoproxil fumarate loaded nanoparticle prepared with PLGA/S-100 in 50/50 weight ratio measured by dynamic light scattering. (B) Typical transmission electron micrograph (Bar=200 nm).

*In vitro* release study

The *in vitro* drug release for a period of 72 hrs in VFS and the mixture of VFS/SFS are shown in **Figure 19 A to D**. For NP with a 75% S-100/25% PLGA weight ratio, there is a 43.6±5.0% and a 78.5±5.8% of the total amount of TNF released in 24 and 72 hrs under pH 7.6, respectively; while there’s only 19.6±4.3% of the total TNF release in 72 hrs under pH 4.2
(Figure 19 A). By reducing the amount of S-100 in that of total polymers, the drug release rate also decreases (Figure 19 B and C). The release rate of TNF and TDF from a 75%/25% S-100/PLGA weight ratio NP under pH 7.6 is also compared (Figure 19 D). The release rate of TDF is lower compared to TNF.

Figure 19. in vitro release profile at pH=4.2 (solid line) and pH=7.6 (dash line) of TNF loaded NP prepared with different weight ratio of S-100 to PLGA, n=3. (A) 75% S-100. (B) 50% S-100. (C) 25% S-100. (D) Comparative in vitro release profile of 75% S-100 NP encapsulated with TNF or TDF under 37°C at pH=7.6, n=3.

Effect of PLGA/S-100 NP on viability of epithelial cell lines and vaginal flora
Figure 20. MTS assay of VK and Endo cells after treated with PLGA/S-100 NP (S-100 at 50%, 75% and 100% w/w) at various concentrations. (A) VK cells at 24 hr, (B) VK cells at 48 hr, (C) Endo cells at 24 hr, (D) Endo cells at 48 hr. The data shown represent the mean ± standard deviation of 3 independent experiments, n=3. Stars indicate significant differences from the controls, with P<0.05.

Our data suggest that a PLGA/S-100 blend NP is safe in both vaginal (VK) and endocervical (Endo) epithelial cell lines up to 10 mg/ml (Figure 20 A through D). Significant reduction of cell viability (p<0.05) has been observed for pure Eudragit S-100 NP at 10 mg/ml on VK cell line after 24 (77.34±7.15%) and 48 hr (71.76±10.60%). This effect is observed on all three formulations (S-100/PLGA 50/50%, 75/25%, 100/0%) at 10 mg/ml on Endo cells. Lactate dehydrogenase (LDH) assay estimates the cell membrane integrity and the release of LDH are markers of non-viable cells. The results of the LDH study correlate with that of the MTS study.
(Figure 21 A through D). PLGA/S-100 NP shows a more toxic effect on Endo cells than VK cells, especially for NP with high S-100 ratio under high concentration (10 mg/ml). However, the highest percent of cell death observed in the whole study is $12.14 \pm 1.36\%$ compared to positive control, suggesting a relatively minor toxic effect.

![Figure 21](image)

**Figure 21.** LDH assay of PLGA/S-100 NP on VK and Endo cells at various concentrations. (A) VK cells at 24 hr, (B) VK cells at 48 hr, (C) Endo cells at 24 hr, (D) Endo cells at 48 hr. The data shown represent the mean ± standard deviation of 3 independent experiments, $n=3$. Stars indicate significant differences from the controls, with $P<0.05$.

The TEER data is shown in **Figure 22**. The medium treated wells varied by less than 13% of their TEER values in a period of 24 hr. The two highest concentrations (1000 and 10,000 µg/ml) of the NP reduced the TEER of Endo cells by a maximum of 9% ($p=0.74$) and 18%
(p=0.76), respectively, indicating a transient reduction of the monolayer integrity. Conversely, the cell monolayer loses its TEER value up to 80% in 24 hr in the positive control. Although there is some reduction of viability on Endo cells at high concentration as shown in the MTS study, the NP does not compromise the integrity of Endo cells monolayer.

Figure 22. Effect of PLGA/S-100 NP on Endo cells monolayer integrity. Endo cells were grown in transwell supports and resistance readings were measured at 30 min, 1, 2, 4, and 24 hr. The data shown represent the mean ± standard deviation of 3 independent experiments, n=3.

Three PLGA/S-100 nanoformulations are tested on *L. crispatus* from 25 to 10,000 µg/ml for a period of 48 hr. No statistically significant loss of viability (P>0.05) is found (Figure 23).
Figure 23. Viability of Lactobacillus crispatus treated with PLGA/S-100 NP (S-100 at 50%, 75% and 100% w/w) after 48 hrs at various concentrations. The data shown represent the mean ± standard deviation of 3 independent experiments, n=3.

Cellular uptake study

Around 50% of the NP can be uptaken by cervicovaginal epithelial cells within 24 hours (See Figure 24). The uptake process is further visualized and proven by a confocal microscope study. Cell nuclei are stained by propidium iodide (PI) and exhibit a red color under red channel while C-6 NP exhibits a green color under green channel. X-Y axis projections are performed using a 0.5 µm interval in an 11 to 18 µm range. Confocal microscopic images (Figure 25) verify that nanoparticles are actually internalized by VK and Endo cells, and distribute throughout the cytoplasm and around nucleus instead of localized on the surface of the cells.
Figure 24. The particle uptake efficiency with incubation time. Each data point shown is the average of three samples. The data shown represent the mean ± standard deviation of 3 independent experiments, n=3.
Figure 25. Confocal fluorescence images of VK and Endo cells with coumarin-6 encapsulated PLGA/S-100 50%/50% NP (C-6 NP) after 4 hr incubation and control. An overlay of red and green channels is shown. (A) Z-stacking of X-Y projection of VK cells treated with C-6 NP. (B) Z-stacking of X-Y projection of Endo cells treated with C-6 NP. (C) Y-Z projection of the optical section of the VK cells incubated with C-6 NP. (D) Y-Z projection of the Endo cells incubated with C-6 NP. (E) Z-stacking of X-Y projection of control VK cells treated with medium alone. (F) Z-stacking of X-Y projection of control Endo cells treated with medium alone.

Figure 26. Effects of inhibitors on the internalization of C-6 NP on VK cells. For all treatments, cells are pre-treated 30 min with the inhibitor prior to treatment with C-6 NP. After 24 h, cells were analyzed by microplate reader. Values indicate means ± standard deviation of 3 independent experiments, n=3. Stars indicate significant differences from the controls, with P<0.05.
The uptake inhibition study shows that the treatment with genistein, an inhibitor of caveolin-mediated endocytosis, has significant reduction of NP uptake on VK cells at all concentrations studied (Figure 26). Interruption of actin microfilament polymerization and depolymerization by cytochalasin D results in significant reduction of uptake on NP concentrations of 250 and 1000 µg/ml, but not on 500 µg/ml. Clathrin-dependent endocytosis and mycropinocytosis are not involved in PLGA/S-100 NP internalization as indicated by PMA and Chlorpromazine treated studies.

4.4 Discussion

PLGA/S-100 NP have an average size between 200-300 nm, which is comparable to previously reported particle size such as the Eudragit RS/RL NP and Eudragit RS/PLGA blend NP, in which the obtained Eudragit RS/PLGA had an average size of 273 nm. The measured zeta potential was around -3.0 mV. This relatively weak negative charge on the surface could be attributed to the presence of ionized carboxyl groups on the particle surface, which is present in both PLGA and Eudragit-S100. The size and zeta potential of the NP can affect toxicity and stability, as well as tissue uptake and interstitial trafficking. It was shown that topical administration of PLGA NP with a strong negative charge (around -20 mV) and size around 200 nm, can display greater permeability deep into vaginal epithelium as well as into the ectocervical tissue. Therefore a close-to-neutral charge and relatively larger size, as obtained in this study, would help reduce particle traversing through the reproductive tract tissue, thus forming a first line of defense of pH-sensitive NP for effective prevention of HIV transmission.
Tenofovir is highly hydrophilic (LogP -1.6)\(^ {93b}\), and therefore it most likely partitions into the aqueous phase during NP preparation, with the incorporation of increasing proportion of the relatively more hydrophilic polymer Eudragit S-100 into the nanoformulation. TDF has higher EE% because it is more hydrophobic (LogP 1.25) compared to TNF\(^ {215}\), and it also has a higher molecular weight (635.52 vs. 287.03 g/mol for TNF)\(^ {93b,215}\). In addition to low molecular weight and relative water solubility of the drug, the high specific surface area of the NP could have also contributed to the low EE%. The result of this study suggests that this drug delivery template works better for the hydrophobic microbicides, and further study is needed to either improve the EE% of the hydrophilic drug, or develop this system using hydrophobic microbicides.

It was hypothesized that the presence of S-100 in the polymeric matrix accelerates the drug release rate in the presence of the semen fluid simulant. The drug release rate from the polymeric shell of NP can be modulated by swelling/deswelling of the polymer. Poly methacrylic acid has a pKa value of 5.65\(^ {216}\), thus it is mostly in ionized form under high pH (such as pH>7) as compared to low pH when it is mostly in its unionized form. Under high pH, the protonated acidic groups will cause swelling of the polymer, thus increase drug release rate\(^ {217}\).

As previously demonstrated, human semen can be detected in vaginal tract up to 48 hours after intercourse, and the cervicovaginal pH remains at a relatively high level within the first 24 hours after the intercourse\(^ {218}\). These observations provide a solid ground for the potential clinical relevance of the observed time dependent pH-responsiveness. Our data support this hypothesis since increasing the percentage of S-100 results in a higher drug release rate, and there is an approximate of 50% drug release within the first 24 hrs. Since the composition of the polymetric matrix is random in NP, lower amounts of S-100 will give the drug less of a chance.
to be in contact with the release medium. Therefore, the drug release rate might become more
dependent on the PLGA degradation rate at a lower S-100 weight ratio. This can explain the
similar release rate shown in Figure 19 C, since it has been shown that PLGA nanoparticles
have a higher drug release rate in an acidic pH environment \(11^2\). Tenofovir has two pKa values at
3.8 and 6.7 \(21^9\). At lower pH, the ratio of unionized drug vs. ionized drug is higher, which has
been identified as a factor that could enhance the permeation of tenofovir through lipophilic
membrane \(21^9\). At higher pH, the degradation of PLGA is controlled by formation of acidic
oligomers, which build up inside the particle and creating water channels \(22^0\). On the other hand,
low pH could catalyze the breakage of ester bond in the polymer backbone, which is considered
to be a controlling effect of the zero-order release phase of PLGA MS \(22^0b\). The average pKa of
PLGA is 3.85 \(\text{(pKa(lactic acid)}=3.86 \text{ and pKa(glycolic acid)}=3.83)\) \(21^9\). A drop in the pH will
also cause a higher acid to base ratio, thus reducing the negative charge of PLGA. Reduced
charge-charge interaction between PLGA membrane and encapsulated drug could also contribute
to the drug release at lower pH. It is also well known that the ratio of lactide to glycolide in
PLGA strongly affects the degradation rate of PLGA \(22^1\). As the hydrophobicity of PLGA
increases, a slower drug release rate is observed because of the reduced water uptake in the
PLGA matrix. Therefore by adjusting the PLGA composition and S-100 percentage, the release
rate of TNF can be further controlled. Given the fact that TDF is more hydrophobic in nature \(93^b,\)
\(21^5\), the TDF release rate is more dependent on its diffusion rate through the polymetric matrix,
while TNF is more exposed to the aqueous phase with release requiring only dissociation from
the NP surface.

The actual drug loading for the 75%/25% S-100/PLGA weight ratio NP is 0.48% w/w for
TNF and 1.20% w/w for TDF, which can be converted to 1.7 µmol and 1.9 µmol per 100 mg NP
for TNF and TDF, respectively. It has been reported that the \textit{in vitro} EC\textsubscript{50} of TNF and TDF was 5.0 ± 2.6 and 0.05 ± 0.03 µM \textsuperscript{222}. Considering that a vaginal suppository weighs approximately 5g, and assuming a 20% w/w of NP in such a suppository, the ultimate vaginal formulation, would be respectively loaded with 17 and 19 µmol of TNF and TDF, leading to a vaginal drug concentration of 5.7 µM and 6.3 µM, given the fact that the average total volume of vaginal fluid and cervical mucus is 3 ml \textsuperscript{223}. Therefore it can be reasonably speculated that a microbicides released from pH-sensitive NP would potentially exhibit an anti-HIV effect. However, this remains to be elucidated in the future in the \textit{in vitro} and \textit{in vivo} efficacy assay.

Another major concern for topical delivery of HIV microbicides formulation is the retention time of the formulation. It has been reported that PLGA nanoparticles loaded with Coumarin-6 can be observed throughout the reproductive tract up to 7 days after only a single vaginal application \textit{in vivo} \textsuperscript{114}. Therefore it is reasonably speculated that the proposed PLGA/S-100 NP has the ability to be retained locally in the vaginal cavity. However, this remains to be further confirmed by \textit{in vivo} biodistribution and retention study.

Topical strategies to prevent HIV infection must have safety profiles that justify its application, especially on mucosal integrity and vaginal ecology \textsuperscript{18}. Since a simple 1% tenofovir gel has been proven to be safe on epithelial cell lines and peripheral blood mononuclear cells (PMBCs) \textsuperscript{6,224}, the purpose of this study is to test the effect of the vehicle PLGA/S-100 NP on the viability of epithelial cells. The observed difference in the viability profile between VK and Endo cells might be due to their difference in cell sensitivity. Columnar endocervical epithelial cells are thought to be more susceptible to toxicant and release more proinflammatory cytokines, such as interleukin 6, 7, and 8, when damaged than do vagina epithelial cells \textsuperscript{70c}. Being only a single cell layer, endocervical epithelium is more vulnerable to pathogen invasion and tissue
injury. Therefore active cytokine production would enable a quick response to infections for maintenance of sterility of the region \(^{70b}\). Based on the data of the MTS, LDH, and TEER studies, the overall toxic effect of PLGA/S-100 NP is transient, especially at concentrations lower than 10 mg/ml. By increasing the amount of PLGA, which is a biodegradable and biocompatible polymer, the cytotoxicity can be further reduced. By comparing the cytotoxicity data, PLGA/S-100 NP has a better safety profile than the carbopol gel formulation \(^{62}\), and has a similar safety profile to that of the terpolymer \(^{62}\) and hydroxyethycellulose gel formulations \(^{224}\). For example, it has been reported that a pH-responsive terpolymer gel formulation can achieve almost 100% viability on a mouse fibroblast cell line at 10 mg/ml, but the viability of cells was less than 20% when treated with carbopol at the same concentration. It is noted that carbopol is widely accepted in commercial vaginal formulations at 0.5-20 mg/ml \(^{225}\).

The normal vaginal flora consists of predominantly *Lactobacillus* species. It is critical that any microbicides formulation to not disturb this normal vagina flora. This is important to maintain the low pH environment and the secretion of hydrogen peroxide (H\(_2\)O\(_2\)), which provides a natural barrier against HIV transmission \(^{21b}\). *Lactobacillus crispatus* was used as a model bacteria since it has been proven to produce H\(_2\)O\(_2\) \(^{226}\). Our data suggested that PLGA/S-100 NP would not disturb the normal vaginal flora. Together with other safety data, PLGA/S-100 NP has the potential to be a safe matrix for vaginal delivery of microbicides. However, further studies are needed to rule out eventual unwanted inflammatory responses.

Nanoparticles encapsulating fluorescent dyes have been frequently used to study cellular uptake, and coumarin-6 was suggested to be a suitable marker for nanoparticles because of their low leaching percentage under various conditions \(^{210}\). Therefore the intracellular fluorescence of C-6 may not be attributed to the uptake of free coumarin-6 released from nanoparticles. A similar
uptake pattern of PLGA NP in C6 glioma cells has been reported, suggesting the capability of endocytosis function of epithelial cells on the reproductive tract. By applying different chemicals to inhibit/promote different endocytic pathways, it is possible to study the specific pathway involved in the internalization of PLGA/S-100 NP. The results indicate that caveolin-mediated endocytosis is clearly involved; with a maximum of 65% reduction observed under genistein treatment. Actin microfilament also contributed in the process, as evidenced by 42% and 36% reduction on 250 and 1000 µg/ml NP, respectively. On the contrast, clathrin-dependent endocytosis is not involved in the internalization. It has been previously reported that the NP size can affect the pathways of internalization, with a cut-off of 200 nm. Particles under 200 nm are more likely to be internalized through clathrin-dependent endocytosis, and caveolin-mediated pathway favors particles over 200 nm. Our data support this hypothesis since the PLGA/S-100 NP has an average size above 200 nm. However, multiple factors can affect NP internalization such as cell line dependence, particle size, and surface composition. Therefore, additional investigations are needed to further the subcellular fate of these NP. It was demonstrated in the *in vitro* release study that over 40% of the drug can be released within the first 24 hours after a contact with the simulated semen. In the case when no sexual activity is immediately engaged, the proportion of NP that is uptaken by the vaginal epithelium would allow the sustained release of microbicides to the basal layer due to the intracellular polymer degradation. Based on our current understanding on how viruses enter animal cells [48], these findings would provide additional physicochemical barrier by outdistancing the virus in regions where the HIV has been shown to transmigrate and infect its target cells. Therefore, these data are important, not only because they elucidate the fate of nanoparticles at site of action, but also reveal the possibility of microbicides localization at its site of action.
4.5 Conclusion

In this study, pH-responsive nanoparticles loaded with tenofovir or tenofovir disoproxil fumarate are formulated. The nanoformulation prepared by 75/25% ratio of S-100/PLGA matrix loaded with TDF (size 336.8 nm, encapsulation efficiency 24.0%, drug loading 1.9% w/w) appears to be the best formulation considering the overall effect based on drug loading, *in vitro* release (pH-responsive release), and safety (up to 10 mg/ml). PLGA/Eudragit S-100 nanoparticles have several advantages, such as small particle size, controlled release of drug in the presence of semen, and they are potentially safe to the vaginal physiological environment. However, the encapsulation efficiency is low. This might be increased by optimization of the preparation protocol or even using a more hydrophobic microbicides in the future study. Future studies are needed to characterize the conditions for optimal formulation stability, vaginal retention time, immunogenicity, *in vivo* safety and efficacy. Collectively, these data suggest the possibility of using nanoparticles as a delivery system for intravaginal delivery of HIV microbicides for the prevention of HIV transmission.
5. SPRAY DRYING TENOFOVIR LOADED MUCOADHESIVE AND PH-SENSITIVE 
MICROSHPERES INTENDED FOR HIV PREVENTION

5.1 Rationale

To this date, due to the problems summarized in the previous chapters, alternative pre-exposure prophylaxis (PrEP) methods are urgently needed, given that antiretroviral therapy is still far from curing the disease and a successful HIV vaccine is yet to be developed. The idea of PrEP methods started with the oral application of antiretroviral drugs, and later focused on the vaginal/rectal application of anti-HIV substances, known as microbicides. A great variety of HIV microbicides candidates have been studied and tested. In terms of formulation, a lot of the emphasis has been put on the first generation gel formulation, and some positive results have been shown. In the CAPRISA004 trial, in which a 1% tenofovir gel was tested, HIV incidence was reduced by 54% in the high gel adherence (>80%) group. This promising result shed light on the prospect of a possible total protection against HIV, if a sustainable concentration of the active drug can be maintained. However, such an aqueous gel system suffers from several disadvantages. Besides its limitation of application towards hydrophobic microbicides, the low retention time requires a high dosing frequency. Such “before and after sex” dosing strategy (otherwise regarded as “coital dependence”) sometimes leads to poor acceptability and adherence. An ideal prevention strategy for women at high-risk of sexually-acquired HIV could be the use of a microbicides formulation that could be administered in a coital-independent fashion (e.g. once a day). To achieve this goal, the ideal formulation should a) have high vaginal
retention time, and b) be able to release a high dose of microbicides when sexual intercourse occurs.

So far, there isn’t much literature on the development of a second generation topical microbicides formulation incorporating a triggering-release mechanism, although many concerns have been given to safe delivery of microbicides using nanotechnology. In a pilot study, Gupta et al. 2007 demonstrated a temperature and pH-sensitive hydrogel taking advantage of the drastic pH change in the presence of human semen as a triggering factor for the burst release of the microbicides. Most recently, Mahalingam et al. 2011 developed a pH-sensitive mucin like polymer that could significantly impede HIV migration at pH ≥4.8. It has recently been shown that PLGA/Eudragit S-100 blend nanoparticle loaded with tenofovir can have a 4-fold increase in the release rate in the presence of semen and is noncytotoxic to vagina epithelium and vaginal flora.

This study reports for the first time the development of a spray dried mucoadhesive and pH-sensitive microspheres (MS) formulation, based on polymethacrylate salt intended for vaginal delivery of HIV microbicides. It has been shown that the sodium or potassium salts of the methacrylic copolymers Eudragit L-100 and S-100 have the potential as a novel low-swellable mucoadhesive material. We postulate that a topical formulation prepared from sodium salt of Eudragit S-100 (EuSNa) may provide site-retentive characteristics as well as pH-sensitive release, which could be a potential alternative solution to current gel formulation. In this study, tenofovir was formulated into a spray dried EuSNa microparticle (EuSNa-MS) formulation. This study encompasses the formulation optimization, characterization, in vitro safety, in vitro mucoadhesion, and immunogenicity testing.
5.2 Materials and Methods

Materials

The Tenofovir (TFN) was purchased from Zhongshuo Pharmaceutical Co. Ltd. (Beijing, China). The Eudragit® S-100 (Methacrylic acid-methyl methacrylate copolymer 1:2) was purchased from Evonik Industries (Darmstadt, Germany). The sodium hydroxide was purchased from Sigma Aldrich (St. Louis, MO, USA). The Oregon green® 488 cadaverin *5-isomer* was purchased from Invitrogen Corp. (Carlsbad, CA, USA). The CytoTox-ONE™ and CellTiter 96™ Aqueous kits were purchased from Promega (Madison, WI, USA). The Milliplex MAP multiplex assay kits were purchased from Millipore (Billerica, MA, USA). All other chemicals used in this study were of analytical grade and used without further purification.

Experimental design

In this study, a $2^{4-1}$ fractional factorial experimental design was used to study the effect of four formulation variables, namely, polymer concentration, polymer to drug ratio, inlet temperature, feed flow rate on drug encapsulation efficiency, yield, and Carr’s index. These independent and dependent variables, and their coded factors, are listed in Table 20. All independent variables in this study were chosen based on preliminary experiments (data not shown).

Microsphere preparation

Eudragit S-100 sodium salt (EuSNa) was prepared according to the method adopted from Cilurzo et al. 2003. Briefly, Eudragit S-100 (S-100) was dissolved in water containing sodium hydroxide (NaOH) pellets to assure complete salification. The ratio of S-100 to NaOH was
calculated based on the acid value of the S-100. The solution was spray dried as described below.

Table 20. Variables and their levels in $2^{4-1}$ design.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Levels</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>$X_1 = \text{Polymer concentration (%)}$</td>
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<td>5</td>
</tr>
<tr>
<td>$X_2 = \text{Polymer : drug}$</td>
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<td>40</td>
</tr>
<tr>
<td>$X_3 = \text{Inlet temperature (\degree C)}$</td>
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<td>140</td>
</tr>
<tr>
<td>$X_4 = \text{Feed flow rate (ml/min)}$</td>
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<td>6.4</td>
</tr>
<tr>
<td>Coded values ($X_1$, $X_2$, $X_3$, $X_4$)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Dependent Variables

$Y_1 = \text{Drug encapsulation efficiency (\%)}$

$Y_2 = \text{Yield (\%)}$

$Y_3 = \text{Carr’s index (\%)}$

To prepare tenofovir-loaded EuSNa microspheres (EuSNa-TNF MS), different amounts of S-100 and TNF were added in 20 ml of deionized water with an appropriate amount of NaOH to achieve complete salification. The solution was then spray dried using a Buchi Mini Spray Dryer, Model 290 (Buchi Laboratoriums - Technik AG, Flawil, Switzerland). The dried MS
were weighed using a Mettler Toledo XS 105 dual range balance (Mettler Toledo Inc., Columbus, USA), and stored in a sealed or capped glass container at 4 °C for further analysis.

Size, zeta potential, and morphology

The size and size distribution of the EuSNa-TNF MS were evaluated using a Scepter 2.0 handheld, automated cell counter (Millipore, Billerica, MA, USA). The spray dried EuSNa-TNF MS were re-dispersed in deionized water at 1 mg/ml after 30 sec sonication, and size was evaluated by Scepter using a 40 µm sensor (measuring range 3-13 µm). The zeta potential was measured at 25 °C using Zeta sizer (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). The surface characteristics and geometry of the MS were analyzed by scanning electron microscopy (SEM). For SEM analysis, a small amount of the powdered MS was put onto a grid. The membrane was mounted on a 1/2” SEM stubs with double-sticky carbon tape. The sample was then sputter coated (Emitech EMS575SX) with ~20 nm thickness of gold and visualized under a Philips SEM 515 microscope (Philips/FEI, Eindhoven, NL).

Yield and powder flowability

The yield of the spray dried MS was simply calculated by the ratio of total mass of the spray dried powder to the total mass of the initial components combined. The powder flowability was measured by calculating Carr’s index (9). Briefly, the bulk density was measured by placing approximately one gram of powder under gravity into a calibrated graduated cylinder and then recording the bulk volume. The tapped density was measured by tapping the graduated cylinder on a wooden platform with an approximate amplitude of 20mm until no further change in powder volume was observed. Carr’s index was calculated through equation (9).
Drug encapsulation efficiency

After spray drying, about 10 mg of EuSNa-TNF MS was dissolved using 5 ml of 95% alcohol at oscillation overnight. Five ml of water was then added into the solution. After vortexed for 1 min, the sample was analyzed using a RP-HPLC method at 259 nm. The HPLC system (Waters, Milford, MA) consisted of a 1575 binary pump system, 717 plus auto sampler, 2487 dual wavelength absorbance detector, and a Bridge™ C18 column (150 mm X 4.6 mm, 5 μm). The results were acquired and processed with Breeze™ software. The mobile phase was water (0.1% triethylamine, pH 5.1 adjusted by orthophosphoric acid) and acetonitrile (35:65 v/v) delivered at a flow rate of 1 mL/min. The standard curve was y=25.267x+79.103, R²=0.9994.

The drug encapsulation efficiency was calculated as:

\[
\text{Drug Encapsulation Efficiency (\%)} = \frac{\text{Actual Loading}}{\text{Theoretical Loading}} \times 100
\]  

(34)

Where actual loading was assessed by HPLC, and theoretical loading was calculated from the mass ratio between TNF and total mass of EuSNa-TNF MS.

*In vitro* release of TNF from EuSNa-TNF MS

To estimate the amount of TNF released from the MS, a protocol similar to the previously chapter was used. Two ml of resuspended EuSNa-TNF MS was put into a dialysis bag (Spectra/Por Float-A-Lyzer G2, MWCO 3.5-5 KD, Spectrum Laboratories Inc. Rancho Dominguze, CA, USA), and maintained in 40 ml of release medium using a shaking water bath (BS-06 Lab. Companion, Jeio Tech Co., LTD, Seoul, Korea) at 37 °C with an agitation speed of 60 rpm. Vaginal fluid simulant (VFS, pH 4.2) mixed with semen fluid simulant (VFS/SFS, pH
7.6) were used as a release medium. At predetermined time intervals (0, 0.25, 0.5, 1, 2, 3, 4 hr) the amount of TNF released was measured by HPLC. Each sample was run in triplicate.

Powder X-ray diffraction analysis

Powder X-ray diffraction (XRD) analysis was performed on raw materials (S-100, TNF), blank EuSNa MS, two EuSNa-TNF MS formulations (high/low drug loading), and a physical mixture of EuSNa and TNF using the same ratio of drug vs. polymer. A MiniFlex automated X-ray diffractometer (Rigaku, The Woodland, Texas, USA) was used for the analysis at room temperature. The diffraction angle covered from 2Θ 5° to 2Θ 40°, and a step of 1° per min was applied. The diffraction patterns were processed using Jade 8+ (Materials Data, Inc., Livermore, CA).

Cell culture

Human vaginal epithelial cell line (VK2/E6E7, ATCC Number CRL-2616), Human Endocervical epithelial cell line (End1/E6E7, ATCC Number CRL-2615), and Lactobacillus crispatus (ATCC Number 33197) were obtained from the American Type Culture Collection (Manassas, VA, USA). Culture medium and reagents were purchased from Invitrogen (Carlsbad, CA, USA). VK2/E6E7 and End1/E6E7 cells were grown and routinely maintained at 37 °C in 75-cm² culture flasks, in keratinocyte-serum free medium supplemented with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, additional calcium chloride 44.1 mg/L, and in an atmosphere of 5% CO₂.

Cellular viability assay on vaginal/endocervical cell lines and vaginal flora
The viability of VK2/E6E7 and End1/E6E7 cells was tested using both CellTiter 96™ Aqueous (MTS) and CellTiter 96™ Aqueous (LDH) assays (Promega, Madison, WI, USA) that were adopted from the previous chapter.

**In vitro** cytokine release study

Cytokine levels were measured in VK and Endo cell culture supernatant by commercially available Milliplex xMAP kits containing the human cytokine antibodies of IL-1α, IL-1β, IL-6, IL-8 and IP-10 (Millipore, Billerica, MA, USA). Similar cytokines have been found to be released by the same cell lines in previous toxicity studies with vaginal microbicidal products \(^{70b}\). For this experiment, VK and Endo cell lines were grown on a 96 well plate until confluence, and then treated with 1 mg/ml EuSNa-TNF for 24 hrs in triplicate. Medium served as negative control and TNF-α at 50 ng/ml was used as positive control. The culture supernatant was collected and incubated with human cytokine antibodies at 4 °C overnight. The plate was analyzed using the Luminex 100 Multiplex system, and the cytokine concentrations were calculated by xPONENT software (Luminex Corp. Austin, TX, USA).

Preparation and characterization of Oregon green conjugated EuSNa MS

Oregon green\(^{®}\) 488 cadaverin *5-isomer* is an amine derivative of Oregon green\(^{®}\) 488 dye, and it was used in this study to form OG conjugated EuSNa MS (EuSNa-OG MS). The synthesis scheme is shown in **Figure 27**.
Figure 27. Synthesis scheme of S-100 conjugated with Oregon green® 488 cadaverin *5-isomer* (OG), molecular weight 496.46.

It has been shown that this derivative can be successfully conjugated with a carboxyl group ended macromolecule, and maintain its fluorescence excitation and emission characteristics. Five hundred mg of Eudragit S-100 with an average molecular weight of 125 KDa (~1.2x10^{-7} M –COOH) was mixed using a magnetic stirrer with 11.5 mg EDC (~6x10^{-6} M) and 33.5 mg sulfo-NHS (~1.5x10^{-6} M) in phosphate buffer (pH 8.0 adjusted with NaOH) and maintained under room temperature for 20 min. OG (0.6 mg, 1:1 ratio of –NH₂ to –COOH) was added to the solution and the reaction was maintained for 24 hrs. The reaction mixture was then put into a dialysis bag (Spectra/Por Float-A-Lyzer G2, MWCO 3.5-5 KD, Spectrum Laboratories Inc. Rancho Dominguze, CA, USA) against 1000 ml of water for 24 hrs under magnetic stirring. The OG conjugated EuSNa was collected by lyophilization and further characterized using a Nicolet iS10 FT-IR Spectrometer (Thermo Scientific, West Palm Beach, FL) and ¹H 400 MHz NMR (Varian Inc., Santa Clara, CA, USA). The FT-IR spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and controlled by OMNIC spectra software. The
transmission mode in the FT-IR spectrometer was utilized to make observations with the sampling area of approximately 1 mm. Analysis was systematically performed between 650 and 4000 cm\(^{-1}\). The background was collected at ambient conditions before analyzing each sample. Spectra were automatically corrected with a linear baseline. No specific sample preparation was used before the FT-IR analyses. For \(^1\)H NMR analysis, all samples were dissolved in 100% D\(_2\)O. The data were recorded with 1024 scans, and the recycle time was set as 1s at 25 °C. All data were analyzed by MestReNova LITE software. The EuSNa-OG MS was prepared using the same method described earlier.

Mucoadhesion study

The scheme of the mucoadhesion test was adopted from published work, and some changes were made for this study \(^{235}\). The study was performed on freshly obtained porcine vaginal tissue (Fairview Farm Meat Co., Topeka, KS, USA) within 2 hours of death of the animal. The tissue was washed with normal saline, snap-frozen in liquid nitrogen, and kept at -80 °C. On the day of study, the tissue was first thawed at 4 °C, brought to room temperature gradually, and then cut into pieces of 8 cm length x 1 cm width. Cyanoacrylate glue previously used in a similar study \(^{236}\) was applied to attach the tissue to a plastic strip, and then was inserted into a tube, which allowed the tissue to be mounted in a 37°C water bath at an angle of 45°. An equilibration period of 5 min with VFS was allowed before administering the MS to humidify the mucosa. The mucosa was then continuously rinsed with 10 ml VFS (pre-equilibrated to 37 °C) containing fluorescent EuSNa-OG MS at 1 mg/ml. The flow rate was set at 1 ml/min using an infusion set. A 1% hydroxyethylcellulose gel, as well as a TNF loaded chitosan nanoparticle formulation \(^{236}\), was used as control. The fluorescent intensities before and after the tissue rinsing
were analyzed by a DTX 800 microplate reader at $\lambda_{\text{ex}}$ 495 nm and $\lambda_{\text{em}}$ 520 nm (Beckman Coulter, Brea, CA, USA). The percent of mucoadhesion was calculated as:

$$\text{Mucoadhesion (\%)} = \frac{F_1 - F}{F_1} \times 100$$  \hspace{1cm} (35)

Where $F_1$ was the initial fluorescent intensity and $F$ was the fluorescent intensity after the treatment.

After the study, the surface of the tissue was cleaned carefully and the tissue was cut into small cubes that were then embedded into Histoprep and frozen using liquid nitrogen. The vertical section from each frozen cube was mounted on a glass slide and examined with a Leica DMI3000 fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a total of 200X magnification.

**CD4 dependent CCR5 tropic cell-free HIV-1 entry assay**

The CD4 dependent cell-free HIV-1 entry assay was performed in cooperation with Southern Research Institute (Durham, NC, USA). This assay is designed to identify compounds that inhibit cell-free HIV-1 entry into HeLa CD4 LTR β-Gal (MAGI) or MAGI-CCR5 cells. The CCR5-tropic HIV-1 entry assay is one of the primary screening assays and is performed as described previously. Briefly, 1.5 mg of EuSNa-TNF MS and 1.3 mg of TNF were dissolved in 1.5 and 64.9 ml of dH$_2$O, respectively. It was calculated that they have an equivalent concentration of TNF (69.7 µM). For pre-treatment test group, at 24 h prior to the assay cells were plated at $10^4$ per well for the CCR5-tropic HIV-1 entry assay. The medium was removed, and the compound diluted in medium was placed on the cells and immediately incubated for 24 hrs at 37°C. On the day of assay, compound was removed and replaced with fresh compound and virus. For standard treatment group, the compound and virus were added to the cells only on the
day of assay. For HIV virus infection, ten 50% tissue culture infective doses of the IIIB or Ba-L strains of HIV-1 were then added to the wells, and incubation was continued for 3 h for the CCR5-tropic viral entry assay. At the end of the incubation, the wells were washed once with cDMEM, and the culture was continued for 48 h at 37°C. At termination of the assay, the medium was removed, and the β-Gal enzyme expression was determined by chemiluminescence using Tropix Gal-Screen (Applied Biosystems) according to the manufacturer’s instructions. dH2O was used as negative control, and TAK 779 and AMD 3100 were chosen to demonstrate specificity for the CCR5 or CXCR4 receptor.

Statistical analysis

The result of the $2^{4-1}$ design was analyzed using JMP software (version 8.0, SAS Institute Inc., Cary, NC, USA). Unless otherwise stated, data were expressed as mean ± standard deviations. Compared to controls, the statistical significant difference of a given mean was determined using a student’s t-test. A P value <0.05 was considered statistically significant.

5.3 Results

Statistical analysis and optimization of EuSNa-TNF MS

Table 21 describes the responses obtained by the $2^{4-1}$ design for the drug encapsulation efficiency ($Y_1$), yield ($Y_2$) and Carr’s index ($Y_3$). The overall drug encapsulation efficiency of EuSNa-TNF is greater than 50%, with a maximal encapsulation efficiency of 95.5%.

The overall yield varies from 53% to 73%, with Carr’s index ranging from 16.7 to 47.9. To check the validity of the model, ANOVA and lack-of-fit test were performed, and the results are shown in Table 22. All three dependent variables have $R^2$ values greater than 0.9. The P
values for ANOVA are less than 0.05, indicating a statistically significant model fit at 95% confidence, with no lack-of-fit (P values for lack-of-fit test are greater than 0.05).

Table 21. Responses values of dependent variables.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Pattern</th>
<th>Polymer Conc. (% w/v)</th>
<th>Polymer: drug ratio</th>
<th>Inlet temperature (°C)</th>
<th>Feed rate (ml/min)</th>
<th>Drug encapsulation efficiency (%)</th>
<th>Yield (%)</th>
<th>Carr's index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++−−</td>
<td>5.0</td>
<td>10</td>
<td>140</td>
<td>1.6</td>
<td>45.7</td>
<td>64.3</td>
<td>16.7</td>
</tr>
<tr>
<td>2</td>
<td>−−−−</td>
<td>2.0</td>
<td>10</td>
<td>140</td>
<td>1.6</td>
<td>67.7</td>
<td>73.2</td>
<td>23.6</td>
</tr>
<tr>
<td>3</td>
<td>−−−−</td>
<td>5.0</td>
<td>10</td>
<td>100</td>
<td>6.4</td>
<td>64.0</td>
<td>53.3</td>
<td>47.9</td>
</tr>
<tr>
<td>4</td>
<td>−−−−</td>
<td>2.0</td>
<td>10</td>
<td>100</td>
<td>6.4</td>
<td>58.6</td>
<td>56.2</td>
<td>37.9</td>
</tr>
<tr>
<td>5</td>
<td>−−−−</td>
<td>2.0</td>
<td>40</td>
<td>100</td>
<td>1.6</td>
<td>82.3</td>
<td>69.7</td>
<td>35.5</td>
</tr>
<tr>
<td>6</td>
<td>−−−−</td>
<td>5.0</td>
<td>40</td>
<td>100</td>
<td>1.6</td>
<td>60.8</td>
<td>54.8</td>
<td>31.2</td>
</tr>
<tr>
<td>7</td>
<td>−−−−</td>
<td>5.0</td>
<td>40</td>
<td>140</td>
<td>6.4</td>
<td>73.5</td>
<td>48.7</td>
<td>25.9</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>3.5</td>
<td>25</td>
<td>120</td>
<td>4.0</td>
<td>63.6</td>
<td>59.2</td>
<td>33.2</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>3.5</td>
<td>25</td>
<td>120</td>
<td>4.0</td>
<td>60.2</td>
<td>63.1</td>
<td>27.7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>3.5</td>
<td>25</td>
<td>120</td>
<td>4.0</td>
<td>66.2</td>
<td>60.9</td>
<td>28.8</td>
</tr>
<tr>
<td>11</td>
<td>−+++</td>
<td>2.0</td>
<td>40</td>
<td>140</td>
<td>6.4</td>
<td>95.5</td>
<td>72.4</td>
<td>40.9</td>
</tr>
</tbody>
</table>

Table 22. The result of ANOVA and lack-of-fit analysis on all dependent variables.

<table>
<thead>
<tr>
<th>Response</th>
<th>R²</th>
<th>P&lt;sub&gt;ANOVA&lt;/sub&gt;</th>
<th>P&lt;sub&gt;lack-of-fit&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug encapsulation efficiency (Y₁)</td>
<td>0.95</td>
<td>0.047 *</td>
<td>0.13</td>
</tr>
<tr>
<td>Yield (Y₂)</td>
<td>0.99</td>
<td>0.0077 *</td>
<td>0.73</td>
</tr>
<tr>
<td>Carr’s index (Y₃)</td>
<td>0.96</td>
<td>0.043 *</td>
<td>0.32</td>
</tr>
</tbody>
</table>
* Statistical significance (α=0.05).

**Figure 28** shows the main effect of all independent variables and their interactions on the dependent variables. The independent variables, which have an effect size (quantified by t-ratio) greater than a tabulated critical t value (t_{crit}=3.18, df=3, indicated by a blue vertical line), are identified as statistically significant. The overall effects of all the independent variables and their interactions are reflected by the following equations:

$$Y_1 = 67.09 - 7.51X_{a1} + 9.51X_{a2} + 2.08X_{a3} + 4.39X_{a4} - 3.36X_{a1}X_{a2} - 3.49X_{a1}X_{a3} + 3.35X_{a1}X_{a4}$$

$$Y_2 = 61.44 - 6.3X_{a1} - 0.18X_{a2} + 3.07X_{a3} - 3.93X_{a4} - 3.35X_{a1}X_{a2} - 1.85X_{a1}X_{a3} - 0.35X_{a1}X_{a4}$$

$$Y_3 = 31.74 - 2.04X_{a1} + 0.92X_{a2} - 5.67X_{a3} + 5.7X_{a4} - 2.8X_{a1}X_{a2} - 3.46X_{a1}X_{a3} + 0.78X_{a1}X_{a4}$$

Where the coded factors $X_{Ai}$ are: $X_{A1} = (X_1 - 3.5)/1.5$, $X_{A2} = (X_2 - 25)/15$, $X_{A3} = (X_3 - 120)/20$ and $X_{A4} = (X_4 - 4)/2.4$, and $X_1$ through $X_4$ stand for the original independent variables in Table 21.
Figure 28. Pareto chart showing the standardized effect of independent variables and their interaction on Drug loading efficiency (top), Yield (middle) and Carr’s Index (bottom). Bars extending past the line indicates statistical significance ($\alpha=0.05$).

The relationship between the independent variables and dependent variables is further investigated using a prediction and desirability plot (Figure 29). In Figure 29, the predicted values of all three dependent variables with their standard deviation values are represented by the solid line and the dotted line, respectively. Our goal was to maximize drug encapsulation efficiency ($Y_1$) and yield ($Y_2$) while minimize Carr’s index ($Y_3$). Based on our goal, the optimum experimental condition is computed as: polymer concentration 2.8%, polymer to drug ratio 40:1, inlet temperature 140 °C, and feed flow rate of 1.6 ml/min, with a maximized desirability value of 0.74. The comparison between the predicted and the actual values of the dependent variables is achieved by a check point analysis shown in Table 23. The differences between the predicted and actual values appear to be statistically insignificant.
Figure 29. Prediction and desirability plot showing the effect of independent variables on Drug loading efficiency, Yield, and Carr’s index.

Table 23. Comparison between predicted and actual values of dependent variables under maximized desirability.

<table>
<thead>
<tr>
<th>Run</th>
<th>$Y_1$</th>
<th>Predicted</th>
<th>P-value</th>
<th>$Y_2$</th>
<th>Predicted</th>
<th>P-value</th>
<th>$Y_3$</th>
<th>Predicted</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP</td>
<td>88.7±2.1</td>
<td>82.1±12.0</td>
<td>0.40</td>
<td>68.9±7.2</td>
<td>73.2±3.9</td>
<td>0.41</td>
<td>28.3±3.3</td>
<td>25.3±7.67</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*OP stands for optimum run

Size, zeta potential, and morphology of EuSNa-TNF MS
As shown in **Figure 30A**, TNF loaded EuSNa MS appear to be well dispersed and spherical in shape. There is a variance in size distribution as shown in **Figure 30B**; however, most particles appear to be greater than 2 µm. The average size of spray dried EuSNa-TNF MS is 4.73 µm as measured by a Scepter 2.0 handheld automated cell counter. The average zeta potential of EuSNa-TNF-MS is -26.3 mV.

![Figure 30A](image1.png)

**Figure 30.** (A) Scanning electron microscopy image of EuSNa-TNF MS. Scale bar set at 5 µm. (B) size distribution of EuSNa-TNF MS measured by Sceptor 2.0 cell counter. Y axis indicates the number of particle counts. M1 indicates a size region with lower limit of 3 µm and higher limit of 9 µm.
In vitro release of TNF from EuSNa-TNF MS

The *in vitro* release profile of TNF loaded EuSNa MS in VFS and a mixture of VFS/SFS is shown in Figure 31. There is 91.7±10.7% (n=3) of TNF released from EuSNa MS at pH 7.6 within 60 min, while there’s only 22.5±8.1% (n=3) of TNF released at pH 4.2 during the same period of time. The simulation of the release profile of TNF to a series of known release kinetic models is performed and the results are shown in Table 24. The release of TNF from the MS at pH 7.6 appears to follow zero-order kinetics, while the release profile under pH 4.2 can be better described by reciprocal powered time model.

Figure 31. *in vitro* release profile of EuSNa-TNF MS at pH=4.2 (blue) and pH=7.6 (red) under 37°C, n=3.
Table 24. Kinetic models used for analysis of TNF loaded EuSNa MS release data and their corresponding $R^2$ values.

<table>
<thead>
<tr>
<th>Model No.</th>
<th>Model name $^a$</th>
<th>Model equation $^b$</th>
<th>$R^2$ (pH 4.2)</th>
<th>$R^2$ (pH 7.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zero order</td>
<td>$F=k_0 t$</td>
<td>0.759</td>
<td>0.983 $^c$</td>
</tr>
<tr>
<td>2</td>
<td>First order</td>
<td>$\ln (1-F)=-k_f t$</td>
<td>0.512</td>
<td>0.933</td>
</tr>
<tr>
<td>3</td>
<td>Higuchi</td>
<td>$F=k_H \sqrt{t}$</td>
<td>0.958</td>
<td>0.574</td>
</tr>
<tr>
<td>4</td>
<td>Power law</td>
<td>$\ln F=\ln k_p + p \ln t$</td>
<td>0.958</td>
<td>0.574</td>
</tr>
<tr>
<td>5</td>
<td>Weibull</td>
<td>$\ln[-\ln(1-F)]=\ln k_w + b \ln t$</td>
<td>0.964</td>
<td>0.862</td>
</tr>
<tr>
<td>6</td>
<td>Reciprocal powered time</td>
<td>$(1/F-1) = m/t^b$</td>
<td>0.969</td>
<td>0.544</td>
</tr>
</tbody>
</table>

$^a$ Models 1-5 were processed by linear regression where model 6 was processed by nonlinear regression.

$^b$ $F$ denotes fraction of drug released at time $t$. $k_0$, $k_f$, $k_H$, $k_p$, $p$, $k_w$, $b$, and $m$ are parameters of the models.

$^c$ last three time points were not considered in the zero order model.

X-ray diffraction analysis
Figure 32. XRD crystallography for drug loaded MS, raw component in the formulation and physical mixture of drug and polymer. From top to bottom, A: pure TNF, B: EuSNa and TNF mixture at 10:1, C: EuSNa and TNF mixture at 40:1, D: EuSNa-TNF MS (polymer to drug ratio 10:1), E: EuSNa-TNF MS (polymer to drug ratio 40:1), F: blank EuSNa MS, G: pure Eudragit S-100.

The result of X-ray diffraction analysis is shown in Figure 32. The characteristic peaks of TNF are found around 2θ = 7°, 15°, 22°, 23.5° and 24.8°. These peaks are not observed in any of the EuSNa MS formulations, while they are still observable in the physical mixture of TNF and EuSNa using the same mass ratio.

Cytotoxicity studies of EuSNa-TNF MS
Our data suggest that EuSNa-TNF MS is safe in both vaginal (VK) and endocervical (Endo) epithelial cell lines (Figure 33). No significant reduction of cell viability was found in MTS assay (Figure 33A) using both blank and drug loaded MS formulation at 1 mg/ml. Lactate dehydrogenase (LDH) is an indicator released from cells with damaged membranes. Minimal amount of LDH was released from cells incubated with blank and drug loaded EuSNa MS, being only 6.9% and 7.7% compared to positive control, respectively (Figure 33B). The result also indicates that EuSNa-TNF MS is not toxic to vaginal flora, as no statistically significant loss of viability was found during the incubation period (Figure 33C).
Figure 33. MTS (A) and LDH (B) assay of VK and Endo cells treated with blank or TNF loaded EuSNa MS at 1 mg/ml over 24 hr. C: Viability of Lactobacillus cripatus treated with blank or EuSNa TNF-loaded MS after 24 hr at 1 mg/ml. The data shown represent the mean ± standard deviation of 3 independent experiments. * Statistical significance (α=0.05).
Cytokine release study

The level of immunogenicity of EuSNa-TNF MS is evaluated using Luminex Multiplex system and the results are shown in Figure 34. The concentration of five cytokines, namely IL-1α, IL-1β, IL-6, IL-8, and IP-10 are shown after 24 hr incubation with EuSNa-TNF MS. In case of IL-6, IL-8, and IP-10, the level of cytokine released is comparable, or even lower, than that of the medium (Figure 34A-C), while significantly lower than the positive control (TNF-α at 50 ng/ml). A similar trend can be observed in cases of IL-1α and IL-1β; however, TNF-α and TNF-β are not able to generate similar level of cytokine as they did in IL-6, 8 and IP-10.
Figure 34. The level of cytokine release from VK and Endo cells treated with TNF-loaded EuSNa MS over 24 hr. A: IP-10, B: IL-8, C: IL-6, D: IL-1α, E: IL-1β. The data shown represent the mean ± standard deviation of 3 independent experiments. * Statistical significance (α=0.05).

Mucoadhesion study

**Figure 35A** shows the FT-IR spectrum of S-100 polymer, EDC, Sulfo-NHS (S-NHS), and S100-OG conjugate. The C=O stretching vibration signal around 1700 cm⁻¹ is reduced and substituted with a peak of similar strength around 1600 cm⁻¹, which is due to the formation of an amide bond. The broad peak around 3300 to 3500 cm⁻¹ may be contributed to NH stretching vibrations. In **Figure 35B**, the existence of Oregon green in S100-OG conjugate is proved by ¹H NMR spectroscopy. The aromatic proton peaks (δ=8.20, 8.05, 7.81, 7.24, 6.69 and 6.58) can be detected in the final product S100-OG, and the line width of the peaks at the aromatic areas is consistent with dye bonding with polymer. In **Figure 36A**, OG conjugated EuSNa MS can be observed with a strong fluorescent signal.

The mucoadhesion of EuSNa MS was evaluated and the results are shown in **Figure 36B-D**. The average mucoadhesion of EuSNa MS is determined to be 8.7%, where mucoadhesion of HEC gel and chitosan nanoparticles is 4.4% and 8.1%, respectively. The mucoadhesion of EuSNa is found to be slightly higher that of chitosan nanoparticles, and it is significantly greater than that of 1% HEC gel formulation.
Figure 35. (A) FT-IR spectrum of the reactants and the product of EuSNa-OG complex. From top to bottom: FT-IR spectrum of pure S-100, Sulfo-NHS, EDC, EuSNa-OG conjugate. (B) NMR spectrum showing the aromatic protons in the EuSNa-OG complex.
Figure 36. A: Fluorescent microscopic image of Oregon green loaded MS. B: Vertical section of porcine vaginal tissue after the mucoadhesion study, arrow showing the surface of the vaginal tissue section. C: Control fluorescent image of the same porcine vaginal tissue represented in figure B, arrow showing the same position as in figure B. D: Percentage mucoadhesion of 1mg/ml HEC gel, chitosan nanoparticles and EuSNa MS. * Statistical significance (α=0.05).

CD4-dependent CCR5 tropic cell-free HIV-1 entry assay

The results are shown in Figure 37. EuSNa-TNF MS was found to have an IC50 value from 0.67-0.78 µM, and that is 3.8-5.8 fold more potent compared to native drug in HIV-1 inhibition.
Table 25. Antiviral evaluation of EuSNa-TNF MS vs. HIV-1 Ba-L in Magi R5 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Units</th>
<th>Assay Type(^1)</th>
<th>IC(_{50})</th>
<th>IC(_{90})</th>
<th>TC(_{50})</th>
<th>TI (TC(<em>{50})/IC(</em>{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EuSNa-TNF MS</td>
<td>µM</td>
<td>Standard</td>
<td>0.78</td>
<td>2.85</td>
<td>&gt;35.0</td>
<td>&gt;44.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-Treatment</td>
<td>0.67</td>
<td>2.71</td>
<td>&gt;35.0</td>
<td>&gt;52.2</td>
</tr>
<tr>
<td>Tenofovir (PMPA)</td>
<td>µM</td>
<td>Standard</td>
<td>4.59</td>
<td>24.7</td>
<td>&gt;35.0</td>
<td>&gt;7.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-Treatment</td>
<td>2.54</td>
<td>20.6</td>
<td>&gt;35.0</td>
<td>&gt;13.8</td>
</tr>
<tr>
<td>dH2O Control</td>
<td>%</td>
<td>Standard</td>
<td>14.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
<td>&gt;3.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-Treatment</td>
<td>13.7</td>
<td>45.6</td>
<td>&gt;50.0</td>
<td>&gt;3.65</td>
</tr>
<tr>
<td>TAK 779</td>
<td>µM</td>
<td>Standard</td>
<td>0.005</td>
<td>0.07</td>
<td>&gt;10.0</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-Treatment</td>
<td>0.004</td>
<td>0.06</td>
<td>&gt;10.0</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>AMD 3100</td>
<td>µM</td>
<td>Standard</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-Treatment</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\(^1\) Assay Type:

- **Standard**: On the day of the assay, compound and virus were added to cells and incubated for ~48 hours.
- **Pre-Treatment**: Cells were pre-treated with drugs 24 hours prior to setting up the assay. On the day of assay, compound was removed and replaced with fresh compound and virus. Plates were then incubated for ~48 hours.

5.4 Discussion

The purpose of the \(2^{4+1}\) fractional factorial design is to screen several key factors contributing to the optimum formulation with high-yield, high drug encapsulation efficiency, and low moisture content during the formulation design and process. For drug encapsulation efficiency (\(Y_1\)), it is found that increasing the polymer to drug ratio, as well as decreasing polymer concentration, can significantly improve drug encapsulation. High drug encapsulation efficiency can be achieved through high polymer to drug ratio, indicating the possibility that the free drug, which is not associated with the polymer, is less likely to be well encapsulated. It is not clear as of how decreasing polymer concentration contributes to high encapsulation efficiency. For yield (\(Y_2\)), it is found that low polymer concentration, low feed flow rate, and high inlet temperature are capable of significantly improving the yield of the spray dried MS.
The effect of inlet temperature and feed flow rate on spray drying is shown in equation (28) and (29). The so-called air/fluid mass ratio represents the energy available for atomization, and decreasing n_{a/f} will result in insufficient drying of particles. When atomizing air flow is fixed, decreasing liquid feeding rate increases n_{a/f}, therefore increasing drying efficiency leads to increased yield. The Carr’s index (Y_3) is an indicator of the powder flowability of the spray dried powder. The value of Carr’s index, which is greater than 25%, indicates a cohesive powder with poor flow characteristics. Increased feed flow rate, as well as low temperature, is found to have a significant effect on increasing Carr’s index. In practice, poor powder flowability is usually correlated with high moisture residue in the powder, which can be an obvious consequence from increased feed flow rate and low temperature as the combination suffers from insufficient drying.

The concept of maximized desirability was used in this study to find the optimum condition. The desirability in optimization of multiple dependent variables is described using a scale of 0 to 1, where 0 is not acceptable and 1 is the perfectly desirable result. The optimum formulation has an average encapsulation efficiency of 88.7% with 68.9% yield, and a Carr’s index of 28.3%. The surface characteristics of the particulate system usually govern its biological fate, especially if delivered topically. In terms of intravaginal delivery, the mucus serves as a barrier that can filter out particles with a large size (>1 µm), rendering these particles only adhering on the mucus surface. Smaller particles may be more capable of diffusing through the mucin mesh, which has been experimentally confirmed that a suitable size range (200-500 nm) is required for muco-penetration. However, in this study, the EuSNa-TNF MS was designed to be responsive to the pH change caused by male ejaculate. Therefore a mucin-adhering, rather than a mucin-penetrating, delivery system may better serve this purpose. Surface charge serves
as another important characteristic that determines whether a particle delivery system will penetrate through the mucin. For example, it has been found that small particles with neutral surface charge have a better chance at reaching deep into the epithelium \(^{239}\). Therefore the relatively large size, combined with strong surface charge, of EuSNa-TNF MS may guarantee that it could be in the right place as a pH-sensitive delivery micro system.

TNF is a hydrophilic molecule (Log P -1.6) with a molecular weight of 287.12. It’s very difficult to achieve high TNF loading using a nanoparticle delivery system prepared by the traditional emulsion-evaporation method \(^{230}\), in which hydrophilic drug like TNF is very likely present in the aqueous phase and therefore being washed out during the preparation process. In this study, the average drug loading of TNF is 2% (w/w), which means that a 1 mg/ml EuSNa-TNF MS suspension is equivalent to a TNF concentration of 69.7 µM. This is almost 10 times higher than the TNF loading in the pH-sensitive nanoparticles prepared by the emulsion-evaporation technique \(^{230}\). Given the fact that the EC\(_{50}\) of TNF is 5.0±2.6 µM, it is reasonably speculated that this MS formulation can provide a sufficient concentration of TNF to exhibit an anti-HIV effect after complete drug release within 2 hours after vaginal pH change occurs \(^{222}\). Spray drying as a quick and easy preparation method, not only produces large and spherical particles that have a good impact on powder flowability \(^{240}\), but also changes the TNF from its crystallized form. As seen in Figure 32, the characteristic peaks of TNF are still observable in the physical mixture of TNF with EuSNa, but almost completely lost in the crystallography of spray dried MS formulations, suggesting that TNF appears to be amorphous in the MS formulation.

Since the altered pH inside the vagina can be maintained for only a couple of hours after ejaculate \(^{218}\), it is critical that any pH-sensitive delivery system has a fast release rate at alkaline
pH. The EuSNa-TNF MS can release almost 90% of its pay load within 60 min at pH 7.6, while only approximately 20% of the TNF is released in the same period at the acidic pH condition of the normal vagina. The portion of drug released at an acidic pH may be due to the surface-associated or free TNF present in the formulation. The TNF release from EuSNa-TNF MS under pH 7.6 appears to follow zero order release kinetic within the first 60 min, while its release under pH 4.2 would be best fit by a reciprocal powered (RP) model. It is possible that under alkaline pH, the fast dissolution of Eudragit S-100 will make the MS a porous matrix; and it has been shown that small molecules released from porous polymer microcarriers are likely to follow zero-order release. In the RP model, not only dissolution and diffusion, but also other time dependent variables, are embedded into the model, such as wettability and degradation of S-100 in this case.

Safety is also an important factor in developing a topical delivery system, as it has been pointed out that a safe microbicides delivery system cannot damage the vaginal/endocervical epithelium, disturb normal vaginal flora, or trigger any immune-response. In this study, MTS and LDH assay show that 1 mg/ml EuSNa-TNF MS is safe to both vaginal and endocervical epithelium with no significant reduction of viability. The normal vaginal flora will not be affected, either. Figure 34 shows the level of inflammatory cytokine release on VK and Endo cell lines. These cytokines are chosen because of their extensive involvement in mucosa toxicity research both in vitro and in vivo. In case of IL-6, IL-8 and IP-10, the level of cytokine triggered by EuSNa-TNF MS is significantly lower compared to the positive control, suggesting its immunogenicity is transient. In case of IL-1α, the overall level of cytokine release is low, suggesting the cells may not be sensitive enough for both TNF-α and EuSNa-TNF MS. However, the level of IL-1α is still significantly lower than TNF-α. The total level of cytokine is
even lower in case of IL-1β. This might suggest that IL-1α and β are not suitable cytokines to be tested *in vitro* on these two cell lines. As in some studies a good correlation between the rabbit vaginal irritation scores and IL-1β levels in vagina lavage has been shown \(^{70c}\), suggesting it might be better detected in future *in vivo* test. However, overall there is no evidence of significant cytokine release triggered by EuSNa-TNF MS in this study.

During the synthesis of EuSNa-OG complex, the dialysis method was applied overnight to ensure the removal of any free OG molecule. As is shown in the FT-IR and NMR spectrum, both amide bond stretch and aromatic protons can be observed in the final synthetically fluorescent MS, which indicates the successful conjugation of OG to the polymethacrylate. These facts together prove that the OG has been successfully covalently attached to EuSNa MS and the fluorescent signal in mucoadhesion study is not due to free OG.

Porcine vaginal tissue was used in this study as it provides a physiologically relevant and sensitive system that can be utilized in *ex vivo* scenario \(^{245}\). It has been reported that the Eudragit salts have a high intrinsic dissolution rate and the dissolved chain could interact strongly with the hydrated mucin glycoprotein \(^{231}\). The *in vitro* mucoadhesion test indeed demonstrates a statistically significant improvement in term of mucoadhesion of EuSNa MS compared to HEC gel (*Figure 36D*). Its mucoadhesion property is even slightly higher than that of nanoparticle formulation prepared by chitosan, whose bioadhesion was tested under the same condition using covalently bonded fluorescent dye \(^{236}\). Our hypothesis in this study is to incorporate a mucoadhesive material to our delivery system, and by doing so prolong the bioretention time of such system. Therefore, when the triggering stimuli is present (semen in this case), it is anticipated that the drug will undergo immediate release at high concentration.
The *in vitro* EC$_{50}$ of TNF has been reported as 5.0 ± 2.6 µM$^{222}$, and it’s well correlated with our study in which the IC$_{50}$ of TNF is 4.59 µM. EuSNa-TNF MS that has an equivalent concentration to free TNF demonstrate a 3.8-5.8 fold higher anti-HIV efficacy. We speculate that the increased hydrophobicity due to the presence of polymer actually increase cellular uptake, therefore boost the anti-viral efficacy. It has been reported that TDF, a more lipophilic prodrug of TNF, can improve the IC$_{50}$ value by 17-90 fold$^{222}$. The increased permeability of EuSNa-TNF MS may help to form a long-lasting barrier of microbicides to prevent HIV transmission in the vaginal epithelium in the event that the semen-triggered release is not happened.

5.5 Conclusion

In this study, spray dried, tenofovir loaded, pH sensitive, and mucoadhesive microspheres are prepared based on polymethacrylate salt. Fractional factorial design has been used to screen and optimize formulation and process parameters. The optimized formulation has an average size of 4.73 µm with a drug loading of 2% (w/w). It has been shown that these microspheres can quickly respond to the pH change, releasing over 90% of drug payload within 60 min. The mucoadhesion property of these microspheres is significantly improved compared to 1% HEC gel formulation. Moreover, the findings in this study reveal that these microspheres are non-cytotoxic and non-immunogenic to vaginal/endocervical epithelial cells. There is also no observable cytotoxic effect on normal vaginal flora. These microspheres are potentially more effective for intravaginal delivery of microbicides, owing to their improved mucoadhesion and pH-responsiveness. Future studies are needed to evaluate their vaginal retention and safety *in vivo*. Collectively, these data present a possible delivery strategy for intravaginal delivery of microbicides for the prevention of HIV transmission.
6. SUMMARY AND CONCLUSIONS

6.1 Summary

Due to the urgent need of stopping HIV transmission and decrease of the number of cases of new HIV infections, topical microbicides prevention strategy is currently under hot pursuit. It is critical to develop a formulation for the topical application of HIV microbicides for PrEP, and the first generation formulation (gels, film, IVRs) either lacks the proper vaginal retention or controlled release of drug substance. These drawbacks lead to low acceptability and adherence to the dosage regime, therefore manifested in insufficient protection of HIV in clinical trials. In this dissertation, we explored the possibility of developing a novel topical formulation that is safe and effective in preventing vaginal transmission of HIV, utilizing the advances in nanoparticles/microparticles formulation technology. Combined with powerful tool of design of experiments, different templates of drug delivery systems such as oily core nanocausles, pH-sensitive nanoparticles, and mucoadhesive/pH-sensitive dual functional microspheres are developed that could be used for lipophilic, or hydrophilic microbicides. Various formulation and process parameters are investigated and optimized using design of experiments, and their physicochemical properties, safety, and efficacy are also evaluated.

In chapter 3, Box-Behnken design enables to elucidate and optimize the size and encapsulation efficiency of IND into spray dried oily core NC prepared by emulsion-diffusion technique. Optimum combination of formulation variables was calculated to be 300 mg of PLA, 0.56 ml of oil, and 239.57 mg of Pluronic F127, which corresponded to particle size of 284.1 nm and encapsulation efficiency of 95.7%. Checkpoint analysis proved the validity of the model and optimum formulation. Morphological and XRD analysis confirmed the existence of oily core structure, and the drug was encapsulated in amorphous form. Fractional factorial design enabled
to investigate and optimize the yield of indomethacin (IND) loaded oily core Nanocapsules (NCs) prepared by emulsion-diffusion technique and spray drying, which led to a yield of 30.8%. Statistical analysis proved the validity of the model and optimization process.

In chapter 4, pH-responsive nanoparticles loaded with tenofovir or tenofovir disoproxil fumarate are formulated. The nanoformulation prepared by 75/25% ratio of S-100/PLGA matrix loaded with TDF (size 336.8 nm, encapsulation efficiency 24.0%, drug loading 1.9% w/w) appears to be the best formulation considering the overall effect based on drug loading, in vitro release (pH-responsive release), and safety (up to 10 mg/ml). PLGA/Eudragit S-100 nanoparticles have several advantages, such as small particle size, controlled release of drug in the presence of semen, and they are potentially safe to the vaginal physiological environment. Collectively, these data suggest the possibility of using nanoparticles as a delivery system for intravaginal administration of HIV microbicides for the prevention of HIV transmission.

In chapter 5, spray dried, tenofovir loaded, pH sensitive, and mucoadhesive microspheres are prepared based on polymethacrylate salt. Fractional factorial design has been used to screen and optimize formulation and process parameters. The optimized formulation has an average size of 4.73 µm with a drug loading of 2% (w/w). It has been shown that these microspheres can quickly respond to the pH change, releasing over 90% of drug payload within 60 min. The mucoadhesion property of these microspheres is significantly improved compared to 1% HEC gel formulation. Moreover, the findings in this study reveal that these microspheres are non-cytotoxic and non-immunogenic to vaginal/endocervical epithelial cells. There is also no observable cytotoxic effect on normal vaginal flora. These microspheres are potentially more effective compared to pure TNF in the in vitro anti-HIV efficacy study.
6.2 Future Directions

Future directions of this work may include: 1) Improve formulation and optimization drawbacks that associated with low encapsulation efficiency, process yield, and size distribution. Encapsulation of hydrophilic small molecules has been a technical difficulty in the development of nanoformulation. Higher encapsulation usually leads to better antiviral efficacy and better protection of drug during transportation and storage. The size of nanoparticles/microparticles need to be precisely controlled to be able to predict the biological interaction between these particles with biological environment. The nanoformulation need to have a narrow size distribution, therefore the effect of burst-release can be avoided. 2) Address unknown issue of bioretention, semen-triggered release, biosafety, and anti-HIV efficacy in vivo using appropriate animal model. In order to advance this work to the clinical setting, critical quality attributes such as biosafety, bioretention, and antiviral efficacy need to be tested on animal models. 3) Expand the application of delivery templates to other microbicides that are currently under clinical trials, and eventually investigate the possibility of developing a topical formulation of combinatorial HIV microbicides that incorporate fusion inhibitors, reverse transcriptase inhibitors, and other microbicides that act by a different mechanism. 4) Study and optimize the manufacturability and address the cost-effectiveness of any of the formulation that has been developed, to eventually meet the actual needs of this kind of formulation.
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VITA

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After graduating with a Bachelor’s degree, Mr. Zhang was accepted into the interdisciplinary Ph.D. program with Pharmaceutical Sciences and Chemistry as his disciplines. Mr. Zhang is a member of American Association of Pharmaceutical Sciences (AAPS) and American Chemical Society (ACS). He was the recipient of Community of Scholars Research Award recognized by University of Missouri Kansas City (2012). He has also received several Travelship Award (2009, 2011, 2012) for excellence in the field of pharmaceutical sciences by various study sections of AAPS annual meeting. He also authored and co-authored several peer reviewed publications in renowned international journals and has presented his work in various annual scientific meetings.